Characterizing Sex Differences in Lipid Metabolism during an Acute, 30-Minute Bout of Moderate-Intensity Continuous (MIC) Exercise

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

INTRODUCTION: Increased intramyocellular lipid (IMCL) size and storage in the subsarcolemmal region of the myocyte are associated with insulin resistance (IR) and the development of type 2 diabetes (T2D). Women have a higher IMCL content than men yet are at a lower risk for T2D due, at least in part, to the fact that they store IMCL as smaller, more numerous droplets than men. Differences in IMCL turnover may also contribute to the decreased T2D risk. Women utilize a greater proportion of lipids as a fuel source during exercise when compared with men, as evidenced by a lower respiratory exchange ratio (RER). However, whether women rely to a greater extent on IMCL during exercise is equivocal with studies showing greater, similar, or lesser IMCL utilization by women during exercise. IMCL utilization during exercise has been shown to be related to basal IMCL content, which would suggest that women would rely to a greater extent on IMCL during the exercise bout given their increased IMCL storage. Some of the conflicting findings may be due to the fact that the sex-comparative studies conducted to date have focused solely on total IMCL utilization and have not examined whether sex influences region-specific IMCL storage or utilization during endurance exercise. **PURPOSE**: The aim of this study was to characterize the sex-based differences in substrate utilization, lipid species profile, and enzymes and proteins related to IMCL, lipid and oxidative metabolism following an acute bout of moderate-intensity continuous (MIC) exercise. **METHODS**: Young, healthy, recreationally active men (n = 12) and women (n = 13) underwent an acute bout of MIC exercise on a stationary cycle ergometer. The MIC exercise session consisted of one, 30-minute cycling bout at 65% VO_2 peak with 5-minute breath collections taken at rest, 12, and 25 minutes of exercise. Muscle biopsies were taken from the *vastus lateralis* muscle prior to and immediately following the exercise bout. All participants were matched for VO_2 peak relative to fat-free mass (FFM) to ensure that they were equally trained. As estrogen is known

to influence fuel utilization during exercise, women were tested in the midfollicular phase of their menstrual cycle (days 4-9). Enzyme activity assays were used to investigate the activity of β-oxidation and tricarboxylic acid cycle (TCA) enzymes, which included β-HAD and CS, respectively. An untargeted macrolipidomics approach was used to determine sex differences in estimates of fatty acid (FA) content of specific lipid species prior to and following endurance exercise using liquid-chromatography tandem mass spectrometry (LC-MS/MS). Western blot analysis was used to determine the content of proteins involved in lipolysis (ATGL, PLIN3), lipid oxidation (β-HAD, CPT1), and oxidative metabolism (CS, COX II-V). **RESULTS**: Sex had no effect on RER ($p = 0.19$), HR ($p = 0.44$), blood lactate ($p = 0.30$), or RPE ($p = 0.48$) throughout exercise. Men oxidized a significantly greater proportion of carbohydrates during the exercise bout ($p < 0.001$), however whole-body lipid utilization was similar between the sexes ($p = 0.92$). Protein content for ATGL ($p = 0.51$), β-HAD ($p = 0.80$), COX II ($p = 0.08$), COX III ($p = 0.13$), COX IV ($p = 0.17$), COX V ($p = 0.94$), CPT1 ($p = 0.25$), CS ($p = 0.70$), and PLIN3 ($p = 0.25$) were all similar between men and women. No significant differences in β-HAD ($p = 0.54$) or CS ($p = 0.56$) enzyme activity were found; however, relative to baseline, CS activity increased to a greater extent in women ($p = 0.04$) during exercise. Sex had no effect on detected PC ($p \geq 0.32$) or PE ($p \geq 0.23$) species. Total abundance of detected TAG content was higher in women than men ($p = 0.018$), and women had a greater proportion of unsaturated TAGs than men ($p = 0.017$). Exercise did not influence the lipid profile of PC, PE or TAG species. **CONCLUSION**: During a 30-minute bout of MIC exercise, whole-body lipid oxidation did not differ between men and women. Furthermore, there was no sex difference in the content of enzymes involved in lipid and oxidative metabolism at rest; however, during the acute bout of MIC exercise CS activity increased to a greater extent in women than men, indicative of a greater increase in oxidative metabolism. Our novel lipidomics analysis provided

exciting new insight into the lipid species profiles of PC, PE and TAGs within human skeletal muscle and provides the first report of sex differences in the global lipidome, and serves as the foundation for future studies to complete subsequent lipidomics analyses in human populations.

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% BF	Percent body fat
AA	Arachidonic acid
Akt	Protein kinase B
AMPK	5’ adenosine monophosphate-activated protein kinase
ATGL	Adipose triglyceride lipase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
β-HAD	3-hydroxyacyl-CoA dehydrogenase
CE	Cholesterol ester
CHO	Carbohydrate
COX	Cytochrome c oxidase
CPT	Carnitine palmitoyltransferase
CS	Citrate synthase
DAG	Diacylglycerol
DDA	Data dependent acquisition
DHA	Docosahexaenoic acid
ETC	Electron transport chain
EM	Electron microscopy
EMCL	Extramyocellular lipids electron microscopy (EM)
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
FA	Fatty acid
FA-CoA	Fatty acyl CoA
$FADH_2$	Flavin adenine dinucleotide
FAT/CD36	Fatty acid translocase/cluster of differentiation
FABPcyt	Cytosolic-associated fatty acid-binding protein
FABPpm	Plasma membrane-associated fatty acid-binding protein
FATP	Fatty acid transport protein
FFA	Free fatty acid
FFM	Fat-free mass
GCN5	General control non-derepressible 5 histone acyltransferase
GLUT4	Glucose transporter 4
GPAT	Glycerol-3-phosphate acyltransferase
GXT	Graded exercise test
^{1}H-MRS	Proton magnetic resonance spectroscopy
HDL	High-density lipoprotein
HFD	High-fat diet
HR	Heart rate
HSL	Hormone sensitive lipase
IMCL	Intramyocellular lipids
IMTG	Intramuscular triglycerides
IR	Insulin resistance
IRS1	Insulin receptor substrate 1

IS	Insulin sensitivity
LD	Lipid droplet
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LCHAD	Long-chain 3-hydroxyacyl-CoA dehydrogenase
LDL	Low-density lipoprotein
LPC	Lyso-phosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPL	Lipoprotein lipase
MAG	Monoacylglycerol
MAGL	Monoacylglyceride lipase
MCAD	Medium-chain acyl CoA dehydrogenase
MCHAD	Medium-chain 3-hydroxyacyl-CoA dehydrogenase
MIC	Moderate-intensity continuous
MS	Mass spectrometry
mtGPAT	Mitochondrial glycerol-3-phosphate acyl transferase
NADH	Nicotinamide adenine dinucleotide
NFD	Normal-fat diet
OXPHOS	Oxidative phosphorylation
pAMPK	Phosphorylated AMP-kinase
PC	Phosphoatidylcholine
PCA	Principal component analysis
PE	Phosphoatidylethanolamine
PFK	Phosphofructokinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGC-1β	Peroxisome proliferator-activated receptor gamma coactivator 1-beta
PL	Phospholipids
PLIN	Perilipin
QToF	Quadropole time-of-flight
R_a	Rate of appearance
RER	Respiratory exchange ratio
SCHAD	Short-chain 3-hydroxyacyl-CoA dehydrogenase
SIRT1	NAD-dependent deacetylase sirtuin-1
SFA	Saturated fatty acid
SM	Sphingomyelin
SREBP-1c	Sterol regulatory element-binding protein
SS	Subsarcolemmal
T2D	Type 2 diabetes
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
UPLC	Ultra performance liquid chromatography
VLCAD	Very-long chain acyl CoA dehydrogenase
VLCHAD	Very-long chain 3-hydroxyacyl-CoA dehydrogenase
VO_2 peak	Maximal oxygen consumption

Chapter 1: Literature Review

1.1 – General Introduction

High intramyocellular lipid (IMCL) content, regardless of sex, is associated with insulin resistance (IR)[1–7] and type 2 diabetes (T2D)[2,3,6,8]. Paradoxically, endurance trained athletes, who are highly insulin sensitive, also have increased IMCL stores[9–12]. This is known as the athlete's paradox and indicates that total IMCL content is not the only factor that contributes to compromised insulin sensitivity (IS) in a diabetic disease state[10]. IMCL are stored in distinct subpopulations within the intermyofibrillar (IMF) and subsarcolemmal (SS) regions of the myocyte[13]. Recent studies have found that it is not total IMCL content, but increased IMCL size and storage in the SS region of the myocyte that are related to IR[9,14,15]. Women inherently store more IMCL than men[11,16–18], yet are more insulin sensitive[19–23]. This is likely, at least in part, due to the finding that increased IMCL storage in women is due to an increased IMCL number, not size[11,16,24]. Distinct differences in IMCL subcellular storage location between men and women have yet to be fully elucidated and may be another reason why women are more insulin sensitive than men, despite having higher total IMCL content.

Sex-based differences in IMCL turnover may also be related to the lower risk of T2D in women. Basal IMCL content has been associated with IMCL utilization during exercise and this would suggest that women would rely more on IMCL during a given bout of exercise[17,18,25]. In line with this hypothesis is the fact that women have a lower RER during exercise than men, indicative of a higher rate of lipid oxidation[11,16,25–40]. However, whether women rely to a greater extent on IMCL during exercise is contentious with studies finding a greater[17,18,25], similar[16,41] or lesser[42] utilization by women during exercise. Yet, following exercise there is a greater percentage of IMCL touching mitochondria in women, but not men, suggesting that women are primed to use IMCL as a fuel source[16]. Since IMCL storage location is related to T2D[9,43], it is possible that IMCL utilization during exercise may not occur

uniformly across the myocyte. Thus, by examining total IMCL utilization during exercise in men and women sex-differences in region-specific IMCL utilization during exercise are missed.

Sex differences in the fatty acid (FA) composition of stored lipids may also play a role in the lower T2D risk in women. Specifically, IMCL saturation is higher in lipodystrophic women with IR, as compared with age- and sex-matched athletes and healthy controls[44]. Furthermore, IMCL saturation was positively correlated with IR and was a better predictor of IR than IMCL concentration alone[45]. Furthermore, a recent study evaluated the subcellular localisation and composition of intramuscular triacylglycerol (IMTG) in lean individuals, endurance-trained athletes, and obese and T2D individuals using liquid chromatography-tandem mass spectrometry (LC-MS/MS) of fractioned muscle biopsies at rest[46]. As suspected this study found that obese and T2D individuals had a higher total IMTG content, as well as, a higher SS IMCL content when compared to athletes and lean controls[46]. The novelty of this study was that they also found that T2D individuals to have significantly greater total saturated IMTG content and saturated IMTG content within the SS region when compared to all other groups, and this was inversely related to IS. These findings suggest that it is saturated IMTG content that is deleterious to skeletal muscle IS and provides key information to better understand the relationship between saturated IMTG and IR in human skeletal muscle. To the best of my knowledge, no study has examined whether there are sex differences in the FA composition of stored lipid or other muscle lipids, which could provide another key piece of information as to why women have a lower risk of T2D despite having a higher IMCL content. Therefore, the purpose of the research to be conducted in this thesis is to determine the effect of sex on region-specific IMCL storage and utilization during moderate-intensity continuous (MIC) exercise, as well as examine if there are sex differences in the muscle lipidome.

1.2 – Intramyocellular Lipid (IMCL) Characteristics, Storage and Metabolism

1.2.1 – IMCL Characterization & Metabolism

Within skeletal muscle, FAs are stored primarily as triacylglycerols (TAGs) in small lipid droplets (LD)[47], known as IMCL. The neutral lipid core of the LD is surrounded by a phospholipid monolayer[9] coated with surface proteins involved in FA uptake into and release from IMCL[48]. The given processes that explain the growth and reduction of IMCL are not fully understood, yet three given processes exist[49]: i) the joining of two IMCL via pores in the phospholipid monolayer that causes fusion and the exchange of lipids, forming a pore with the monolayer of a different IMCL or the outer monolayer of a bilayer, ii) IMCL enrichment due to exchange of lipids from one IMCL to another, causing a linear increase in IMCL size and phospholipids in the monolayer and a decrease in size of the other IMCL, and iii) IMCL growth by TAGs synthesis caused by excess FAs present in the cell.

IMCL pools were previously thought to be static storage depots for excess FAs[50]; however, it is now known that IMCL pools are highly dynamic[9] and can contribute to oxidative metabolism[10]. That being said, FAs, primarily derived from adipocyte triacylglycerol (TAG) lipolysis and dietary consumption, are transported into skeletal muscle by passive diffusion and FA transporters: fatty acid translocase/cluster of differentiation (FAT/CD36), plasma membrane-associated fatty acid-binding protein (FABPpm), and FA transport proteins (FATP1/FATP4)[51]. FA is then converted to FA-CoA by acyl-CoA synthetase once within the myocyte, where it can either flux towards storage as IMCL or flux towards oxidation by entering into the mitochondria[52,53]. In order to be stored as IMCL, three FAs must be added to a glycerol backbone, a process that is regulated by glycerol-3-phosphate acyl transferase (GPAT)[54]. A family of proteins known as perilipins also play a key role in regulating IMCL metabolism. Perilipin 2 (PLIN2) plays an important role in TAG metabolism by inhibiting lipolysis and promoting TAG synthesis[55]. PLIN3 is involved in IMCL metabolism by promoting TAG breakdown by delivering FAs to mitochondria (PLIN3). PLIN5 facilitates both IMCL synthesis and breakdown depending on whether the muscle is in the rested or stimulated state. In the rested/basal state, PLIN5 inhibits lipolysis

by inhibiting ATGL; however, in the stimulated state, such as during exercise, PLIN5 promotes lipolysis by mediating IMCL-mitochondrial apposition (PLIN5)[55–57]. IMCL breakdown is primarily controlled by adipose triglyceride lipase (ATGL), with hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) also contributing[58,59]. Finally, to undergo β-oxidation, FAs are transported by carnitine palmitoyltransferase 1 (CPT1) and CPT2 into the mitochondria[53]. Once within the mitochondria, the FA can undergo β-oxidation with each cycle yielding 1 acetyl CoA, 1 $FADH_2$ and 1 NADH. The acetyl CoA can then enter the tricarboxylic acid (TCA) cycle whereas the $FADH_2$ and NADH go directly to the electron transport chain. In total, each turn of β-oxidation yields ~12 ATP.

CPT1 is the rate limiting step of fat oxidation, whereas 3-hydroxyacyl-CoA dehydrogenase (β-HAD) is the rate-limiting step of β-oxidation. β-HAD has different isoforms that are specific to fatty acid chain length including short-chain (SCHAD), medium-chain (MCHAD), long-chain (LCHAD) and very-long chain (VLCHAD). Of these, SCHAD is the most widely measured enzyme to determine the rate of β-oxidation in human muscle tissue.[60]. Thus, to understand sex differences in lipid metabolism, it is important to have a measure of CPT1 and β-HAD. Citrate synthase (CS) is the first enzyme in the TCA cycle that catalyzes the reaction between acetyl-CoA and oxaloacetate to produce citrate. Citrate is an important regulatory metabolite as it inhibits phosphofructokinase (PFK) activity, thus decreasing the rate of glycolysis. Thus, high CS activity could be one mechanism by which women rely to a greater extent on lipid than men during exercise. Mitochondrial cytochrome c oxidase subunit IV (COX IV) is the rate-limiting step of the electron transport chain (ETC) and determines the flux of oxidative phosphorylation. It is known that women can initiate aerobic metabolism more effectively than men[61], thus examining sex differences in ETC capacity can provide important information regarding the mechanisms that underpin sex differences in metabolism. An overview of FA and IMCL metabolism is shown in Figure 1.

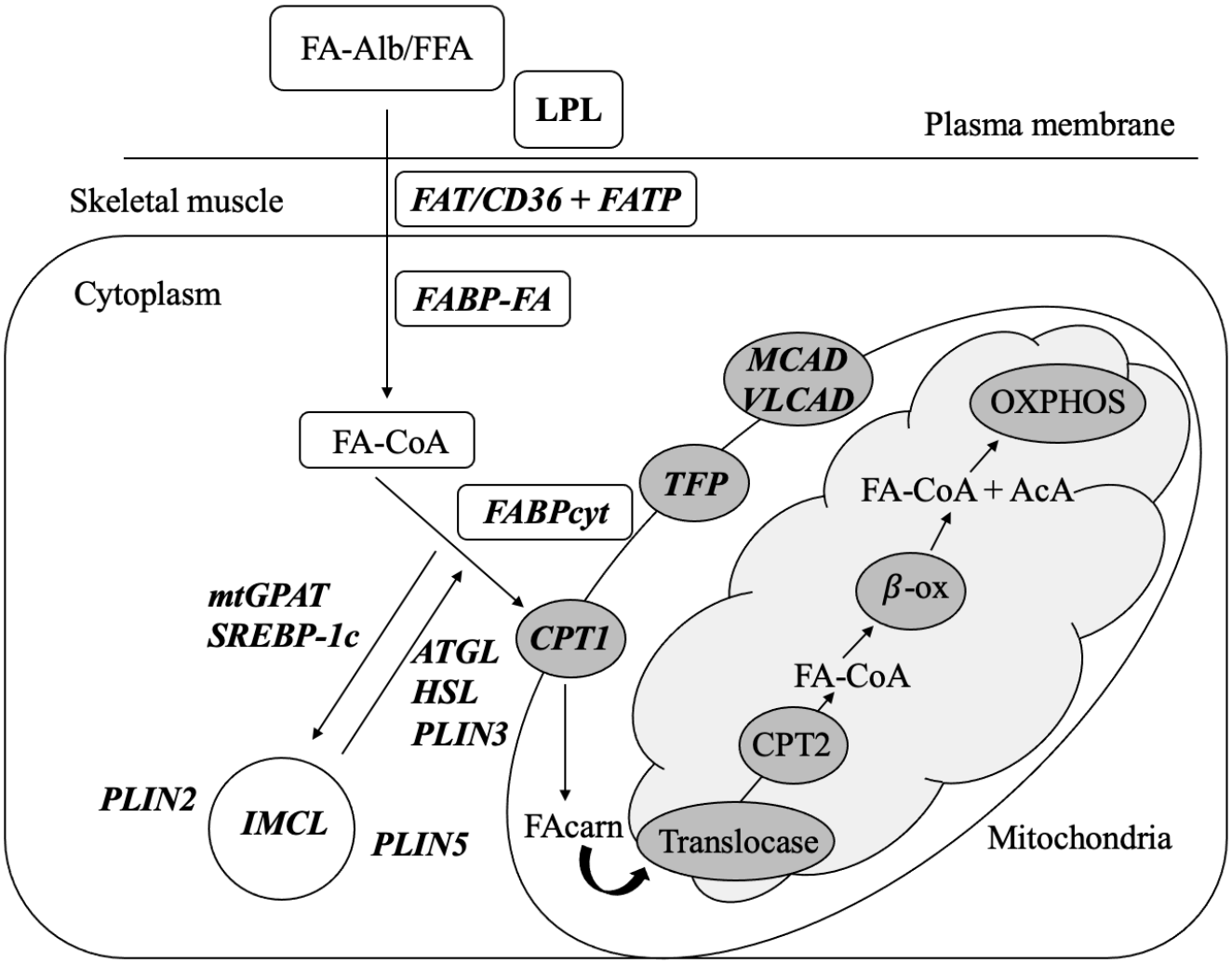

Figure 1. Regulation of IMCL and lipid metabolism, adapted from Beaudry & Devries (2018)[13]. Bolded and italized text represents proteins that are greater in women as compared with men.

1.2.2 – IMCL Storage

Skeletal muscle is comprised of two distinct compartments, the IMF and SS regions[13], where IMCL and mitochondria are stored. IMCL can be found within the IMF region alongside the contractile units in close proximity to mitochondria, or near the muscle cell membrane in the SS region. The given region within skeletal muscle where IMCL and mitochondria are located has proven to have an association with IS[47]. In general, neutral lipid accumulation in the IMF region of type I muscle fibres is a feature of insulin-sensitive, endurance-trained athletes[9–12]. Alternatively, increased IMCL storage within the SS region has been linked to IR in obese individuals[15] and the IMCL stored in the SS region in older

overweight, insulin-resistant individuals are larger than those of older lean individuals[14]. As well, individuals with T2D store fewer, larger IMCL primarily in the SS regions of type II fibres[9]. Taken together, it may be inferred that larger IMCL droplets stored in the SS region, particularly of type II fibres, interferes with insulin signaling and promotes IR due to their instability and the insufficient lipolytic capacity of the muscle[62,63]. On the other hand, athletes store numerous, smaller IMCL primarily in the IMF region of type I fibres, which are markedly more oxidative and insulin sensitive than type II fibres[9]. Furthermore, exercise training studies have shown that exercise is a potent way to decrease IMCL content in the SS region by reducing IMCL number[64–66] and size[65,66], inducing a redistribution of IMCL from the SS to the IMF region of the myocyte[64,66]. IMCL stored within the IMF region allows for the interaction of IMCL with cellular components related to insulin signaling, substrate delivery, TAG synthesis and oxidative degradation of FAs during TAG hydrolysis[47] and is a more favorable storage location for IMCL to meet metabolic demands during exercise. In conclusion, for a given amount of IMCL, it is advantageous to have more numerous, smaller IMCL droplets[11,16,24] as opposed to fewer, larger IMCL droplets as smaller IMCL droplets will have a greater surface area to enable interaction between the IMCL and proteins involved in IMCL turnover[55]. Therefore, it is important to characterize location-specific IMCL storage differences in the IMF and SS regions due to its relationship with lipid metabolism and mitochondrial function.

1.2.3 – IMCL Utilization During Acute Exercise

IMCL are an important fuel source during acute endurance exercise[16–18,25,41,42,67–70], suggesting an increased rate of IMCL turnover within the muscle during exercise[68]. Since IMCL are an important fuel during exercise it is not surprising that, as noted previously, IMCL content is higher in athletes[9–12] and has been found to increase in response to endurance training[11,71–75]. Furthermore, endurance training alters the storage pattern of IMCL within the myocyte, redistributing IMCL from the SS to the IMF

region[64,66]. These data suggest the potent effects of endurance exercise to shift IMCL content to a more favorable region of the muscle to be readily oxidized and used as a fuel source during exercise, since it has been reported that increased IMCL in the SS region is related to IR[9,14,15,47]. Furthermore, within the IMF region, endurance training has been found to shift IMCL storage from the central region of the IMF to the periphery of the IMF[76]. This finding is important as it has also been shown that mitochondrial content is highest in the peripheral IMF region of highly-trained endurance athletes[77], suggesting that endurance training increases proximity of IMCL to mitochondria, where they can be oxidized more effectively during exercise. Indeed, endurance training has been found to increase the percent of IMCL in direct proximity with mitochondria[11,64,66]. Together, these findings provide significant support for the role IMCL play as an energy source during exercise.

During endurance exercise carbohydrates and fats are the two main fuel sources. The given contribution and the source depend on the duration and the intensity of the exercise bout[78–80]. Lipid oxidation contributes optimally during moderate-intensity exercise (40-65% VO_2 peak) contributing between 40-60% of total ATP production[70,80]. Of total fat oxidation, plasma free fatty acids (FFAs)[70,79,80] and IMCL[16] contribute roughly 40-60% and 30-50%, respectively. The pattern of fuel utilization changes over the course of the exercise bout with muscle sources (IMCL) being used predominately at the onset of exercise and plasma sources (FFAs) becoming the main source of fuel utilization with progressing exercise duration[17].

There are numerous methods available to determine IMCL content and utilization, including proton magnetic resonance spectroscopy (^{1}H-MRS), electron microscopy (EM), histochemical staining, and biochemical extraction[81]. The gold-standard method of IMCL quantification is ^{1}H-MRS[82]; however, this method is not commonly used due to its high cost[83]. Historically, histochemical staining and biochemical extraction were the primary methods used to determine IMCL utilization during

exercise[17,18,25,37,65,79–88]; however, methodological issues, particularly contamination of the sample with extramyocellular lipids (EMCL)[94], make the validity of these methods questionable. Indeed, while IMCL are recognized as an important fuel source during exercise, contributing 30-50% of total fat oxidation, several studies using either histochemical or biochemical extraction methods have reported that IMCL content does not change following a bout of endurance exercise in men[17,18,25,88,90,95]. EM has also been used to examine IMCL utilization during exercise[14,16,84,85,96,97] and has been found to correlate well with ^{1}H-MRS[96]. Furthermore, EM allows for examination of subcellular IMCL storage pattern and individual IMCL characteristics[11,16,24,66,67]. Given that IMCL storage pattern and characteristics are related to IR, examining how exercise influences region-specific IMCL utilization and IMCL characteristics can provide rich information to improve our understanding of the IMCL-athlete paradox. As such, EM is an ideal method to use to characterize IMCL utilization during acute exercise.

1.3 – Sex Differences in Metabolism

1.3.1 – Sex-Based Differences in IMCL Storage

Studies consistently show women store more IMCL[11,16–18], yet have a lower risk for IR and T2D[19–23]. EM allows for the determination of IMCL droplet number, size, and subcellular location[11,16,24,66,67], which has revealed how IMCL storage differs between men and women. Using EM, it has been shown that women have a greater number of IMCL, but not IMCL size, when compared with men and this contributes to a greater IMCL area density[11,16,24]. Interestingly, in one study that examined IMCL storage distribution between the sexes, it was found that women stored more IMCL within the SS region of the myocyte as compared with men[24]. Given the relationship between increased SS IMCL content and IR/T2D, this is a surprising finding given that the risk for IR and T2D is lower in women[19–23]. Importantly, the increased SS IMCL content in women was due to a greater number of IMCL, not an increased IMCL size[24]. These findings suggest that it is storage of large IMCL in the SS region, not total

SS IMCL content, that is related to IR; however, this remains to be tested. However, it is important to note that the aforementioned study did not determine whether men and women were equally trained by matching them for VO_2 peak relative to fat-free mass (FFM), which limits the ability to interpret these findings. Despite having greater IMCL content in the SS region of the myocyte, indices of glycemic control and IS including fasting glucose, insulin and homeostatic model assessment of insulin resistance (HOMA-IR) were similar between the sexes. These findings suggest that women can store more IMCL within a given area of skeletal muscle, either in the total area[11,16–18] or within the SS region[24], without it negatively impacting IS. More research comparing IMCL storage characteristics between equally trained men and women is required to determine location-specific IMCL storage patterns.

1.3.2 – Sex-Based Differences in Fuel Utilization During Acute Exercise

It has been very well established that women rely to a greater extent on fat as a fuel source during exercise, as evidenced by a lower RER during endurance exercise[11,16,25–40]. Furthermore, glycerol rate of appearance (R_a), an indicator of whole-body lipolysis, is significantly higher in women than men during MIC exercise[35]. Whether their increased reliance on lipids during exercise is due to an increased reliance on adipose tissue/plasma FFA or IMCL remains controversial. As previously mentioned, women naturally store more IMCL within their muscle fibres than men[11,16–18], which suggests that women are better able to use IMCL as a fuel source during exercise since IMCL use during exercise is proportionate to starting concentration[17,18,25]. Furthermore, as women store IMCL as more numerous, smaller IMCL[11,16,24], they possess a larger surface area and can be more easily oxidized as a fuel source during exercise[55]. Lastly, women have a greater ability to bring IMCL into direct contact with mitochondria following exercise[16], suggesting a greater capacity to shuttle FA from IMCL into the mitochondria.

However, despite this apparent greater capacity for women to utilize IMCL, whether its use during exercise differs between men and women is controversial. Studies have shown that women rely

to a greater[17,18,25], similar[16,41] or lesser[42] extent on IMCL during endurance exercise as compared with men. Furthermore, IMCL use during endurance exercise remains equivocal. Most studies show IMCL being used as an important fuel source during MIC exercise[16–18,25,41,42,67–70], however, others have not[88,90,98,99]. In addition, other studies suggest that only women use IMCL during endurance exercise[17,18,25]. While this is in line with women relying more on lipids to fuel exercise[11,16,25–40], it is unlikely that men do not oxidize any IMCL at all during endurance exercise[17,18,25]. These discrepant findings between the aforementioned studies are likely in part due to methodological differences when quantifying IMCL content as discussed above. For example, three studies that used the biochemical extraction method to determine IMCL utilization during a 90-minute cycling protocol at ~60% VO_2 peak found that women used significantly more IMCL than men during the exercise bout[17,18,25]. However all three studies also found that men did not use IMCL at all during exercise. Given the potential for EMCL contamination with the biochemical extraction method[94], it is possible that IMCL utilization during exercise by men was missed. In fact, in one study showing that men did not utilize IMCL during the exercise bout, roughly 28% of total lipid oxidation was unaccounted for in men[17]. It is likely that this missing 28% was IMCL oxidation but EMCL contamination disguised its contribution to lipolysis. Alternatively, the only study investigating sex differences in IMCL utilization during acute exercise using EM found no effect of sex on net IMCL use during a 90-minute cycling bout at 63% VO_2 peak[16], despite a greater rate of lipid oxidation in women throughout exercise. Only two trials have been conducted using ^{1}H-MRS to investigate sex differences in IMCL utilization and these trials have differential findings. In the one study that involved a 60-minute cycling bout at 65% VO_2 peak both men and women were found to utilize IMCL during exercise, with no difference between the sexes[41]. On the other hand, the other study, which utilized a 3-hour cycling bout at 60-65% VO_2 peak, found that IMCL utilization was greater in men as compared with women[42]. However, it is important to note that the

participants in this trial were not appropriately matched for fitness, with the men having a higher VO_2 peak relative to FFM than women (~72 mL/kg FFM/min vs. ~58 mL/kg FFM/min). Given that training increases the ability to utilize fat as a fuel source, it is not surprising that IMCL oxidation was greater in men than women in this trial. Given that ^{1}H-MRS is the gold-standard method for quantifying IMCL content and EM correlates highly with this method[96], the findings from sex-comparative studies that used these two methods may be more valid than those that used the biochemical extraction method. Thus, collectively, these studies suggest that during exercise bouts of at least 1-hour in duration at a moderate intensity, IMCL utilization does not differ between men and women when they are appropriately matched for fitness and when appropriate methods of IMCL determination are used.

To date the studies that have examined sex differences in IMCL utilization during exercise have used exercise protocols of at least 1-hour in duration. Since muscle sources of fat are used preferentially at the onset of exercise, longer exercise protocols may miss sex differences in IMCL utilization. To our knowledge, the shortest exercise bout investigating sex differences in IMCL utilization used a 60-minute bout of submaximal cycle ergometry at 65% VO_2 peak as determined by ^{1}H-MRS[41]. The authors found no significant sex differences in IMCL utilization during exercise (men: -11.5% vs. women -17.1%); however, these findings are confounded by the small sample size (n = 6 men and 8 women). The authors explained that there was a large degree of variability in the magnitude of IMCL use, potentially contributing to a lack of difference between the sexes, particularly given the small sample size. Importantly, the authors did find that the extent of IMCL utilization was correlated with initial IMCL storage, again suggesting that since women store more IMCL than men[11,16–18], that they may use more IMCL than men during exercise. Most studies conducted examining sex differences in IMCL utilization during exercise have used exercise protocols of 90 minutes in duration[16–18,25]. Interstingly, sex differences in plasma FFA oxidation become apparent at the later stages (i.e., last 30 minutes of a 90

minute bout) of an exercise bout[17], suggesting that the site of increased lipid utilization by women during exercise may change across the exercise bout. Thus, we hypothesize that the site of increased lipid utilization during exercise is temporal with sex differences in IMCL utilization potentially being more apparent earlier on during the exercise bout and sex differences in plasma FFA utilization being seen later as the predominant site of fat oxidation shifts from muscle stores to plasma FFA to support energy demands as exercise duration progresses. However, to date no study has examined sex differences in IMCL utilization during an exercise bout shorter than 60 minutes in duration.

1.3.3 – Sex-Based Differences in Metabolic Enzymes and Proteins

Metabolically women have a greater capacity to utilize fats during exercise as evidenced by a greater expression, content and activity of enzymes and proteins related to fat uptake, storage and oxidation. With respect to fat uptake into skeletal muscle, it has been found that FAT/CD36 protein content is roughly 50% higher in women[100] and sarcolemmal fatty acid transporter (FATP-1) mRNA content is twofold higher in women as well[101]. Furthermore, women have significantly higher skeletal muscle lipoprotein lipase (LPL) mRNA content, but there is no sex differences in LPL activity[100]. Together these findings indicate that women have a greater ability to bring fat into the muscle. Once in the muscle women also have a greater capacity to store, breakdown and oxidize lipid. Indeed, sterol regulatory element-binding protein (SREBP-1c)[102] and mitochondrial glycerol-3-phosphate acyltransferase (mtGPAT) expression[102] and PLIN2 protein content[50] are higher in women, indicative of a greater ability to synthesize IMCL. Furthermore, ATGL activity[103], HSL mRNA expression and protein content[25], and PLIN2, 3 and 5 content[50] have all been found to be higher in women as compared with men, indicative of a greater lipogenic and lipolytic capacity. Interestingly, while it has been consistently shown that women have higher HSL mRNA and protein content than men, HSL activity seems to be higher in men following an acute 90-minute bout of exercise due to a greater increase in

phosphorylated AMP-kinase (pAMPK)[104]. HSL translocates to LDs during muscle contraction or β-adrenergic stimulation in skeletal muscle to facilitate TAG hydrolysis[105]. However, since IMCL breakdown is primarily mediated by ATGL[58,59], it is unclear whether higher HSL activity would have an impact on the rate of IMCL lipolysis. Research also shows that women have a greater capacity to oxidize lipid within skeletal muscle as evidenced by greater CPT1, trifunctional protein β and very long-chain acetyl-CoA dehydrogenase (VLCAD)[102,106,107]. Furthermore, despite no difference in SCHAD, MCAD and VLCAD mRNA expression[60], protein content for MCAD and VLCAD, but not SCHAD, were determined to be roughly 2.5-fold higher in women than in men and both were significantly correlated with a lower RER during a 90-minute cyling bout at 65% VO_2peak[60]. Overall, the cumulative body of evidence demonstrates that women have a greater capacity for fat uptake, IMCL storage and lipolysis, and β-oxidation within skeletal muscle.

Previous research has shown that women are better able to initiate aerobic metabolism than men[61] and rely to a greater extent on lipid[11,16,25–40]; however, whether there are sex differences in TCA and ETC function are not well understood. A recent study investigating sex differences found that mitochondrial respiration, assessed using isolated mitochondria, was higher in women than in men, and was similar to that of trained men[108]; however, as men and women were not properly matched for training status, it is more likely that the training status of the women more closely matched that of the trained men, thus the validity of these findings is questionable. Furthermore, another study investigating sex differences in mitochondrial respiratory function showed similar content of ETC complexes (COX I-III, V) and mitochondrial biogenesis proteins (PGC-1α, PGC-1β, GCN5 and SIRT1) in men and women[109]. In addition, the men and women in this study also had similar COX I-II respiration rates and maximal ADP-stimulated respiration; however, the women had lower ADP sensitivity and lower respiration at physiological concentration of ADP[109]. However, the findings from this study are also

questionable as the men and women were not accurately matched for training status. When men and women are appropriately matched for training status it has been found that resting mRNA content of CS and β-HAD were 27% and 48% higher in women than in men; however, the activity of these enzymes did not differ between the sexes[110]. Interestingly, it has also been found that CS and β-HAD activity increases more robustly following training in men (26% for CS and 39% for β-HAD) when compared to women (3% for CS and 13% for β-HAD), with no sex differences in maximal activities for either enzyme[11]. Given the controversy regarding whether sex influences mitochondrial function, it is important to examine how sex influences the content and activity of these proteins.

1.4 – Combination of FAs in TAGs in Relation to IR

IMCL consist predominantly of stored FAs [palmitate (16:0), stearate (18:0), and oleic acid (18:1)], that are stored as LDs in the cytoplasm near mitochondria and provide energy for skeletal muscle during endurance exercise[41]. The determination of specific lipid species in skeletal muscle has emerged as another potential mechanism to explain the IMCL-athlete paradox[44]. Saturated fatty acids (SFAs) impair insulin signaling in animal and cell culture studies by activating pro-inflammatory signaling pathways and/or providing substrates for the synthesis of lipotoxic intermediates[111–114]. SFAs are also substrates for ceramides, diacylglycerols (DAGs) and phospholipids, which may also impair insulin action, further highlighting that SFAs can impair IS via multiple pathways[115]. Ceramides and DAGs have been found to inhibit insulin signaling action in various cell lines, including 3T3-L1 adipocytes and C2C12 myotubes[116–122]. These studies investigated if saturated FFAs are either antagonists of insulin signaling and/or inducers of ceramide and diacylglycerol (DAG) accumulation. It was determined that long-chain FFAs, such as palmitate (16:0), stearate (18:0), arachidate (20:0), and lignocerate (24:0), were able to effectively induce ceramide and DAG synthesis thereby inhibiting activation of the Akt/protein kinase B (PKB) pathway in C2C12 myotubes. On the other hand, saturated FFAs with shorter carbon chains, such

as laurate (12:0) and myristate (14:0), did not induce ceramide and DAG synthesis or inhibit the Akt/PKB pathway in either cell type. However, in 3T3-L1 adipocytes, although palmitate was a potent inhibitor of the Akt/PKB pathway, it failed to induce ceramide and DAG accumulation or inhibit the insulin pathway[116–122]. Together it seems as though long-chain saturated FFAs are potential antagonists to the insulin signaling pathway in skeletal muscle myotubes, but not adipocyte cells, due to their ability to cause the synthesis of lipotoxic intermediates.

The saturation index of IMCL may also be related to IR. A recent study investigating the saturation index of IMCL between lipodystrophic individuals compared with age and sex matched athletes and healthy controls found that the IMCL compositional saturation index (CH_2:CH_3) was related to IR and was significantly higher in the lipodystrophic individuals when compared to controls and athletes[44]. Athletes and controls proved to have a similar IMCL compositional saturation index adjusted for quantity (CH_2:CH_{3adj}), as a strong inverse relationship between CH_2:CH_{3adj} with VO_2max was found, which indicates that fitness is associated with a relatively lower saturation index of IMCL as fitter individuals had less saturated IMCL for the same absolute quantity of IMCL[44]. The CH_2:CH_{3adj} was also found to have a stronger relationship to IR than IMCL concentration alone. These data suggest that the accumulation of saturated IMCL, not the concentration alone, may be related to the early-onset of skeletal muscle IR. However, this study did not test for palmitic acid content, which is the most abundant saturated fat within muscle TAGs and is thought to activate stress-responsive serine kinases that interfere with insulin signaling and downstream signaling molecules such as insulin receptor substrate 1 (IRS-1) and PKB/Akt[123]. Overall, these signalling impairments may result in impaired insulin-stimulated glucose transporter type 4 (GLUT4) translocation, subsequently causing IR[53].

Since FA composition of stored lipids may be important to the onset of IR, understanding whether sex influences FA composition of stored lipids is important as it may relate to sex differences in

disease risk. Paradoxically, women store more IMCL than men[11,16–18], yet have a lower risk for IR and T2D[19–23]. Furthermore, women also store more IMCL within the SS region of the myocyte as compared with men[24], yet SS IMCL storage has been shown to be related to IR[9,14,15,47]. While the decreased risk of T2D in women may be related to the fact that women store their IMCL as more numerous, smaller IMCL[11,16,24], the decreased risk may also be related to differences in the FA composition of stored lipids, which has not been studied. Furthermore, whether exercise preferentially mobilizes specific FAs from IMCL and whether this differs between men and women is also unknown.

1.5 – Lipidomics

Lipidomic analyses can allow for the identification of the class, subclass, molecular species and regiospecificity of isomers of lipids[124–126]. The precise molecular information available from lipidomic approaches is an important new tool for studying the regulation and dysregulation of lipid metabolism in diseases such as obesity and T2D[127]. As such lipidomics has also been proposed as an important tool in the study of the role of IMCL in exercise and their implication in IR[128]. To date the application of lipidomic techniques have been focused on targeted analyses of DAG and ceramides[129] while the use of discovery oriented, untargeted lipidomics have been limited. The TAG lipidomic composition of subcellular fractions of skeletal muscle from athletes, lean and obese individuals and type 2 diabetics have been profiled at the sum compositional or brutto level of information[46], but medio level lipidomic data confirming the identity of specific fatty acyl chains was not available. A few studies have used lipidomics to examine the relationship between the fatty acid composition of PC, PE and TAG species and IR[44,46]; however, this area of investigation is still in its infancy. Furthermore, no studies have been done comparing sex differences in skeletal muscle TAG fatty acid composition despite significant sex differences being observed in plasma lipidomic profiles[130–136]. Nor has there been any research conducted looking at changes in stored lipid composition following an acute bout of exercise, which

would provide valuable information about what FA species are preferentially oxidized during exercise. However, there are numerous studies using fatty acid analyses to examine skeletal muscle in relation to IR, sex differences, exercise and diet which are summarized in sections 1.5.1-1.5.4.

1.5.1 – Fatty Acid Composition of Lipid Species in Relation to IR

Many aspects of the onset of T2D still remains unclear but it is known that a common pathological feature is hepatic and skeletal muscle IR with impaired insulin signaling[137,138]. Previous studies have shown that IR is associated with the accumulation of lipid species (i.e., DAGs, ceramides, fatty acyl CoAs, TAGs) in hepatocytes and skeletal muscle[138], which can selectively interfere within insulin signaling[139–141]. However, a consistent association between specific lipid species causing IR has yet to be fully elucidated. A recent study investigating the effects of lipotoxic lipids on human cultured hepatocytes (HepG2) in relation to insulin sensitivity using a lipidomics analysis found that a 24-hour palmitate treatment caused increased DAG and TAG content[137]. Upon further analysis the authors found that the accumulated lipids were highly saturated, namely TAG 16:0/16:0/16:0 increased 32-fold and DAG 16:0/16:0 increased ~9-fold, with no significant changes in ceramide content[137]. Furthermore, when hepatocytes were pre-treated with conditioned medium from palmitate loaded macrophages or Kupffer cells that were loaded with either palmitate or oleate divergent effects on insuling signaling were found[142]. Specifically, when exposed to palmitate loaded cells, stress kinase phosphorylation and endoplasmic reticulum stress signaling increased, whereas insulin signaling decreased[142]. Conversely, when exposed to oleate loaded cells, enhanced insulin signaling insulin signaling was found[142]. Together these results suggest that changes in FA composition due to lipotoxic stress, specifically the accumulation of SFAs in cultured human hepatocytes, are involved in the onset of hepatic IR and may be deleterious to insulin sensitivity in other tissues as well.

At the muscle level, the FA profile of stored lipids has recently emerged as another factor that may influence the relationship between IMCL and IR. A recent study investigating the effect of subcellular localization and composition of IMTG found that the accumulation of saturated IMTG in human skeletal muscle was associated with impaired IS. Using LC-MS/MS of fractioned muscle biopsies, it was found that individuals with T2D had a greater total and SS IMTG content as compared to obese individuals, athletes and lean controls[46]. Of particular importance, the authors showed that T2D individuals also had significantly greater abundance of saturated IMTG in the whole muscle, as well as within the SS region when compared to all other groups, and saturated IMTG was inversely correlated to IS. These results suggest that it is saturated IMTG that is harmful to skeletal muscle IS and provides key information to better understand the relationship between IMTG and IR in human skeletal muscle. However, this study was limited to the brutto species level of lipids, which details the lipid class and the total number of carbons and double bonds across the fatty acyl chains[143]. Performing a lipidomics analysis capable of identifying lipids at the medio level would provide key information concerning the FA constituents (fatty acyl/alkyl composition) of complex lipids[143] and would provide valuable insight into how the global lipidome may change following an exercise intervention and how it may differ between men and women. However, the use of lipidomics analyses to determine the effect of sex and exercise on the specific FA composition of stored lipids within skeletal muscle has not been investigated.

1.5.2 – Sex Differences in the Fatty Acid Composition of Skeletal Muscle PC, PE and TAG Species

While little is known about sex differences in skeletal muscle TAG FA composition, there has been work done looking at sex differences in PL composition. A recent study investigating sex differences in the association of PL with components of metabolic syndrome found that men have lower concentrations of sphingomyelin (SM) and higher concentrations of lysophosphatidylcholine (LPC),

which is line with LPCs being associated with higher low-density lipoprotein (LDL) and lower high-density lipoprotein (HDL)[144]. Higher plasma circulating levels of LPC have been mostly associated with lecithin-cholesterol acyltransferase (LCAT) activity[145], which has also been shown to be related to phospholipase A2 (PLA2) activity as well, which mainly associates with LDL and hydrolyses the FA at the *sn*-2 position from PL. The reaction by LPC followed by PLA2 is thought to play an important role in the early onset of cardiovascular diseases[146]. Previous studies have suggested that PLA2 activity is higher in men, which is in line with their finding that LPC concentration was higher in men when compared to women[147].

Furthermore, many recent studies in humans and animal models indicate that sex differences exist in the composition of membrane and plasma PL. Women have higher serum concentrations of DHA (22:6n-3) in PL than men[130–133]. It has also been shown that female rats have higher DHA in both the liver and plasma[134–136], and in erythrocytes[148] when compared to their males counterparts. Arachidonic acid (AA; 20:4n-6) content of rat liver PC is also higher in females, but decreases following ovariectomy, while estrogen supplementation following orchiectomy to males increases hepatic AA[149], suggesting that these observed sex differences are mediated by estrogen. AA and DHA are both substrates for more complex lipids and can be synthesized from their respective FA precursors linoleic (18:2n-6) and α-linolenic (18:3n-3) acids by sequential desaturation and carbon chain elongation reactions[150]. In humans, the conversion of α-linolenic to DHA is very limited in men, but is significantly higher in women[151,152] and remains higher in women when taking an estrogen-based oral contraceptive[152]. Female rats also possess greater hepatic mRNA expression of linoleoyl-CoA desaturase, the rate-limiting enzyme in AA and DHA synthesis when compared to males. It may be inferred that enhanced biosynthesis of α-linolenic to DHA is related to estrogen signaling pathways[153]. This hypothesized relationship is supported by a study investigating pregnancy in rats that found

increased hepatic and plasma DHA levels with pregnancy, with a large increase in plasma DHA between day 15 and 20, which was mainly attributed to an increase in 16:0/DHA PC in both liver and plasma[154]. This was accompanied by increased protein content of Δ6 desaturase and phosphatidylethanolamine methyltransferase (PEMT) at day 20, as well as increased *Pemt* expression and PEMT activity at day 15, suggesting that DHA and 16:0/DHA PC synthesis are upregulated during pregnancy. Thus, there is significant evidence to support that biological sex influences the specificity of FA precursor synthesis, the separation of dietary lipids between lipid classes, and PC and PL biosynthesis in different tissues; however, sex differences in PC, PE and TAG fatty acid composition in skeletal muscle has not been studied yet.

1.5.3 – Influence of Exercise and Diet on the Fatty Acid Composition of Skeletal Muscle PC, PE and TAG Species

Little information is known regarding how exercise and diet influences the FA composition of PC, PE and TAG species. Previous research investigating the effect of chronic exercise and diet in sedentary, trained, normal-fat (NFD) and high-fat (HFD) diet rats using thin layer chromatography found that PL, such as PC and PE, did not differ in total content and that there was a tendency toward decreased TAG content in the training group and increased levels in the HFD group[155]. Using imaging mass spectrometry the authors also quantified three groups of PC and PE species, namely 1) linoleic acid (LA) and mono-unsaturated fatty acid (MUFA)-containing, 2) arachidonic acid (AA)-containing, and docosahexaenoic acid (DHA)-containing and found that the lipid ratios in sedentary and training rats did not change[155]. However, when comparing differences between NFD and HFD rats, the percentages of PC and PE containing AA were significantly higher in HFD rats and LA- and MUFA-containing levels were decreased in HFD rats[155]. These findings suggest that HFD-induced lipid compositional changes occur at the molecular-species level and it may be inferred that the accumulation of AA-containing PC

and PE is representative of a more pathological storage pattern that is associated with obesity, particularly given its role as an inflammation-associated lipid mediator[155]. Thus, future studies should examine the relationship between AA-containing PC and PE and insulin resistance. Furthermore, seeing as chronic exercise did not influence the ratios of AA- or LA-containing PC or PE species, it would be interesting to see how acute exercise influences PC and PE fatty acid composition.

1.5.4 – Current Gaps in the Literature

To date the gaps in the literature are related to the effect of acute exercise and sex on lipid species profiles within human skeletal muscle of young, healthy, untrained individuals. Very few studies to date have attempted to gain insight into the human global lipidome; however, some studies have advanced our understanding of the changes in lipid composition within skeletal muscle by measuring IMCL saturation index[44] and the subcellular localization of total and saturated IMTG and its effects on IS[46]. Nonetheless, it is known that FA remodeling can alter lipid metabolism and that such changes have been associated with a decreased risk for T2D[156–158]. To the best of our knowledge, no studies have investigated sex differences in FA composition of PC, PE and TAG species in young, healthy, untrained individuals following acute exercise. Thus, gaining insight into these metabolic adaptations will advance our knowledge of FA composition and patterns of remodeling in response to acute exercise and may be able to explain differences in insulin sensitivity between men and women.

Chapter 2: Study Rationale, Purpose, Objectives, and Hypotheses

2.1 – Rationale

Lipids stored within human skeletal muscle are known as intramyocellular lipids (IMCL). While IMCL pools were previously thought to be static storage depots for excess fatty acids (FAs)[50], it is now known that IMCL pools are highly dynamic[9] and contribute substantially to oxidative metabolism[10]. However, high IMCL content is associated with insulin resistance (IR)[1–7] and type 2 diabetes (T2D)[2,3,6,8]. Paradoxically, endurance trained athletes, who are highly insulin sensitive, also have higher IMCL stores[9–12], a phenomenon known as the athlete's paradox. The athlete's paradox indicates that it cannot be total IMCL content that is the only factor that contributes to compromised insulin sensitivity (IS) in a diabetic disease state[10]. IMCL are stored in distinct subpopulations within the intermyofibrillar (IMF) and subsarcolemmal (SS) regions of the myocyte[13]. Recent studies have found that it is not total IMCL content, but increased IMCL size, storage of IMCL in the SS region of the myocyte, and saturation of the fatty acids that comprise the IMCL that are related to IR[9,14,15].

Women inherently store more IMCL than men[11,16–18], yet are more insulin sensitive and have a lower risk for T2D[19–23]. This is likely, at least in part, due to the finding that the increased IMCL storage in women is due to an increased IMCL number, not size[11,16,24]. Furthermore, women also have a greater ability to oxidize lipids during endurance exercise as evidenced by a lower RER[11,16,25–40] and greater expression/content/activity of enzymes involved in fat oxidation[16,44,102,105,106], which suggests that women are better able to use IMCL as a fuel source during exercise. This hypothesis is supported by the fact that since women store IMCL as more numerous, smaller IMCL[11,16,24], their IMCL have a larger surface area, suggesting that they can be more easily oxidized as a fuel source during exercise[55]. Distinct differences in IMCL storage location between equally trained men and women have yet to be fully elucidated and may be another reason why women are more insulin sensitive than men, despite having

higher total IMCL content. Interestingly though, in the one study that has compared IMCL storage location between men and women, it was found that women stored more IMCL in the SS region of the myocyte than men[24]. Importantly, the increased storage was due to an increased IMCL number, not an increased IMCL size[24]. This finding is perplexing given that SS IMCL storage has been associated with IR; however, in this study IS was similar between men and women despite women having a higher SS IMCL content. These findings need to be interpreted with caution though as it is unclear whether the men and women were matched for training status. More work is needed to examine sex differences in IMCL storage pattern. Additionally, whether sex influences the site of IMCL oxidation during endurance exercise remains unexamined and may explain why IMCL storage within the SS region in women is not associated with IR[64].

Despite women relying to a greater extent on fat during exercise[11,16,25–40] and having a greater capacity for fat, particularly IMCL, oxidation[16], whether women oxidize more IMCL during exercise is controversial. Studies have found greater[17,18,25], similar[16,41] or lesser[42] IMCL utilization by women as compared with men during endurance exercise. While methodological limitations associated with the quantification of IMCL may, at least in part, explain these discrepant findings, the duration of the exercise bout may also play a role. Muscle fuel sources are used predominately at the onset of exercise and as exercise duration increases there is a shift towards an increased reliance on plasma fuel sources[17]. The studies that have examined sex-differences in IMCL utilization during exercise have used exercise protocols of at least 60 minutes in duration, which if the site of increased lipid utilization by women during exercise is temporal, may be too long to observe sex differences in IMCL utilization. Indeed, while no overall effect of sex on plasma FFA oxidation during exercise was found, women oxidized 47% more FFA during the last 60 minutes of a 90 minute bout of endurance exercise than men[17]. The findings from this study suggest that sex differences in plasma fatty acid (FA) oxidation may only

become apparent during the later stages of exercise when plasma fuel oxidation predominates. Perhaps sex differences in IMCL utilization during exercise may only be observed during shorter bouts of endurance exercise.

IMCL consist predominantly of triacyglycerols (TAGs) that are stored as LDs within skeletal muscle. These TAGs are predominately composed of palmitate (16:0), stearate (18:0) and vaccenic acid (18:1)[41]. The fatty acid composition of TAGs may also play a role in the relationship between IMCL storage and IR. Saturated fatty acids (SFAs) impair insulin signaling in animal and cell culture studies by activating pro-inflammatory signaling pathways and/or providing substrates for the synthesis of lipotoxic intermediates[111–114]. Furthermore, saturated IMCL content is higher in T2D as compared with athletes, lean sedentary and obese individuals, further suggesting that saturated FA storage within IMCL may contribute to the development of IR and T2D[46]. SFAs are also substrates for ceramides, DAGs and PL, which can also impair insulin action, highlighting that SFAs can impair IS via multiple pathways[115]. The relationship between the accumulation of saturated TAGs within skeletal muscle and IR highlights a potential early pathological marker and reveals a potential site for therapeutic action for the prevention of T2D. Whether the estimates of FA content of TAGs differs between men and women remains unknown and may provide further insight into the mechanism by which women store more IMCL than men yet remain more insulin sensitive.

2.2 – Purpose

The purpose of the research conducted in this thesis was to characterize sex differences in IMCL storage and estimates of FA content of lipid species, specifically TAGs, as well as, sex differences in IMCL utilization and fat metabolism during exercise.

2.3 – Objectives

The objectives of the trial were to determine:

a) If IMCL storage location differs between men and women using electron microscopy.
b) Location specific IMCL utilization during an acute bout of MIC exercise and whether it differs between men and women using electron microscopy.
c) If location specific IMCL utilization differs between men and women during an acute bout of MIC exercise using electron microscopy.
d) If sex influences the content of proteins involved in lipid and oxidative metabolism using Western blot analysis.
e) If sex influences the enzyme activity of key TCA cycle and β-oxidation enzymes using enzyme activity assays.
f) Estimates of FA content of PC, PE, and TAG lipid species in both men and women using a non-targeted macrolipidomics approach.
g) How exercise influences the estimates of FA content of PC, PE, and TAG lipid species in both men and women using a non-targeted macrolipidomics approach.

2.4 – Hypotheses

We hypothesized that:

a) Women would store more lipids in the IMF region of the myocyte and would have a greater IMCL area density due to an increased IMCL number, but not size.
b) There would be a greater reliance on IMF as compared with SS lipids during exercise in both men and women.
c) Women would have a greater reliance on IMF and SS IMCL during exercise than men.
d) Women would have a higher protein content of enzymes and proteins related to lipid breakdown (ATGL, PLIN3), and oxidation (CS, CPT1, β-HAD).

e) β-HAD and CS enzyme activity would increase to a greater extent in women as compared with men during exercise.

f) Women would have a higher abundance of total and unsaturated TAG species than men.

g) Exercise would not affect the abundance of PE or PC species in either men or women, but exercise would lower the abundance of TAGs in both sexes, but to a greater extent in women.

2.5 – COVID-19 Pandemic

The pandemic meant that I was unable to perform any wet lab work between mid-March and the start of August. While I was able to complete almost all of the experiments that I had planned to do prior to the pandemic, I was unable to complete the TEM analysis to determine IMCL storage location or utilization during exercise.

Chapter 3: Methods

3.1 – Participants

Young, healthy, recreationally active men (n = 12) and women (n = 13) were recruited to participate in this study. The study was approved by the Research Ethics Committee (ORE #22477) at the University of Waterloo and was performed in agreement with the Tricouncil policy statement: Ethical conduct for Research Involving Humans – TCPS2[159]. Men and women were matched based on maximal O_2 consumption (VO_2 peak) relative to fat-free mass (FFM) ($mL \cdot kg\ FFM^{-1} \cdot min^{-1}$) in order to ensure that they were equally trained[160]. All women were tested in the mid-follicular phase of their menstrual cycle (day 4-9) as determined by the start of menses. Women taking either triphasic/non-hormonal contraceptives were eligible to participate. All procedures, risks and benefits of the study were explained and written consent was obtained before beginning data collection.

3.2 – Study Procedures

On the first visit, a detailed explanation of the experimental procedures were given, and any questions and/or concerns related to the study were addressed. Anthropometric measurements (height and weight) were taken and a graded exercise test (GXT) was completed on a stationary cycle ergometer (Ergoline GmbH, Bitz, DE) to determine cardiorespiratory fitness (VO_2 peak). The VO_2peak test was performed using a Vmax metabolic cart (Vyaire Medical Inc., Illinois, USA) and the data was used to determine the intensity corresponding to 65% of the participants's VO_2peak to be used during the MIC exercise bout. The GXT began with a two minute warm-up at 50 watts (W), after which the workload increased by 1 W every 2 seconds until the participant could no longer maintain 60 revolutions per minute (RPM) for more than 3 seconds or the participant terminated the exercise test. Once the GXT was complete, a cool-down at 50 W was initiated until the participant felt comfortable to end the test. Throughout the GXT, heart rate (HR) was measured by a HR monitor chest strap (Polar Electro, Kempele, FN) and wristwatch

every 30 seconds. Blood lactate levels were measured by a Lactate Scout+ monitor (EKF Diagnostics, Cardiff, Wales) and lactate sensor strips (EKF Diagnostics, Cardiff, UK) every 3 minutes.

On the second visit, a dual-energy X-ray absorptiometry (DXA) scan was done to determine FFM and % body fat. Participants will given instructions on how to complete a 3-day food log and a 7-day physical activity log. The purpose of the 3-day food log was to allow for the comparison of habitual diet between men and women.

On the third visit, each participant underwent a familiarization trial to ensure that they were exercising at the desired intensity of 65% of VO_2 peak from the initial VO_2 peak test. The familiarization was done on the cycle ergometer (Ergoline GmbH, Bitz, DE) and was completed at 65% of VO_2 peak at a given wattage, as calculated in the equation below.

$$W = (\left[\frac{(VO2 - 7}{1.8}\right] \; x \; mass \; (kg)) \; / \; 6$$

Participants then cycled at this calculated wattage for at least 6 minutes and the real-time respiratory data was investigated to ensure that the participant were working at the desired 65% of VO_2 peak workload. If not, the wattage was adjusted to elicit the desired workload, and the participant continued cycling for blocks of 6 minutes until 65% of VO_2 peak is obtained.

On the fourth visit, participants reported to the laboratory after a 12-hour overnight fast to perform the 30-minute bike ride on a cycle ergometer at 65% of their predetermined VO_2 peak with 5-minute breath measurements taken at rest, 0, 12, and 25 minutes during the exercise bout to allow for determination of whole-body substrate utilization. Participants were instructed to refrain from any physical activity at least 72 hours before the MIC exercise trial and dietary intake on the day before the exercise trial was determined to ensure that men and women had similar dietary intakes leading up to the acute exercise bout. Prior to and following exercise, a muscle biopsy was taken from the *vastus lateralis* muscle 10 cm proximal to the knee joint using a sterile custom suction-modified Bergström needle (5

mm diameter)[161,162]. Specifically, two sites ~3 cm apart were chosen for the biopsies, the skin surface cleaned, and the skin and subcantenous tissue was infiltrated with 1% xylocaine (50% with epinephrine and 50% without epinephrine; AstraZeneca, UK) using a 26G needle. After ~2 minutes, further local anesthetic was infiltrated into the fascia using a 22G needle. After ~ 5-10 minutes, a sterile scalpel was used to create an incision through the skin, subcutaneous tissue and fascia at the two predetermined biopsy sites. The site for the post exercise biopsy was immediately covered with protective Tegaderm film (3M, MN, USA). The suction-modified Bergström needle was inserted into the pre-biopsy incision site perpendicular to the *vastus lateralis* muscle until the fascia was reached, then the needle was firmly advanced through the fascia and was then turned to move up the leg running parallel to the muscle fibres. The inner trochar of the needle was then pulled back until the cutting port was completely open, the assistant was instructed to pull suction with the 60 mL syringe. The needle was closed while maintaining its position within the muscle, then it was rotated several times and the suction process repeated to obtain ~75-150 mg of tissue. The needle was removed, the biopsy was inspected for adequacy, and it was then flash frozen in liquid nitrogen. Finally, the incision was closed using tissue adhesive (3M, MN, USA) and covered with a protective Tegaderm film (3M, MN, USA). The muscle biopsy was sectioned into pieces to allow for multiple analyses. One piece was to be used for Western blotting, enzyme activity, and lipidomics analyses. A second piece was fixed in glutaraldehyde to be used for TEM analysis.

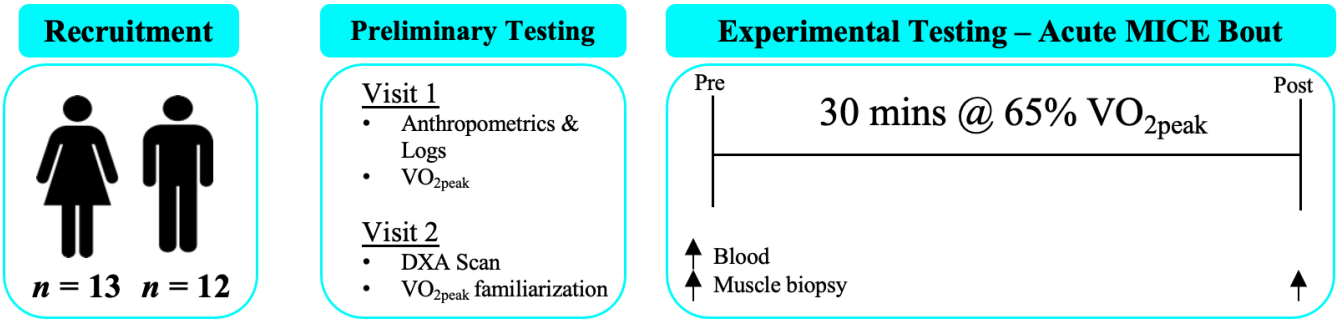

Figure 2. Overall study schematic.

3.3 – Western Blot Analysis

Muscle samples were homogenized in ice cold 25 mM Tris buffer [25 mM Tris, 0.5 % (v/v) Triton X-100, and phosphatase and protease inhibitor tablets (Roche Diagnostics, Laval, CA)]. Muscle samples were added to Eppendorf microtubes (Eppendorf, Hamburg, DE) with Tris buffer and a homogenization bead (Qiagen, Hilden, DE), then homogenized using a TissueLyser II (Qiagen, Hilden, DE) run at 20 cycles/second for 40 seconds or until samples were fully homogeneous. The beads were removed, the samples were centrifuged for 10 minutes at 1500 g and 4°C, the supernatant and myofibrillar pellet were separated and subsequently flash frozen and stored in separate Eppendorf microtubes (Eppendorf, Hamburg, DE) at -80°C. Total protein content of each muscle sample homogenate was determined using the bicinchoninic acid assay (BCA) technique. Thereafter, samples were prepared for Western blotting analysis using Laemmli buffer (0.5 M Tris-HCl, glycerol, 10% SDS, 1% bromophenol blue, β-mercaptoethanol, and ddH_2O) and stored at -80°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was used to investigate proteins involved in lipolysis, lipid oxidation and oxidative metabolism (see Table 1). Equal amounts of protein (10 µg) from each sample were run on 4-15 % Criterion TGX Stain-Free protein gels (BioRad, Hercules, USA) at 200 V for 40 minutes. A protein ladder (Precision Plus Protein Kaleidoscope Standard, BioRad, Hercules, USA) and standard curve pooled from all samples was run on every gel, and all proteins were then transferred to a PVDF membrane. Total protein content was determined post-transfer using a ChemiDoc MP imaging system (BioRad, Hercules, USA). The membranes were blocked in the corresponding blocking solution (see Table 1) for 1 hour. Membranes were then immunoprobed at 4°C for at least 12-hours with the corresponding primary antibody (see Table 1), washed 3 x 5 minutes in 1X TBST, then immunoprobed with the corresponding secondary antibodies: goat anti-rabbit IgG (H+L)-HRP (Biorad #1706515) and goat anti-mouse IgG (H+L)-HRP (Biorad #1706516). Finally, the membranes were

washed again for 3 x 5 minutes in 1X TBST then incubated with Clarity or Clarity Max Western ECL Blotting substrates (BioRad, Hercules, USA) before being imaged on the ChemiDoc MP imaging system (BioRad, Hercules, USA). Bands were quantified using Image Lab (BioRad, Hercules, USA) and protein content was normalized using total protein content obtained from the gel.

Table 1. Western blot analysis antibodies and the corresponding blocking agents, primary (1°), and secondary (2°) antibodies to be used for incubation.

Pathway	Protein	Molecular Weight (kDa)	Antibody Provider	Host	Isotype	Monoclonal or Polyclonal	1°/2° Solutions
Lipolysis	PLIN3	47	Abcam 47638	Rabbit	IgG	Polyclonal	1 : 1000 / 1 : 10000
	ATGL	54	Cayman Chemical 10006409	Rabbit	IgG	Polyclonal	1 : 500 / 1 : 10000
Lipid oxidation	CPT1	88	Abcam 128568	Mouse	IgG2b	Monoclonal	1 : 1000 / 1 : 10000
	β-HAD	34	Abcam 154088	Rabbit	IgG	Polyclonal	1 : 1000 / 1 : 10000
Oxidative metabolism	CS	47	Abcam 96600	Rabbit	IgG	Polyclonal	1 : 2000 / 1 : 15000
	COX (II-V) cocktail	29 (II) 48 (III) 22 (IV) 54 (V)	Abcam 110411	Mouse	IgG	Monoclonal	1 : 1000 / 1 : 10000

3.4 – Enzyme Activity Assays

All samples were homogenized in mannitol buffer [70 mM sucrose, 220 mM mannitol, 10 mM HEPES, 1mM EGTA, supplemented with protease inhibitors (Roche Diagnostics, Laval, CA)]. Buffer was added in a 1:20 dilution then samples were homogenized using a TissueLyser II (Qiagen, Hilden, DE) run at 20 cycles/second for 40 seconds or until samples were fully homogeneous. For the determination of β-HAD activity, 800 uL of buffer (50 mM Tris-Cl, 1 mM EDTA, pH 7.0, 0.2% Triton X-100), along with

10 uL of 5 mM NADH and 25 uL of muscle lysate were added to a cuvette. The cuvette sat for 4 minutes at 37°C, after which 10 µL 5 mM acetoacetyl-CoA was added to initiate the reaction. All samples were analyzed in duplicate on a UV spectrophotometer (SpectraMax Plus 384, San Jose, USA). Absorbance was recorded at 340 nm continuously for 2 minutes and data was expressed as nmol·min^{-1}·mg wet weight tissue^{-1}. The intra-assay coefficient of variation for all samples was less than 10%. For the determination of CS activity, 15 µL of muscle homogenate was added to a cuvette containing: 825 µL 0.1M Tris Buffer (pH 8.0), 100 µL 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 5 mg/10 mL 0.1M Tris Buffer) and 10 µL acetyl CoA (10 mg/1.6 mL 0.1M Tris Buffer), then 50 µL of oxaloacetate (6.1 mg/5 mL 0.1M Tris buffer) was added to initiate the reaction. All samples were analyzed in duplicate using a UV spectrophotometer (SpectraMax Plus 384, San Jose, USA) warmed to 37°. Absorbance was recorded at 412 nm for 2 minutes and the slope between 1 minute and 2 minutes was determined, and data was expressed as nmol·min^{-1}·mg wet weight tissue^{-1}. The intra-assay coefficient of variation for all samples was less than 10%.

3.5 – Lipidomics Analysis

Four men and women matched exactly for VO_2 peak/FFM were selected for the lipidomics analysis. Approximately 5-10 mg of muscle tissue from pre- and post-exercise samples were homogenized as described previously in 3.3 – Western Blot Analysis, however the muscle homogenate was not centrifuged following tissue lysis. A 10 µL aliquot of the muscle homogenate was transferred into cell culture tubes and 3 mL of 2:1 chloroform to methanol solution was added and vortexed for 1 minute at 2500 rpm. Once all samples were vortexed, 500 µL of 0.2 M NaH_2PO_4 buffer in ddH_2O was added and culture tubes were inverted several times before being centrifuged for 5 minutes at 3000 rpm to induce phase change. The organic phase containing suspended lipids was extracted using a pastor pipette and transferred into other cell culture tubes, and 2 mL of chloroform was added. All samples were dried

using nitrogen gas for 30-45 minutes or until all chloroform was evaporated. The remaining lipids were resuspended in 100 µL of 65:35:5 'Solvent X' (isopropanol : acetyl nitrile : H_2O + 0.1% formic acid + 0.1 % chloroform). Samples were spiked with an EquiSPLASHTM LIPIDOMIX® Quantative Mass Spec Internal Standard (Avanti Polar Lipids, AL, USA) internal standard mix to provide 0.25 μg of each of the containing a mixture of 13 different deuterated lipids at relevant physiological concentrations and vortexed for 10 minutes at 2500 rpm. Samples were then transferred into LC vials for subsequent LC-MS/MS analysis at the University of Waterloo Mass Spectrometer Facility (UWMSF) to allow for an untargeted macrolipidomics analysis of the relative abundance of detected phosphatidylcholine (PC), phosphatidylethanolamine (PE) and TAG species as described below.

Tandem MS/MS was completed using a Waters Acquity UPLC system coupled to a Waters Synapt G2Si Quadrupole-Time-of- Flight mass spectrometer (QToF; Waters Corporation, MA, USA) using a novel retention time-based electrospray ionization polarity-switching method to characterize both polar (PC and PE in the negative mode) and non-polar lipids (TAG and CE in the positive mode) within a single UPLC-MS/MS analytical run[163]. UHPLC was completed using the binary multi-step gradient using 60:40 acetonitrile/water (v/v) + 10 mM ammonium formate + 0.1% formic acid, and 90:10 isopropanol/acetonitrile (v/v) + 10 mM ammonium formate + 0.1% formic acid phases with a Waters Acquity UPLC Charged Surface Hybrid (CSH), 1.7 µm x 2.1 mm x 150 mm columns as described previously[163]. The spray voltages were -2.25 kV and +2.25 kV for the negative and positive-ion modes, respectively. High-resolution mode (continuum; approximately 42,000 resolution), scan range *m/z* 100 to 1200, scan time 0.2 s/scan, cone voltage 40 V, cone gas flow 100 L/hr, desolvation gas flow 600 L/hr, nebulizer gas flow 7.0 bar, source temperature 140 ºC, desolvation temperature 400 ºC were used. Spectra were lock mass-corrected using leucine enkephalin (*m/z* 554.2615 for [M-H]- and m/z 556.2771 for [M+H]+). Mass spectra were acquired under DDA conditions for top-5 ions with a ±

1.0 Da isolation window, scan frequency 0.1 s/scan with the collision energies in the transfer cell being ramped from 30 V to 45 V at low mass (*m/z* 100) and 35 V to 60 V at high mass (*m/z* 1200) for negative-ESI, and from 30 V to 50 V at low mass (*m/z* 100) and 40 V to 60 V at high mass (*m/z* 1200) for positive ESI. Peak areas from QToF-based analyses were integrated using MassLynx software (version 4.1; Waters Corporation, Milford, MA, USA) and quantitated by comparison to the area under the curve for the internal standard(s). Progenesis QI software (version 2.3; Nonlinear Dynamics, Waters Corporation, Milford, MA, USA) was used for preliminary automated compound identifications using 25 ppm and 5 ppm precursor and product mass tolerances, respectively, with the ChemSpider[164] and LipidBlast[165] databases with final identifications confirmed by manual inspection.

3.6 – Calculations

Respiratory measures for 5-minute breath collections at rest, 0, 12, and 25 minutes of exercise were collected using a Vmax metabolic cart (Vyaire Medical Inc., Illinois, USA). Whole-body carbohydrate (CHO) and lipid oxidation rates were estimated from expired VO_2 and VCO_2 using the non-protein respiratory quotient, as described by Péronnet and Massicotte[166]:

$$\text{CHO oxidation} = (4.585 \times VCO_2 - 3.226 \times VO_2),$$

$$\text{Lipid oxidation} = (1.695 \times VO_2 - 1.701 \times VCO_2),$$

In addition, substrate oxidation and the relative contributions to total energy expenditure (TEE) were calculated using modified stoichiometric equations, which assumed negligible protein oxidation, as described by Elia and Livesey[167]:

$$\%\,\text{CHO} = (5.045 \times \text{RER} - 3.582) / (0.36 \times \text{RQ} + 1.103)$$

$$\%\,\text{Lipid} = 1 - \%\,\text{CHO},$$

Lipid and CHO oxidation were expressed in grams/minute or as a percentage of TEE.

3.7 – Statistical Analysis

Baseline characteristics and dietary intake differences between men and women were investigated using an unpaired t-test. A two-way mixed model ANOVA with sex (men/women) as the between-participants variable and time (pre-exercise/post-exercise) as the within-participants variable was used for all other experimental measures. Significance was set at $\alpha \leq 0.05$. If significance was reached, a Tukey's HSD post-hoc test was conducted.

Principal component analysis (PCA) was also completed on the lipidomic data using Progenesis QI to determine group clustering for sex and pre/post exercise.

3.8 – Summary of Experimental Measures

Table 2. Summary of experimental measures of the current study.

Enzyme Activity Assays	**Lipidomics**	**Western Blot**
β-HAD and CS activity	Detected PC, PE, TAG species	Lipolysis, lipid oxidation, oxidative metabolism proteins
	Saturation index for TAG species	
	Pre vs. post-exercise differences of lipid species profile in men and women	

Chapter 4: Results

4.1 – Baseline Characteristics

Participant characteristics data is presented in Table 3. There was no significant difference in age between men and women ($p = 0.46$). Men were taller ($p < 0.001$), heavier ($p < 0.05$), had a higher amount of FFM ($p < 0.001$), and lower % body fat ($p < 0.001$) compared to women. Men had a higher VO_2 peak relative to total body weight ($p < 0.001$); however, when VO_2 peak was expressed relative to FFM, no sex differences was found ($p = 0.19$).

Table 3. Participant characteristics for 12 men and 12 women who completed the study.

Participant Characteristics	Men	Women	p-value
Age (years)	22 ± 1	21 ± 1	0.46
Height (cm)	177.9 ± 2.0	161.6 ± 2.0*	< 0.001
Weight (kg)	76.6 ± 2.4	62.6 ± 3.3*	< 0.05
BMI (kg/cm^2)	23.7 ± 0.8	22.9 ± 0.7	0.45
% BF	20.9 ± 1.6	32.9 ± 1.2*	< 0.001
FFM (kg)	55.1 ± 2.2	37.6 ± 2.0*	< 0.001
VO_2 peak (mL/kg/min)	44.4 ± 1.6	35.3 ± 1.4*	< 0.001
VO2 peak FFM (mL/kg FFM/min)	61.3 ± 1.5	58.3 ± 2.0	0.19

Data are expressed as means ± SEM. FFM, fat-free mass. BF, body fat. BMI, body mass index.
*Significantly different from men, $p < 0.05$.

Dietary intake data is presented in Table 4. Men consumed more energy ($p < 0.001$), carbohydrates ($p < 0.01$), total fat ($p = 0.001$), saturated fat ($p = 0.031$), monounsaturated fat ($p = 0.001$), polyunsaturated fat ($p = 0.004$) and protein ($p < 0.001$) when compared to women. However, when expressed relative to % daily energy intake, carbohydrates ($p = 0.47$), total fat ($p = 0.38$), saturated fat ($p = 0.96$), monounsaturated fat ($p = 0.23$), polyunsaturated fat ($p = 0.28$) and protein ($p = 0.26$) consumption were similar between the sexes. Men and women also consumed similar amounts of protein in grams per kilogram of BW per day ($p = 0.11$).

Table 4. Dietary intake for 12 men and 12 women who completed the study.

Fuel Source		Men	Women	p-value
Energy intake (kcal)		2008 ± 119	1507 ± 60*	< 0.001
Carbohydrate intake	Absolute (g)	234.0 ± 15.8	185.6 ± 9.2*	< 0.01
	% Daily kcal	48.0 ± 2.7	50.6 ± 2.3	0.47
Total fat intake	Absolute (g)	79.1 ± 5.6	56.5 ± 3.6*	0.001
	% Daily kcal	36.3 ± 2.1	33.8 ± 1.9	0.38
Saturated fat intake	Absolute (g)	25.0 ± 2.3	18.9 ± 1.5*	0.031
	% Daily kcal	11.2 ± 0.8	11.3 ± 0.8	0.96
Monounsaturated fat intake	Absolute (g)	30.9 ± 2.5	20.9 ± 1.6*	0.001
	% Daily kcal	14.2 ± 1.0	12.6 ± 0.9	0.23
Polyunsaturated fat intake	Absolute (g)	16.1 ± 1.4	11.3 + 0.9*	0.004
	% Daily kcal	7.6 ± 0.7	6.7 ± 0.5	0.28
Protein intake	Absolute (g)	96.4 ± 7.8	65.0 ± 3.4*	< 0.001
	% Daily kcal	19.6 ± 1.4	17.6 ± 1.0	0.26
	g / kg BW / day	1.3 ± 0.1	1.1 ± 0.1	0.11

Data are expressed as means ± SEM. *Significantly different from men, $p < 0.05$.

4.2 – RER During Exercise

There was a significant main effect of time ($p < 0.001$; Figure 3) showing that RER decreased steadily across the exercise bout in both men and women. However, there was no main effect of sex ($p = 0.19$; Figure 3), nor was there a sex by time interaction ($p = 0.48$; Figure 3).

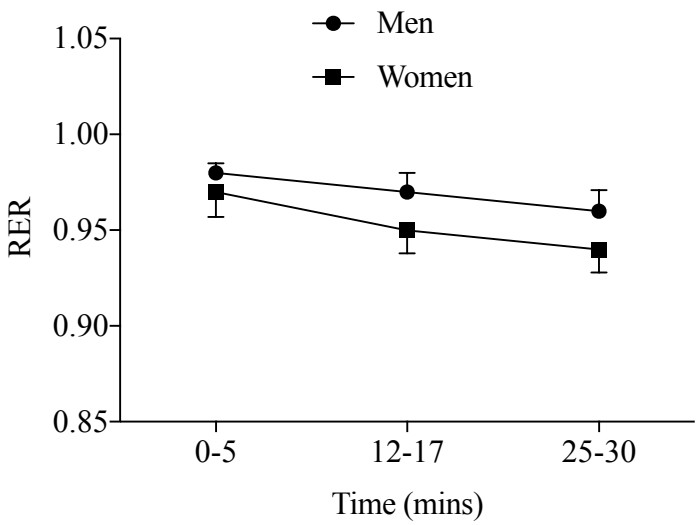

Figure 3. RER during MIC exercise. Data are expressed as means ± SEM.

4.3 – Carbohydrate & Lipid Oxidation During Exercise

There was a significant main effect of time ($p < 0.001$; Figure 4a) showing that the rate of CHO oxidation increased steadily throughout the exercise bout in both men and women. In addition, there was also a significant main effect of sex ($p = 0.007$; Figure 4a), where the rate of CHO oxidation was higher in men during the exercise bout when compared to women. However, no significant interaction effect between sex and time was detected ($p = 0.26$; Figure 4a). When examined across the entire exercise bout, the total grams of CHO oxidized was greater in men than women ($p < 0.001$, Figure 4b). When expressed relative to body weight, the total grams of CHO oxidized during the exercise bout was significantly greater in men when compared to women ($p = 0.007$; Figure 4c). However, when expressed relative to FFM, the total grams of CHO oxidized was similar between men and women during exercise ($p = 0.98$; Figure 4d).

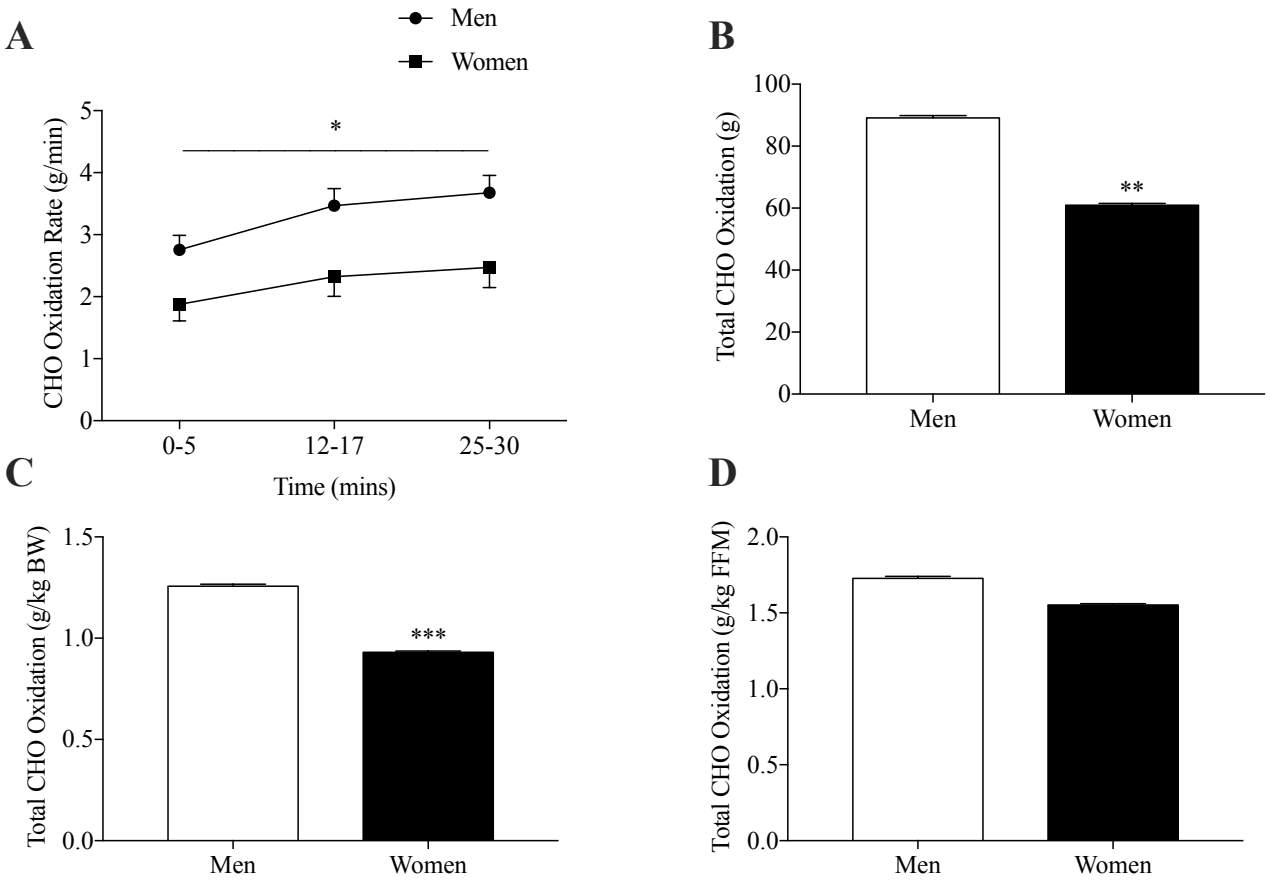

Figure 4. a) Rate of carbohydrate oxidation, b) Total CHO oxidation, c) Total CHO oxidation relative to BW, d) Total CHO oxidation relative to FFM during MIC exercise. Data are expressed as means ± SEM. * Greater than women at all time points, $p < 0.001$. ** Significantly different from men, $p < 0.001$. *** Significantly different from men, $p = 0.007$.

For fat oxidation, there was a significant main effect of time ($p < 0.001$; Figure 5a) showing that the rate of fat oxidation increased steadily during the exercise bout in both men and women. However, there was no main effect for sex ($p = 0.92$; Figure 5a) nor was there an interaction effect between sex and time ($p = 0.85$; Figure 5a). When examined across the entire exercise bout, the total grams of fat oxidized did not differ between men and women ($p = 0.89$, Figure 5b). When expressed relative to body weight, the total grams of fat oxidized during the exercise bout did not differ between men and women ($p = 0.51$; Figure 5c). When expressed relative to FFM, the total grams of fat oxidized was similar between men and women during exercise ($p = 0.15$; Figure 5d).

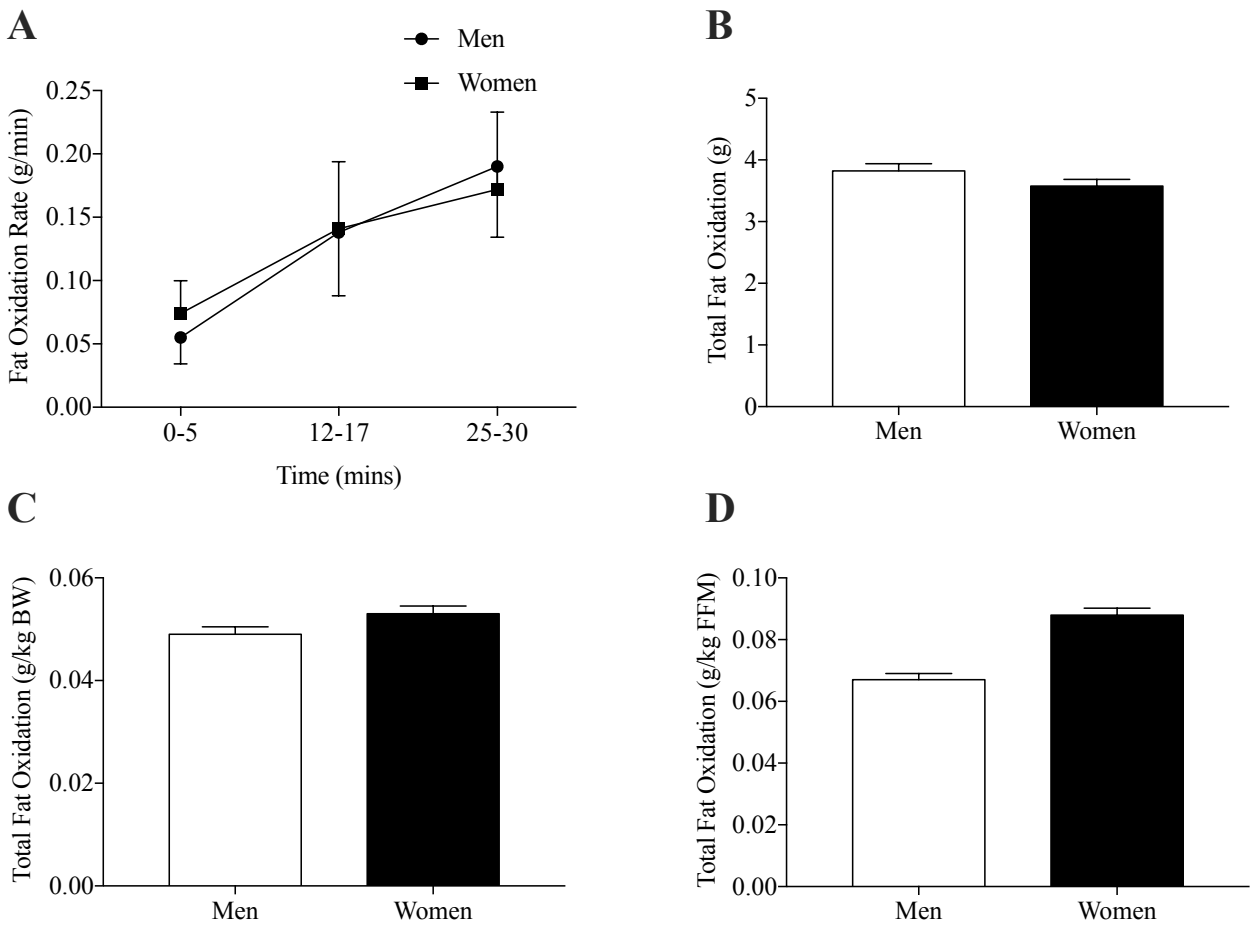

Figure 5. a) Rate of fat oxidation, b) Total fat oxidation, c) Total fat oxidation relative to BW, d) Total fat oxidation relative to FFM during MIC exercise. Data are expressed as means ± SEM.

4.4 – Oxygen Consumption & Total Energy Expenditure During Exercise

Men consumed a greater total amount of oxygen when compared to women during the exercise bout ($p = 0.002$; Figure 6a). When expressed relative to total body weight, total oxygen consumption remained significantly higher in men ($p = 0.024$, Figure 6b); however, when expressed relative to fat-free mass, total oxygen consumption was similar between men and women ($p = 0.61$; Figure 6c).

Total energy expenditure was greater in men than women ($p = 0.002$; Figure 6d) during the acute MIC exercise bout. When expressed relative to total body weight, total energy expenditure remained significantly greater in men ($p = 0.024$, Figure 6e); however, when expressed relative to FFM, total energy expenditure was not different between men and women ($p = 0.61$; Figure 6f).

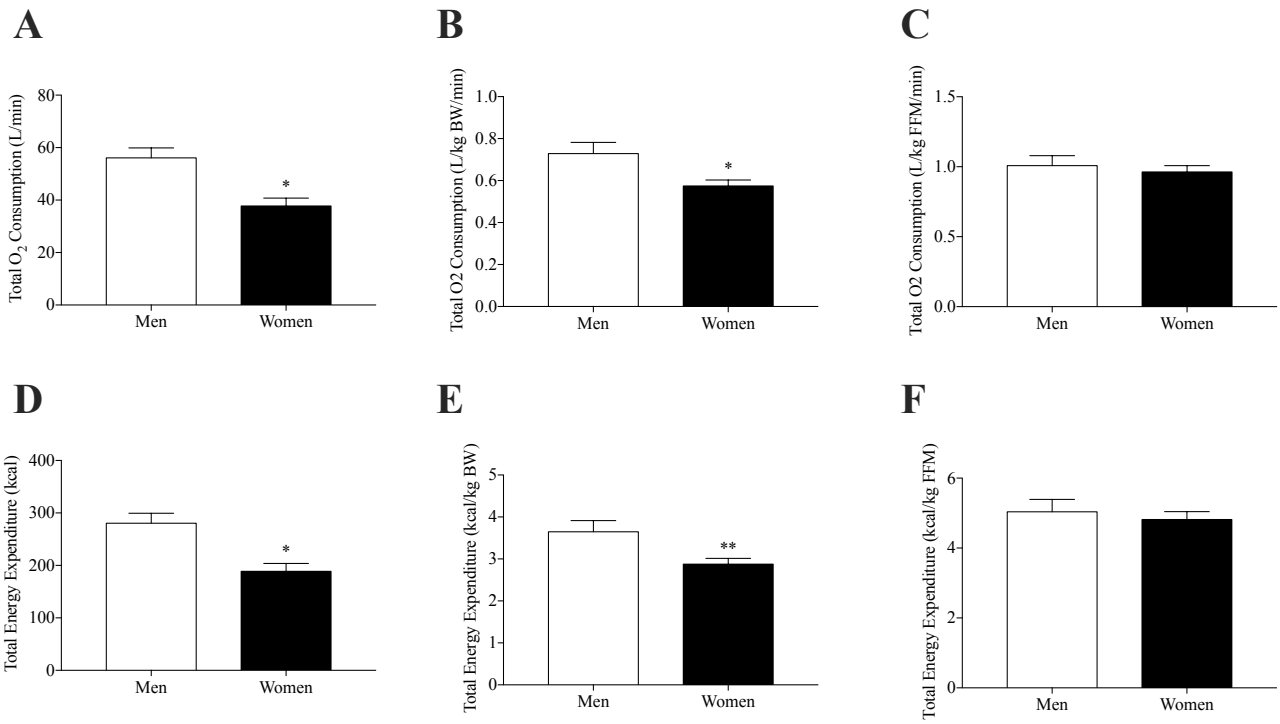

Figure 6. a) Difference in total oxygen consumption, b) Difference in total oxygen consumption relative to body weight, c) Difference in total oxygen consumption relative to FFM, d) Difference in total energy expenditure, e) Difference in total energy expenditure relative to body weight, f) Difference in total energy expenditure relative to FFM during MIC exercise. Data are expressed as means ± SEM.
* Significantly different from men, p = 0.002. ** Significantly different from men, p = 0.024.

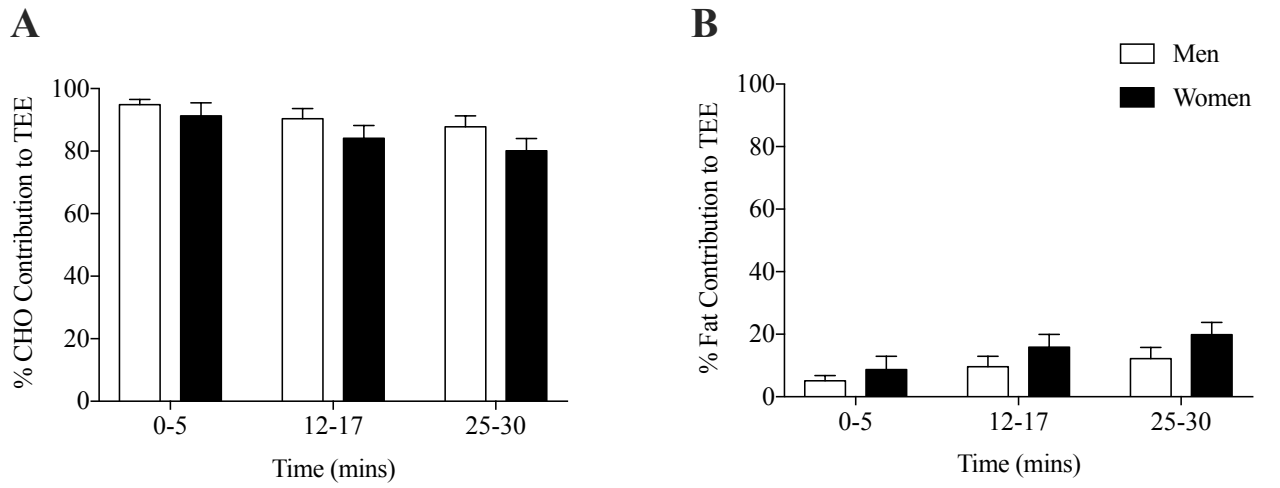

Figure 7. a) Difference in % CHO contribution to TEE, b) Difference in % fat contribution to TEE during MIC exercise. Data are expressed as means ± SEM.

4.5 – Heart Rate, Lactate, and RPE Response During Exercise

HR increased throughout the exercise bout in both men and women ($p < 0.001$; Figure 8a); however, there was no difference in HR response between men and women ($p = 0.44$; Figure 8a), nor was there a sex by time interaction ($p = 0.44$; Figure 8a). Plasma blood lactate concentration increased during exercise ($p < 0.001$, Figure 8b); however, there was no difference between men and women ($p = 0.30$; Figure 8b), nor was there a sex by time interaction ($p = 0.84$; Figure 8b). Sex also had no effect on peak lactate concentration ($p = 0.36$; Figure 8d), blood lactate AUC with baseline values included ($p = 0.18$; Figure 8e) or blood lactate AUC when controlled for resting values ($p = 0.17$; Figure 8f). RPE increased throughout the exercise bout in both men and women ($p < 0.001$; Figure 8c); however, sex had no effect ($p = 0.48$; Figure 8c), nor was there a sex by time interaction ($p = 0.98$; Figure 8c).

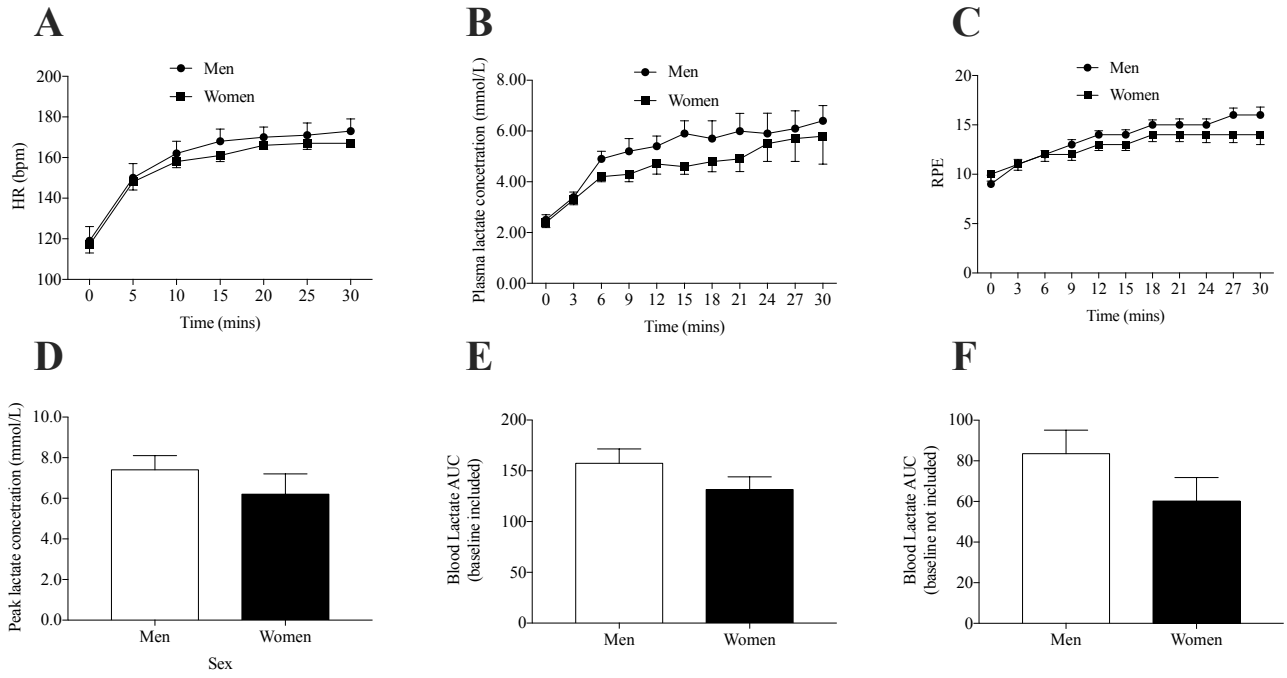

Figure 8. a) Heart rate response, b) Plasma lactate concentration, c) RPE, d) Peak lactate concentration, e) Blood lactate AUC with baseline values included, f) Blood lactate AUC without baseline values included during MIC exercise. Data are expressed as means ± SEM.

4.6 – Western Blot Analysis

4.6.1 – Lipolysis Proteins

There was no significant difference in ATGL ($p = 0.51$) nor PLIN3 ($p = 0.25$) protein content between men and women at rest (Figure 9).

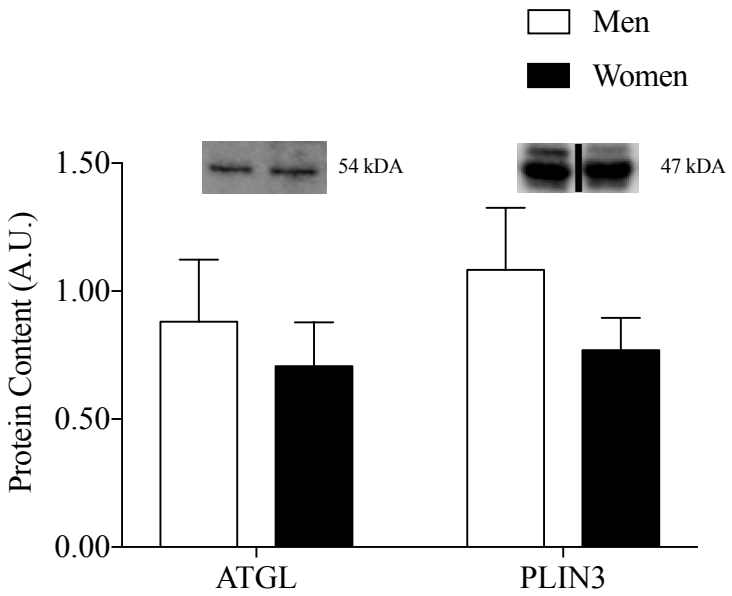

Figure 9. ATGL and PLIN3 protein content between men and women at rest. Data are expressed as means ± SEM. Gels for PLIN3 were originally run as women pre, women post, men pre, men post and had to cut the gel to get the representative blot for PLIN3. The line denotes where the gel was cut.

4.6.2 – Fat Oxidation Proteins

There was no significant difference in β-HAD ($p = 0.80$) or CPT1 ($p = 0.25$) protein content between men and women at rest (Figure 10).

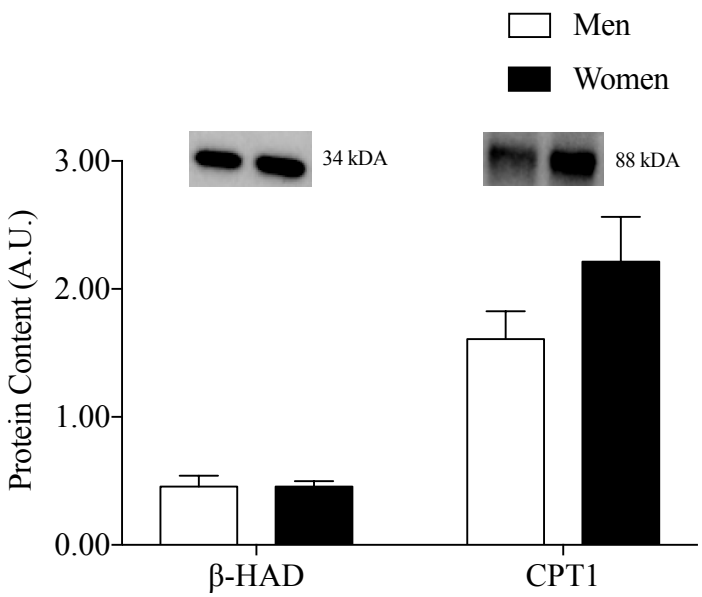

Figure 10. β-HAD and CPT1 protein content between men and women at rest. Data are expressed as means ± SEM.

4.6.3 – Mitochondrial Electron Transport Chain and TCA Cycle Proteins

There was a tendency for COX II to be higher in women (p = 0.08), but no differences in COX III (p = 0.13), COX IV (p = 0.17), COX V (p = 0.94) or CS (p = 0.70) protein content between men and women at rest (Figure 11).

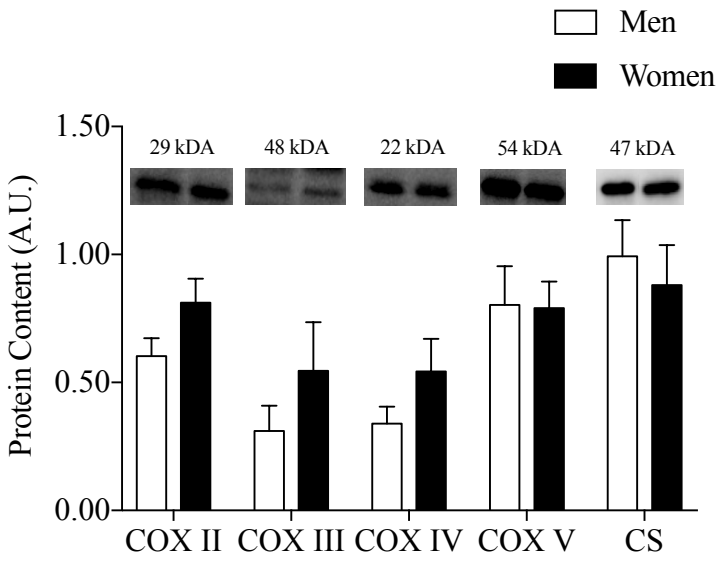

Figure 11. COX II-V and CS protein content between men and women at rest. Data are expressed as means ± SEM.

4.7 – Enzyme Activity Assays

β-HAD enzyme activity (Figure 12a) increased during exercise in both men and women ($p < 0.001$); however there was no sex difference in β-HAD activity ($p = 0.54$) and no sex by time interaction ($p = 0.29$). When expressed relative to baseline, β-HAD activity increased similarly in men (42 ± 12 %) and women (25 ± 7 %) during MIC exercise ($p = 0.19$, Figure 12c). CS enzyme activity (Figure 12b) increased during exercise in both men and women ($p < 0.001$); however, there was no sex difference in CS activity ($p = 0.56$). Interestingly, while the sex by time interaction only tended to be significant ($p = 0.09$), when compared relative to baseline, CS activity increased to a greater extent in women (38 ± 11 %) as compared with men (20 ± 4 %) during exercise ($p = 0.04$, Figure 12d).

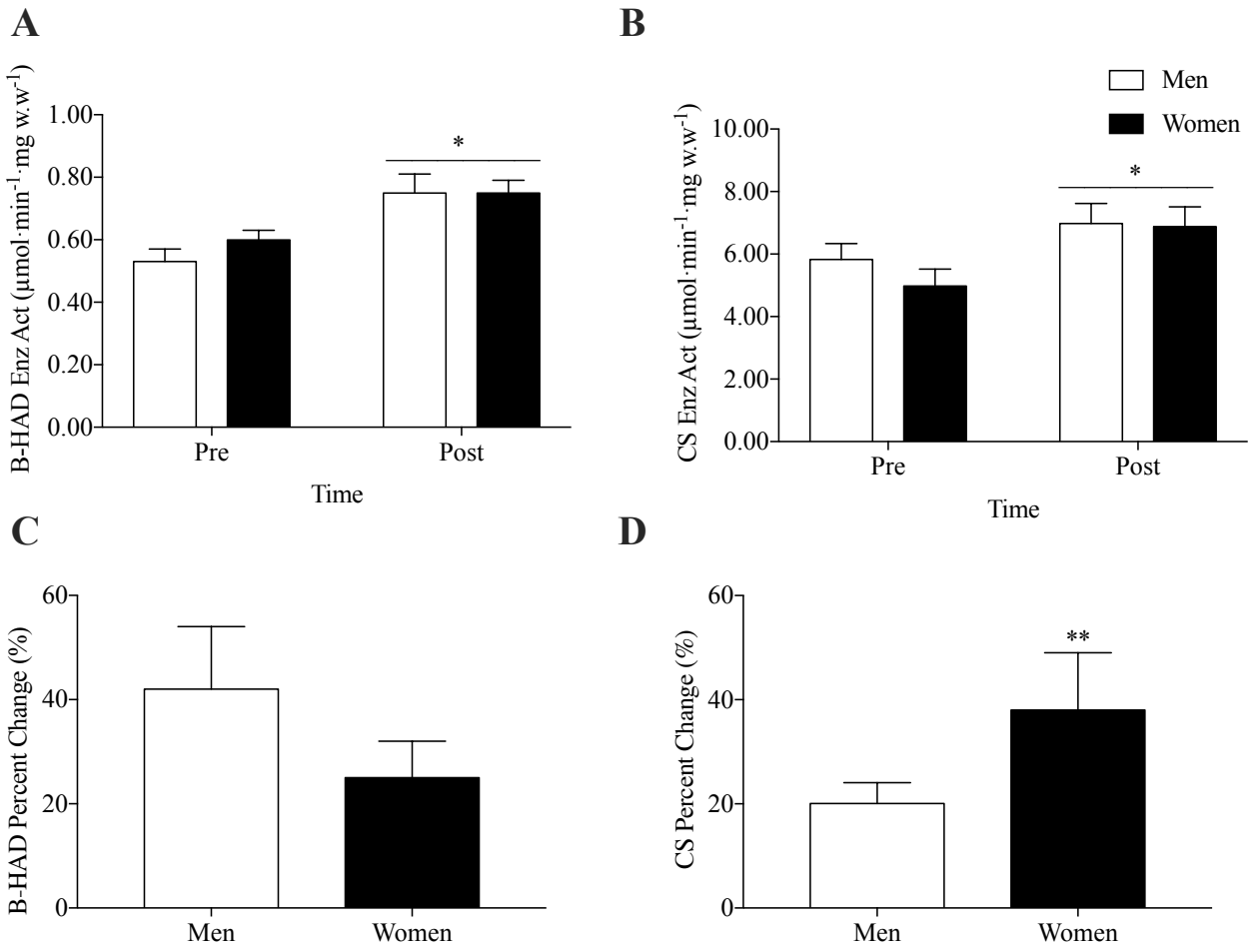

Figure 12. A) β-HAD enzyme activity, B) CS enzyme activity, C) % change of β-HAD enzyme activity, D) % change of CS enzyme activity in men and women prior to and following an acute bout of MIC exercise. Data are expressed as means $\pm$ SEM. * Greater than rest, $p < 0.001$. ** Significantly different from men, $p = 0.04$.

4.8 – Lipidomics Analysis

4.8.1 – PCA Analysis

PCA in the negative-ion mode (Figure 13), showed partial clustering of men versus women with some overlap, while there was no evidence of separation of pre/post exercise. This shows that in the lipids detected by the negative mode which includes FA, PL, and sphingolipids there may be a sex effect but it may be difficult to detect. The PCA in the positive-ion mode (Figure 14) again indicated no clustering of pre/post exercise lipids, but the men and women clustered separately without any overlap. This

indicates that lipids detected in the positive mode which include TAGs and cholesteryl esters had a distinct sex profile.

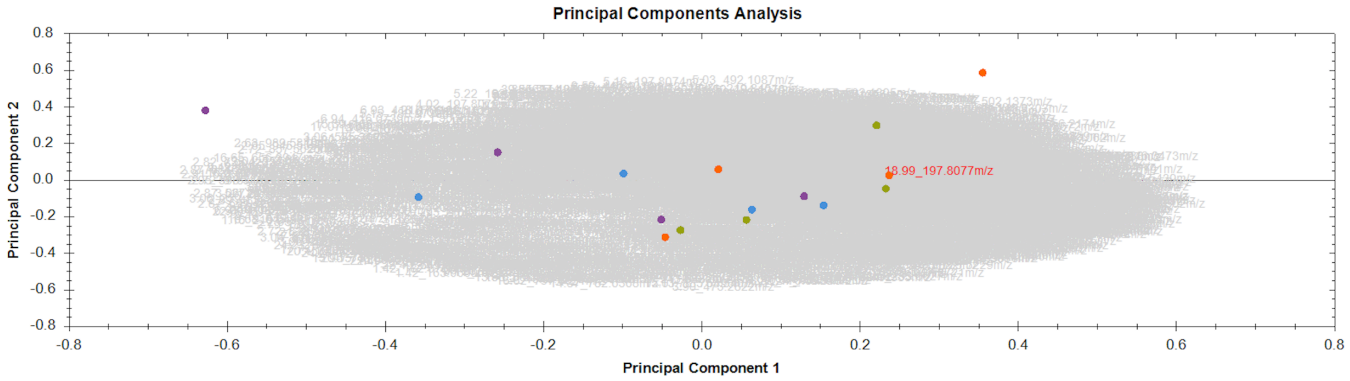

Figure 13. Representative image of the principal component analysis in the negative-ion mode. Men pre, blue. Men post, purple. Women pre, orange. Women post, green.

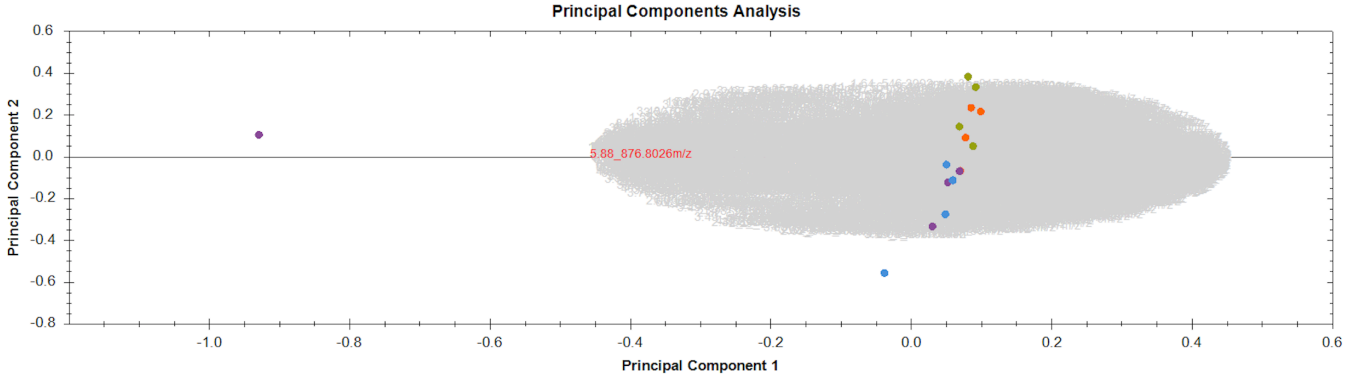

Figure 14. Representative image of the principal component analysis in the positive-ion mode. Men pre, orange. Men post, green. Women pre, blue. Women post, purple.

4.8.2 – Phosphatidylcholine (PC) Identification and Abundance

Six different PC species were identified with a high level of confidence (Table 5). There was no effect of sex for any of the detected PC species ($p \geq 0.32$). Exercise increased the abundance of PC P-16:0_18:2 ($p = 0.046$), but did not affect any other PC species. In addition, there no significant interaction effect between exercise and sex for 5 detected PC species ($p \geq 0.17$). However, a significant interaction effect between sex and time was found for PC 18:0_18:2 ($p = 0.046$), where the relative abundance increased following exercise in men and decreased in women. It is interesting that in women, although not significant, PC 18:1_18:2 also tended to decrease with exercise while those containing 16:0

tended to remain the same or increase slightly. Ad hoc analysis of the sum of 16:0-containing PC species indicated that they tended to be higher in women ($p = 0.098$) and with exercise tended to increase in women, and decrease in men following exercise ($p = 0.074$). There was also no effect of sex ($p = 0.93$), time ($p = 0.63$) or sex by time interaction ($p = 0.80$) on the total abundance of detected PC species. The most abundant PC species was PC 16:0_18:2 in both men and women.

Table 5. Amount of detected PC species between men and women pre and post exercise (nmol/mg tissue).

Detected Lipid	Men PRE	Men POST	Women PRE	Women POST	p-value (S, T, S x T)
PC 16:0_18:1	1.23 ± 0.10	1.32 ± 0.13	1.26 ± 0.10	1.28 ± 0.10	0.98, 0.63, 0.76
PC 16:0_18:2	3.01 ± 0.36	2.92 ± 0.27	3.22 ± 0.19	3.34 ± 0.09	0.32, 0.95, 0.60
PC 16:0_20:4	0.40 ± 0.05	0.37 ± 0.07	0.33 ± 0.03	0.32 ± 0.03	0.37, 0.35, 0.61
PC 18:0_18:2	0.86 ± 0.03	1.04 ± 0.11	0.94 ± 0.05	0.88 ± 0.07	0.69, 0.29, 0.046
PC 18:1_18:2	0.68 ± 0.09	0.65 ± 0.09	0.77 ± 0.05	0.59 ± 0.04	0.47, 0.63, 0.77
PC P-16:0_18:2	1.00 ± 0.15	1.19 ± 0.18	0.88 ± 0.09	0.92 ± 0.12	0.93, 0.046, 0.17
Total abundance	7.18 ± 0.58	7.48 ± 0.57	7.23 ± 0.21	7.33 ± 0.27	0.93, 0.63, 0.80

PRE, pre-exercise. POST, post-exercise. S, sex. T, time. S x T, sex x time. Data are expressed as means ± SEM.

4.8.3 – Phosphatidylethanolamine (PE) Identification and Abundance

Seven different PE species were identified with a high level of confidence (Table 6). There was no effect of sex ($p \geq 0.23$), time ($p \geq 0.11$), nor was there a significant interaction effect between exercise and sex ($p \geq 0.12$) for any of the detected PE species. There was also no effect of sex ($p = 0.52$), time ($p = 0.24$) or sex by time ($p = 0.24$) on the total abundance of detected PE species. The most abundant PE species was PE 18:0_20:4 in both men and women.

Table 6. Amount of detected PE species between men and women pre and post exercise (nmol/mg tissue).

Detected Lipid	Men PRE	Men POST	Women PRE	Women POST	p-value (S, T, S x T)
PE 18:0_18:2	0.55 ± 0.15	0.41 ± 0.09	0.64 ± 0.10	0.62 ± 0.08	0.33, 0.21, 0.38
PE 18:0_20:4	0.95 ± 0.19	0.68 ± 0.06	0.94 ± 0.22	0.85 ± 0.14	0.71, 0.11, 0.34
PE P-16:0_18:2	0.45 ± 0.14	0.29 ± 0.09	0.41 ± 0.10	0.47 ± 0.09	0.63, 0.47, 0.12
PE P-16:0_20:4	0.65 ± 0.08	0.55 ± 0.04	0.56 ± 0.06	0.59 ± 0.03	0.71, 0.11, 0.34
PE P-16:0_22:5	0.88 ± 0.19	0.61 ± 0.14	0.77 ± 0.10	0.87 ± 0.10	0.65, 0.47, 0.14
PE P-18:0_18:2	0.44 ± 0.13	0.31 ± 0.08	0.61 ± 0.14	0.59 ± 0.14	0.23, 0.19, 0.30
PE P-18:0_20:4	0.63 ± 0.13	0.53 ± 0.08	0.69 ± 0.16	0.63 ± 0.10	0.68, 0.19, 0.73
Total abundance	4.56 ± 0.96	3.38 ± 0.47	4.62 ± 0.82	4.62 ± 0.63	0.52, 0.24, 0.24

PRE, pre-exercise. POST, post-exercise. S, sex. T, time. S x T, sex x time. Data are expressed as means ± SEM.

4.8.4 – Triacylglycerol (TAG) Identification and Abundance

Lipidomics analysis allowed for identification and quantification of 15 TAG species with a high level of confidence (Table 7). There was no effect of time ($p \geq 0.25$) nor was there a sex by time interaction ($p \geq 0.20$) for any of the detected TAG species. Women had a higher abundance of TAG 16:0_18:1_18:1, TAG 16:0_18:1_18:2, TAG 18:0_18:1_18:1, TAG 18:0_18:1_18:2, TAG 18:1_18:1_18:2, and TAG 18:1_18:1_20:1 than men ($p \leq 0.02$). Additionally, women tended to have a higher abundance of TAG 16:0_16:1_18:1 than men ($p = 0.06$) Alternatively, men had a higher abundance of TAG 16:0_18:0_18:0 than women ($p = 0.03$). The abundance of all other species did not differ between men and women. There was a main effect of sex whereby women had a greater total abundance of TAG species as compared with men ($p = 0.02$); however, men had a greater abundance of saturated TAG abundance when compared to women ($p = 0.02$). There was no effect of time on the total abundance of TAG species ($p = 0.80$), nor was there a significant interaction effect of sex by time ($p = 0.75$). The most abundant TAG species was TAG 16:0_18:1_18:1 in both men and women.

Table 7. Amount of detected TAG species between men and women pre and post exercise (nmol/mg tissue).

Detected Lipid	Men PRE	Men POST	Women PRE	Women POST	p-value (S, T, S x T)
TAG 14:0_16:0_18:1	0.36 ± 0.08	0.36 ± 0.05	0.54 ± 0.17	0.48 ± 0.07	0.26, 0.72, 0.72
TAG 14:0_16:1_18:1	0.45 ± 0.05	0.65 ± 0.18	0.66 ± 0.22	0.71 ± 0.21	0.57, 0.25, 0.51
TAG 16:0_16:0_18:1	0.66 ± 0.12	0.63 ± 0.08	0.84 ± 0.21	0.75 ± 0.11	0.32, 0.71, 0.87
TAG 16:0_16:1_18:1	0.88 ± 0.18	0.91 ± 0.11	1.83 ± 0.54	1.57 ± 0.28	0.06, 0.72, 0.63
TAG 16:0_18:0_18:0	0.19 ± 0.02**	0.26 ± 0.06‡	0.14 ± 0.03	0.12 ± 0.01	0.03, 0.51, 0.20
TAG 16:0_18:0_18:1	0.43 ± 0.07	0.40 ± 0.03	0.60 ± 0.17	0.60 ± 0.11	0.23, 0.81, 0.81
TAG 16:0_18:1_18:1	1.73 ± 0.41	1.48 ± 0.27	3.56 ± 0.92*	3.09 ± 0.51†	0.01, 0.62, 0.88
TAG 16:0_18:1_18:2	1.47 ± 0.33	1.39 ± 0.24	3.78 ± 1.00*	3.21 ± 0.52†	0.009, 0.64, 0.72
TAG 16:0_18:1_22:5	0.55 ± 0.05	0.59 ± 0.07	0.47 ± 0.04	0.58 ± 0.14	0.64, 0.33, 0.68
TAG 16:0_18:2_18:2	0.52 ± 0.08	0.62 ± 0.08	1.33 ± 0.48	1.29 ± 0.34	0.12, 0.82, 0.61
TAG 18:0_18:1_18:1	0.67 ± 0.07	0.69 ± 0.04	1.10 ± 0.18*	1.17 ± 0.17†	0.02, 0.64, 0.82
TAG 18:0_18:1_18:2	1.16 ± 0.17	1.10 ± 0.14	2.51 ± 0.30*	2.28 ± 0.34†	< 0.001, 0.68, 0.82
TAG 18:1_18:1_18:2	0.99 ± 0.13	0.99 ± 0.12	2.20 ± 0.34*	1.99 ± 0.20†	< 0.001, 0.73, 0.72
TAG 18:1_18:1_20:1	0.23 ± 0.01	0.27 ± 0.03	0.37 ± 0.04*	0.38 ± 0.04†	0.007, 0.52, 0.67
TAG 18:1_18:2_18:2	0.58 ± 0.05	0.70 ± 0.10	0.92 ± 0.19	0.99 ± 0.19	0.16, 0.25, 0.72
Total abundance	10.86 ± 1.83	11.05 ± 0.58	20.84 ± 4.84*	19.22 ± 3.24†	0.02, 0.80, 0.75
Saturation index (%)	33.25 ± 0.28	33.25 ± 0.43	31.10 ± 1.28*	31.01 ± 0.34†	0.02, 0.89, 0.93

PRE, pre-exercise. POST, post-exercise. S, sex. T, time. S x T, sex x time. Data are expressed as means ± SEM. *Significantly different from men PRE, $p \leq 0.02$. †Significantly different from men POST, $p \leq 0.02$. ** Significantly different from women PRE, $p = 0.03$. ‡ Significantly different from women POST, $p = 0.03$.

When individual TAG species were expressed as the percentage of total identified TAG (data not shown) there was a significant main effect of sex indicating differences in the lipidomic profile beyond higher levels of TAG in women. Men had higher % of several specific fatty acids as compared with women. This included TAG 16:0_16:0_18:1 (men pre and post: 6.01 ± 0.31 and 5.67 ± 0.32 % vs women pre and post: 3.96 ± 0.43 and 3.95 ± 0.42 %; $p = 0.006$), TAG 16:0_18:0_18:0 (men pre and post: 1.80 ± 0.23 and 2.44 ± 0.55 % vs women pre and post: 0.71 ± 0.10 and 0.65 ± 0.11 %; $p = 0.001$), TAG 16:0_18:1_22:5 (men pre and post: 5.39 ± 0.82 and 5.42 ± 0.55 % vs women pre and post: 2.48 ± 0.40 and 3.15 ± 0.75 %; $p = 0.005$), and TAG 18:1_18:1_20:1 (men pre and post: 2.28 ± 0.26 and 2.46± 0.07 % vs women pre and post: 1.88 ± 0.22 and 1.97 ± 0.08 %; $p = 0.033$). In contrast women had a higher % of TAG 16:0_18:1_18:2 (men pre and post: 13.16 ± 0.88 and 12.50 ± 1.29 % vs women pre

and post: 17.76 $\pm$ 0.75 and 16.56 $\pm$ 0.90 %; $p = 0.001$). The relative abundance of all other species did not differ between men and women. There was no effect of time ($p \geq 0.16$), nor was there a sex by time interaction ($p \geq 0.16$) for any of the detected TAG species.

Chapter 5: Discussion

5.1 – Overall Summary

The current study examined sex differences in lipid and oxidative metabolism and muscle lipid FA composition in relation to fuel utilization during an acute bout of MIC exercise. We determined that during exercise RER did not differ between men and women, but that men had significantly higher rates of CHO oxidation than women. Furthermore, resting content for proteins involved in lipolysis (ATGL, PLIN3), lipid oxidation (β-HAD, CPT1), and oxidative metabolism (CS, COX II-V) were all similar between men and women. Interestingly, despite there being no differences in CS or β-HAD activity between the sexes, CS activity increased to a greater extent in women during exercise. Our novel lipidomics analysis found no significant effect of sex or exercise on detected PE species. Interestingly, lipidomics analysis revealed that while sex did not influence the abundance of PC species, exercise increased the abundance of PC-P-16:0_18:2 in both men and women and affected the abundance of PC-18:0_18:2 differently in men and women (increased in men, decreased in women). Unsurprisingly, women had a higher detected TAG abundance than men. Interestingly, examination of the type of FAs comprising the identified TAGs revealed that men had a higher saturated TAG abundance than women. All in all, these findings reveal that there are differences in lipid storage and muscle metabolism during a short bout of endurance exercise between men and women.

5.2 – Sex Differences in Whole-body Fuel Utilization During Exercise

Most trials that have been conducted examining sex differences in fuel utilization during exercise have employed longer duration (i.e., 60-120 min) bouts[16–18,25,41,42]. These longer duration studies have consistently shown that men utilize a greater proportion of CHO and a lesser proportion of lipids during exercise[11,28,30–32,35,37,39,40]. In contrast to studies involving longer duration exercise bouts where RER is lower in women during exercise[11,16,25–40], our study found no difference in RER during exercise between

men and women. We used a short acute bout of MIC exercise since muscle fuel sources are the first fuels to be used at the onset of exercise and thus we believed that sex differences in muscle fuel utilization may be more apparent during shorter duration exercise. When examining the findings from longer duration MIC exercise trials it is apparent that sex differences in RER do not become apparent until later in the exercise bout and are similar between men and women at 30 minutes of exercise[17,37]. Thus, it is not surprising that we did not see an effect of sex on RER in the current trial.

Despite no difference in RER, men did have a greater rate of CHO oxidation and a greater total CHO oxidation during exercise, which is in line with previous studies[11,28,30–32,35,37,39,40]. However, it has been well established that women rely to a greater extent on fat stores during exercise[11,16,25–40], which was not confirmed by the current study. These findings are somewhat surprising due to the aforementioned studies, but perhaps the duration of the exercise bout blunted any sex-differences in substrate utilization as fat oxidation increases with increasing exercise duration, and thus perhaps similar to RER, it does not become significantly different between the sexes until later in the exercise bout.

5.3 – Sex Differences in Muscle Metabolism at Rest and During Exercise

5.3.1 – Sex Differences in Lipolysis

In the current trial, we measured ATGL and PLIN3 content as indicators of lipolytic capacity in men and women, finding no sex difference in the content of either protein at rest. A recent study in men only found that PLIN3 content increased following a long duration exercise bout and that the increase was in line with increased whole-body lipid oxidation[168], suggesting a role for PLIN3 in promoting lipid oxidation. If PLIN3 content is related to lipid oxidation rate, then the finding that PLIN3 did not differ between men and women in the current study is in line with the finding that we did not find an effect of sex on lipid oxidation during exercise. However, our finding that PLIN3 did not differ between men and women is in contrast to findings from another study showing that PLIN3 content is higher in women

than men[50]. This discrepancy is hard to reconcile given that in both studies men and women were matched for cardiorespiratory fitness relative to FFM and were of similar training status (though endurance training has not been found to influence PLIN3 protein content[50]) and suggests that further examination to determine the influence of sex on PLIN3 protein content is required.

Similar to PLIN3, we expected that ATGL protein content would be higher in women than men; however, we found that it did not differ between the sexes. Upon reflection, the lack of sex difference in ATGL content is not necessarily surprising as a previous study also found no sex difference in resting ATGL content[109]. However, previous work has shown that ATGL activity is higher in women than men[103], indicating that women have a higher lipolytic capacity. The discrepant findings related to whether content or activity are measured highlight the importance of including measurements of both protein content and enzyme activity where possible to fully understand the effect of sex on muscle metabolism. Unfortunately, we were unable to measure ATGL enzyme activity in the current study. It would have been very interesting to see if women had higher ATGL activity than men during our 30 minute exercise bout. As noted, previous work has shown that ATGL activity is higher in women than men; however, this measurement was conducted at rest[103]. Given that we found no difference in the rate of lipid oxidation during exercise between men and women during the 30 minute exercise bout, it would be interesting to see if sex differences in ATGL activity persisted during exercise. Future work should examine sex differences in ATGL enzyme activity at rest, during shorter exercise bouts (when differences in whole-body fat oxidation may not be apparent) and during longer duration exercise bouts (when differences in whole-body fat oxidation are almost always observed) in order to improve our understanding of how sex influences the regulation of skeletal muscle lipolysis during exercise.

5.3.2 – Sex Differences in Lipid Oxidation

Previous work has shown that women have a higher capacity for β-oxidation, as evidenced by significantly greater protein content for trifunctional protein, MCAD and VLCAD[60]. However, no difference in the expression of SCHAD has been found[60]. In the current trial we found no effect of sex on SCHAD or CPT1 protein content, which is in line with the finding that SCHAD mRNA does not differ between men and women, but discordant from the finding that trifunctional protein, MCAD and VLCAD protein content are higher in women. However, in line with the findings from our trial, a previous trial found that accurately matched men and women have similar enzyme activity for β-HAD at rest[169]. Unfortunately this study did not examine whether β-HAD activity differed between men and women during exercise. However, another study that found no difference in β-HAD activity at rest between men and women, also found no sex difference in β-HAD activity during exercise[110]. However, unlike our trial where β-HAD increased during exercise, they found no change in β-HAD activity in response to exercise. Differences in the length of the intervention (30 vs 90 minutes) and the participants used (untrained and endurance trained vs. recreationally active) could explain these differences, but this is not clear. Taken together these findings suggest that while there are differences in the protein content of some of the β-oxidation enzymes, the activity of β-HAD does not differ between men and women and thus, sex differences in lipid oxidation during exercise are not mediated by β-HAD.

Given that CPT1 is the rate limiting step of β-oxidation, it is unclear what sex differences in the content or activity of other β-oxidation enzymes mean and whether they would actually influence the rate of fat oxidation. Since CPT1 determines the flux of FAs into the mitochondrial matrix and the rate of β-oxidation, sex differences in its activity are more likely to be related to sex differences in fat oxidation during exercise. However, while previous work has determined that women have significantly higher mRNA expression for CPT1 when compared to men, there is no significant sex difference in

CPT1 content or activity[170]. Therefore, it appears as though CPT1 is not regulating the increased rate of lipid oxidation seen in women. However, previous research has highlighted the importance of mitochondrial FAT/CD36 association with CPT1 in regulating lipid oxidation[171–173]. Exercise training has been found to alter the localization of FAT/CD36 and thereby increase its association with CPT1, which was indicative of enhanced lipid oxidation[174]. Thus, future studies should investigate the effect of sex on the interaction between CPT1 and FAT/CD36 during acute exercise to see if it is the interaction between the two that explains the greater rate of fat oxidation in women during exercise.

While we were surprised by the finding that SCHAD and CPT1 protein content did not differ between men and women in the current study, these findings are in line with the fact that we found no effect of sex on rates of lipid oxidation during exercise. However, while breath measurements to assess substrate utilization are useful, it would have been interesting to measure FA oxidation rate using stable isotope tracers and to measure CPT1 enzyme activity (and its relationship with FAT/CD36) as well to better understand the effect of sex on rates of mitochondrial FA influx and oxidation.

5.3.3 – Sex Differences in Mitochondrial Content and Function

In the present study, we measured resting COX II-V and CS content as indicators of mitochondrial content and function in men and women, finding no sex difference in the content of any of these proteins. A previous study investigating the effect of exercise and sex in untrained and endurance-trained men and women determined that resting CS mRNA content was 27% higher in women than men, regardless of training status, but that CS enzyme activity was similar at rest between the sexes[110]. However, in response to 90 minutes of endurance exercise they found that CS activity increased in women only[110]. The authors indicated that the acute increase in CS activity in women was not related to CS mRNA content, suggesting that the translational efficiency of CS may be higher in women than men during exercise. Similar to these findings, our study found that resting CS enzyme content and activity

were both similar between the sexes. However, while not significant when analyzed using an ANOVA, when expressed relative to baseline, CS activity increased to a greater extent in women than men, in line with the findings of previous research[110]. Although we were unable to measure sex differences in fibre type distribution, this beneficial metabolic adaption in women is also supported by a study showing that women have significantly more type I fibres than men[175], which suggests greater mitochondrial content and relative oxidative capacity following a 30-minute bout of MIC exercise. The greater increase in CS activity relative to baseline in women would also suggest that women are better able to flux through the TCA cycle, causing greater citrate production, thereby inhibiting PFK activity, which in turn would slow down the rate of glycolysis and may explain why men relied to a greater extent on CHO as a fuel source during exercise.

A recent study investigating sex differences in mitochondrial respiratory function at rest, specifically complexes I-III and V, found similar similar protein expression of ETC complexes as well as similar maximal respiration rates between men and women[109]. However, this study did not properly match men and women for cardiorespiratory fitness relative to FFM and training status, bringing into question the validity of the findings. Our findings that protein content of resting ETC complexes did not differ between men and women is rather surprising seeing as it has been shown that women have a greater proportion of type I fibres[175] and enhanced relative CS activity during exercise, suggesting that the women in our study should have greater oxidative capacity. Unfortunately, we were unable to measure COX IV enzyme activity, but it would be very interesting to see if COX IV activity differed between men and women at rest or increased differentially during exercise, as was seen with CS activity. This measurement is particularly important as it could explain why no sex difference in COX protein content was found, particularly since we know that protein content is not always indicative of enzyme

activity. Thus, future work should examine sex differences in COX IV activity and mitochondrial respiration at rest and during exercise.

Our finding that the content of proteins related to lipolysis, lipid oxidation and mitochondrial TCA cycle and ETC did not differ between men and women are particularly surprising. While we did not measure fibre type composition, previous work has found that women have a greater proportion of type I fibres than men[175] and this should, at least in theory, be accompanied by a greater oxidative genotype; however, this was not the case in the current trial. Briefly, type I fibres have greater whole-muscle oxidative capacity as evidenced by greater mitochondrial content and greater content of proteins and enzymes related to oxidative metabolism[176]. Possibly differences in oxidative capacity and content and activities of oxidative enzymes are only apparent when there are significant differences in fibre type composition but this is unclear at this time as we did not measure fibre type composition in the current trial. The findings from our trial could mean two things: 1) if fibre type distribution was not different then perhaps sex differences in protein content of proteins involved in oxidative metabolism are only apparent when women have a greater proportion of type I fibres or 2) if women did more higher type I fibres, then it's unclear why this would not be accompanied by a greater oxidative phentotype and could be related to slight differences in the type of training between men and women. High-intensity endurance training is known to enhance mitochondrial content and oxidative capacity in type II fibres[177]. Our participants were recreationally active individuals matched for aerobic capacity relative to FFM; however, it is unclear whether there were differences in the intensity of their habitual activities that would make their type II muscle fibres more oxidative in nature. If our men participated more often in higher intensity activities, their type II muscle fibres may have been more oxidative than those of the women, which could explain the lack of difference in the content or activity of enzymes related to lipolysis, β-oxidation or oxidative metabolism as these were measured in whole homogenate. Indeed, the

only metabolic difference we found was the greater relative increase in CS activity during exercise in women than men. As noted, protein content is not necessarily reflective of enzyme activity and the lack of difference in the content of lipolytic and oxidative proteins between the sexes warrants further investigation where both content and activity of rate-limiting enzymes are measured. Where possible, future work should try to examine the content and activity of these proteins in type I and II muscle fibres separately as it would be interesting to see if fibre type composition could have explained the lack of sex differences between our participants and if other factors irrespective of sex could explain differences in oxidative capacity.

5.4 – Sex Differences in Lipid Species Profiles Prior to and Following Exercise

One of the purposes of this study was to characterize the muscle lipid species profiles of young, healthy, recreationally active men and women prior to and following an acute MIC exercise bout. In fact, we are the first study to compare sex differences in estimates of FA content for PC, PE, and TAG species in human skeletal muscle and this was achieved using a lipidomics analysis utilizing LC-MS. This novel technique allowed us to take a discovery-based approach and we were able to identify 28 different phospholipid and TAGs species with confidence in the highest relative abundance.

PC and PE species are the most abundant PL in mammalian cell membranes[178,179]. We did not find that sex influenced PC or PE abundance. Phospholipids are a key component of the membrane constituents that influence the physical properties of membrane function such as fluidity, permeability, membrane-protein dynamics, and insulin-receptor kinetics[158,180]. Specifically, the FA composition of skeletal muscle PL is closely related to insulin action[181–185] and has also been linked to obesity and IR, with a higher proportion of more saturated FAs linked to adverse outcomes[186]. It has been observed that estrogen can influence the synthesis of PC from PE by upregulating PE methyltransferase[154]. Thus it is somewhat surprising that significant sex differences in individual and/or total abundance of PC or PE

species were not observed. Given the relationship between saturated PE and PC species, a greater saturated PC and PE abundance in men could provide evidence as to why men are at higher risk for T2D than women. The fatty acid composition of PC synthesized *de novo* through the Kennedy pathway or by PC generated from PE methyltransferase can differ[187]. In the current analysis, we found that 16:0 containing PC species tended to be higher in women than men. While this finding was not significant, it will be important to follow up to examine PC synthesis and content in regard to PC 16:0 content and the impact on insulin resistant and type 2 diabetic men and women to examine the relationship between saturated PC and IR. Furthermore, monitoring how the saturation of PC and PE species changes over time in men and women would provide valuable information related to T2D risk.

Interestingly, we found that the abundance of PC 18:0_18:2 increased in men and decreased in women following exercise. Furthermore, while not significant, we found that 16:0 containing PC tended to be higher in women and tended to increase in women, but decreased in men following exercise. Previous studies have shown that skeletal muscle phospholipids undergo remodeling in response to exercise[156,188]; however, this divergent response between men and women is surprising and it is unclear as to why this would change differently during exercise in men and women. It could be speculated that preferential oxidation of FAs could modify the FA composition of stored fat within the muscle, and may subsequently affect muscle membrane phospholipid FA composition. While our lipidomics analysis was limited to the medio level, the observations of a possible sex difference involving 16:0 on PL suggests that these differences involve the *sn*-1 position of the phospholipid[189] therefore analyses capable of regioisomeric discriminations should be considered in the future to examine sex differences. For example, the activity of PLA2, which is responsible for the hydrolysis of glycerophospholipids at the *sn*-2 position and results in the formation of LPC, is higher in men and is in line with previous findings showing higher LPC concentrations in men when compared to women as well[147]. Thus, it may inferred

that since the saturated 16:0 fatty acids of PC species in the *sn*-1 position increased following exercise in women, that they relied to a greater extent on *de novo* PC synthesis via the Kennedy pathway[187,190].

Unsurprisingly we found that TAG abundance was greater in women than men. Notably, saturated TAG storage was greater in men when compared to women. These differences in TAG lipid composition occurred despite similar relative intakes of total, saturated and unsaturated FAs. Recent trials have found that individuals with T2D have a higher IMCL saturation index and that IMCL saturation index is associated with IR[44,46]. To the best of our knowledge, no study has examined the effect of sex and exercise on estimates of FA lipid composition or saturation index. Our results showing that the greater TAG content in women is comprised of a greater amount of unsaturated lipid species provides exciting new data to explain how women store more TAGs without being at a greater risk for T2D[19–23]. These findings pair well with previous work that has shown that while women store more IMCL[11,16–18], they are not at a greater risk for T2D as the greater IMCL content is due to a greater IMCL number, not a greater IMCL size[24].

An inherent limitation of our analysis is that there could have been other lipid species identified within the muscle samples, however they were not identified due to the top 5 DDA screening method and untargeted approach. In addition, our analysis inhibited the identification of DAG species, due to the absence of a permanent charge on the molecule and their relatively low abundance under normal biological conditions[191]. Briefly, DAG species are relatively polar and their retention time results in them being identified simultaneously during the negative-ion mode with phospholipids species before the instrument switches to the positive-ion mode. Resting intramyocellular DAG and ceramide content and estimates of FA content have been found to be similar between men and women[103]. Of particular importance, when the authors looked into the combination of FAs of identified lipid species, they found a significantly higher amount of oleic acid (18:1)-containing DAG species and saturated ceramide

species in obese vs. non-obese participants, suggesting that DAG and ceramides containing these fatty acids may be related to dysregulated insulin signaling; however, more work is needed. Future studies should utilize a targeted positive-ion mode analytical technique to specifically identify DAG species since they are relative low abundant lipids and may provide key information to better understand sex differences in estimates of lipid species profiles within skeletal muscle.

5.5 – Strengths & Limitations

The novelty of this study is that we used a shorter exercise protocol, as compared to longer duration acute endurance exercise protocols that have been typically conducted, to examine whether sex differences in metabolism are apparent early on during an exercise bout. Since muscle fuel utilization predominates at the start of exercise, we felt that we may be best able to detect differences in IMCL utilization and lipid metabolism using a shorter exercise bout. Unfortunately due to COVID-19, we were unable to determine whether sex influenced IMCL utilization during the exercise bout; however, this work is ongoing. The strengths of this trial include that men and women were matched properly based on aerobic capacity since endurance training is known to alter substrate utilization during exercise[28,35,72,192–194]. Secondly, women were tested in the midfollicular phase of their menstrual cycle since it is know that estrogen can strongly influence fuel utilization, specifically lipid utilization, during exercise[20,37,60,92,106,160,195–198] and thus by controlling for menstrual phase, we can minimize variability in the data. Thirdly, all participants underwent a familiarization session to ensure that they were exercising at the desired intensity of 65% of VO_2 peak determined from the VO_2 peak test. Finally, both men and women provided food logs to allow for the comparison of habitual dietary intake and when expressed relative to % daily kcal macronutrient consumption were similar between the sexes. Furthermore, given our lipidomic approach, it was also important to compare the intake of saturated, monounsaturated and polyunsaturated fats between men and women as the type of dietary fat consumed could influence the

FA storage pattern within PE, PC and TAG species. Importantly we show that men and women had similar intakes of saturated, monounsaturated and polyunsaturated FAs, and thus differences in PC, PE and TAGs lipid species profile are likely the result of differences between men and women, not differences in diet.

However, our study is not without limitations. The main limitation of this study is that measures of IMCL content and utilization using TEM were not obtained due to the COVID-19 pandemic. Women have consistently shown that they store more IMCL than men[11,16–18], yet are more insulin sensitive[19–23]. This is most likely due to the finding that increased IMCL storage in women is due to an increased IMCL number, not size[11,16,24]. Distinct differences in IMCL storage location between men and women have yet to be fully elucidated and may be another reason why women are more insulin sensitive than men, despite having higher total IMCL content. Sex differences in IMCL utilization may be more apparent earlier on during the exercise bout with sex differences in plasma FFA utilization being seen later as the specific site of utilization for preferential fat oxidation shifts from muscle stores to plasma FFA. Previous studies have found greater[17,18,25], similar[16,41] or lesser[42] IMCL utilization by women as compared with men during endurance exercise. Thus, characterization of sex differences in IMCL content, storage location and utilization, specifically during a 30-minute acute bout of MIC exercise, has yet to be done and will hopefully provide key information to explain the current controversial results surrounding IMCL utilization. Distinct differences in IMCL storage location between equally trained men and women have yet to be fully elucidated and may explain why women are more insulin sensitive than men, despite having higher total IMCL content. Finally, the site-specific IMCL content and utilization patterns during an acute 30-minute bout of MIC exercise will hopefully clarify the discrepancies in the literature and will strongly compliment the data of the present study.

5.6 – Conclusions

In conclusion, during an acute bout of MIC exercise, whole-body lipid oxidation was similar between the sexes, but men had significantly higher rates of CHO oxidation. This difference in fuel utilization occurred despite no differences in the content of proteins related to lipolysis, lipid oxidation or mitochondrial function. However, CS activity did increase to a greater extent in women than men, which given the role of citrate to inhibit glycolysis, fits with our findings. While, due to COVID-19, we were unable to determine the effect of sex on IMCL storage localization and site of subcellular utilization during exercise, we are the first study to investigate the effect of sex and acute exercise on the lipid species profile of human skeletal muscle. The significantly greater abundance of unsaturated TAG species in skeletal muscle of women improves our understanding of how women can store more IMCL than men and yet be more insulin sensitive. Our results suggest that a short bout of MIC exercise may not be sufficient to detect robust sex differences in fuel metabolism between men and women; however, it is sufficient to induce a greater increase in CS activity in women. Furthermore, sex differences in the lipid species profile of TAG species may relate to differences in the risk for T2D between men and women and require further examination. All in all, these findings lay the foundation for future studies to investigate sex differences in skeletal muscle metabolic properties in response to a relatively short exercise effort, and provides key information for enzymes and proteins related to lipolysis, fat oxidation and mitochondrial respiratory capacity, as well as the first study to quantify the difference in estimates of FA content of specific lipid species of men and women prior to and following exercise.

5.7 – Future Directions

In order to understand the effects of a short acute bout of MIC exercise, more research is needed. To the best of our knowledge, we our the first study to characterize sex differences during a moderate-intensity continuous exercise bout less than 60 minutes in duration. Future studies should investigate these

differences in endurance trained individuals as a means of comparison to understand if the lack of significance in most of our results is due to the study population and/or the duration of exercise. Perhaps performing the exercise bout at a higher relative intensity would be needed to show any sex differences during a relatively short exercise bout. Indeed, during a single 30s all out sprint on a cycle ergometer, it has been found that glycogen utilization in type I muscle fibres is 50% less in women than men, with no difference in type II muscle fibre glycogen utilization[199]. Given the orderly recruitment of muscle fibres based on task intensity, these findings suggest that there is the potential to detect differences in muscle glycogen utilization during moderate intensity continuous exercise as type I fibres are the main fibres recruited to generate force during this type of exercise. Given that men relied to a greater extent on carbohydrates during the exercise bout, it is possible that they used more muscle glycogen. Future work should investigate if muscle glycogen utilization differs during short bouts of moderate intensity endurance exercise.

Seeing as estrogen is known to impact substrate utilization, specifically lipid oxidation during exercise[20,37,60,92,106,160,195–198], it would be interesting to compare women in the follicular phase vs. the luteal phase, and to men to see if differences in fuel utilization during short duration exercise bouts are more prevalent in the luteal phase when estrogen is high. Additionally, supplementing men with estrogen to elucidate the role of estrogen mediating fuel utilization during a shorter exercise bout would also provide key information as to whether estrogen mediates differences in fuel utilization during short duration bouts of MIC exercise.

Endurance training robustly alters substrate utilization[28,35,72,192–194], alongside improvements in endurance capacity and mitochondrial content and function[11,169,200]. The effects of endurance training have also proven beneficial for the redistribution of IMCL from the SS to the IMF region[64,66], increasing IMCL content[11,71–75], and enhancing IMCL-mitochondrial juxtaposition[11,64,66]. Furthermore, endurance

training induces improvements in skeletal muscle CHO (PDHE1α) and lipid oxidation (β-HAD maximal activity)[201]. Thus, it may be advantageous to investigate sex differences in these proteins (ATGL, β-HAD, CPT1, PLIN3) prior to and following an endurance training program to better understand the cellular adaptations of skeletal muscle given their beneficial implications in both lipid and IMCL metabolism. Future studies should also investigate differences in trained men and women to better understand the acute effects of exercise on β-HAD and CS activity in trained participants, seeing as exercise training is capable of robustly influencing key enzymes and proteins involved in mitochondrial respiration. It would be beneficial to investigate these differences using different exercise modalities as well, such as high-intensity interval training and low-load, high-repetition resistance training, as most endurance training trials involve traditional MIC exercise on a cycle ergometer.

As noted, the goal of our study was to characterize sex differences during a 30-minute exercise bout in this specific population, but it would beneficial to conduct a longer duration exercise bout with biopsies taken throughout the exercise bout to capture differences over time to better understand the magnitude of change for various markers of substrate utilization, lipid species profiles, and enzymes and proteins involved in mitochondrial respiration and overall lipid metabolism between men and women. Many gaps in the literature concerning sex-based differences in skeletal muscle metabolic properties in response to an acute bout of MIC exercise remain; however, the present study provides exciting data and direction for future studies.

Chapter 6: Significance of Research

Metabolically, women have a distinct advantage over men by having an enhanced capacity to utilize lipids as a fuel source during exercise. Nevertheless, the normal physiological adaptations to a short, acute bout of MIC exercise remains undefined in a young, healthy population. Undoubtedly, endurance training is known to alter substrate utilization during exercise[28,35,72,192–194] and has been shown to contribute to many beneficial adaptations, such as a greater content, activity and expression of proteins, enzymes and genes related to lipid oxidation and oxidative metabolism, respectively. In the present study, we sought to characterize the influence of sex and acute exercise on specific metabolic adaptations pertaining to whole-body substrate utilization and the molecular mechanisms associated with lipid metabolism. The novel aspect of our study is that we wanted to characterize the effect of sex during a 30-minute bout of exercise, instead of the longer and more traditional MIC exercise bouts researched previously, as it is more representative of the duration of exercise performed by most people. Thus, our study bridges the gap between clinical populations and research examining trained athletes by identifying the normal physiological responses to acute exercise and makes our findings more appropriate to the general population of untrained, healthy individuals. While there are no reported sex differences that have been reported with respect to improvements in cardiorespiratory fitness, mitochondrial content or muscle metabolism induced by training, there is significant data to suggest that exercise training improves insulin sensitivity in men, but not women[64,66,202–204], a phenomenon hypothesized to be mediated by differences in metabolism or signaling induced by acute exercise. If differences in metabolism or exercise-induced signaling underpin differential adaptations to exercise training, it is imperative for us to examine how sex influences metabolism during bouts representative of what is being done by the general population. Thus, the findings from our trial provide the first evidence characterizing how sex influences muscle metabolism during shorter bouts of endurance exercise.

Furthermore, the results of the present study set the foundation for future sex comparative studies investigating short, acute bouts of exercise. We found that many enzymes and proteins related to lipolysis, lipid and oxidative metabolism did not differ between men and women during the acute bout of MIC exercise, which was unexpected. In addition, we found that sex did not have an effect on whole-body lipid oxidation, which is in disagreement with previous literature suggesting that women rely to a greater extent on fat as a fuel source during exercise, as evidenced by a lower RER during endurance exercise[11,16,25–40]. Although these results have been very well defined, all of these studies used exercise bouts longer than 60-minutes in duration. Thus, the significance of our results pertaining to similar RER during exercise will serve as a means of comparison for future studies and perhaps the lack of difference between the sexes is due to any distinctions not being able to be captured during a short exercise bout as fat oxidation increases as exercise duration increases. Unfortunately due to the COVID-19 pandemic, differences in IMCL storage and utilization patterns were not able to be determined at this time and limits the impact of the present study. Sex differences in IMCL storage and utilization patterns have never been characterized during a short bout of acute exercise and may help clarify the contentious findings of studies showing greater[17,18,25], similar[16,41] or lesser[42] utilization by women during exercise. Furthermore, given that muscle fuel sources are the first to be used at the onset of exercise, perhaps we could have observed differences in IMCL utilization during exercise between men and women despite not finding an effect of sex on whole-body lipid oxidation. Finally, these data would also provide key information into IMCL storage and utilization patterns in men and women, which could provide further data to explain how women store more IMCL, but are more insulin sensitive than men.

Lastly, we are the first study to characterize sex differences in lipid species profiles in human skeletal muscle. We were able to utilize a novel lipidomics analysis to accurately identify and compare differences in estimates of FA content for PC, PE and TAG species between men and women at rest and

following acute exercise. The pathological implications of storing higher saturated TAG within skeletal muscle has emerged as an important indicator of the onset of IR. The finding that women store more unsaturated TAG species than men provides key evidence to explain how women can store more IMCL while having greater insulin sensitivity than men. Although we were unable to quantify differences in DAG species, a lipotoxic intermediate, our results set the foundation for future studies to better understand how sex influences the relationship between IMCL and IR in humans, and also provide insights into the global lipidome of young, healthy men and women.

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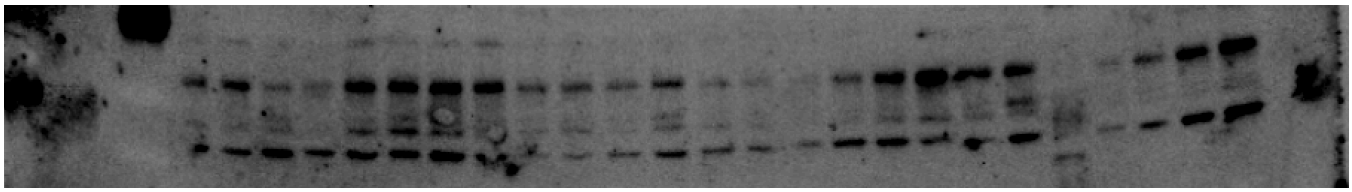
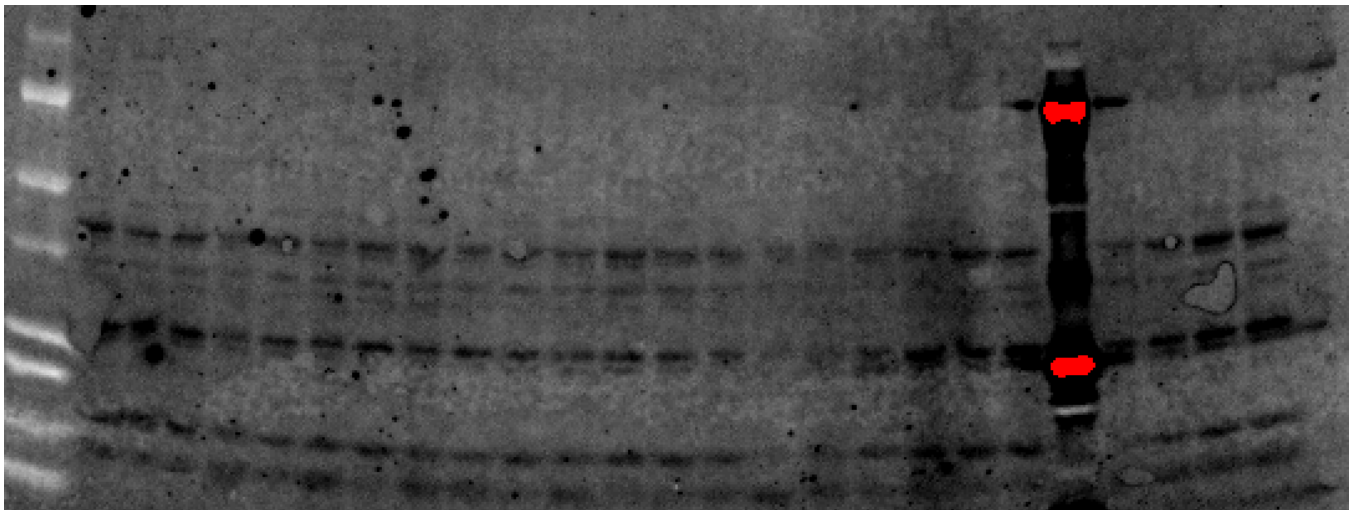
Appendix

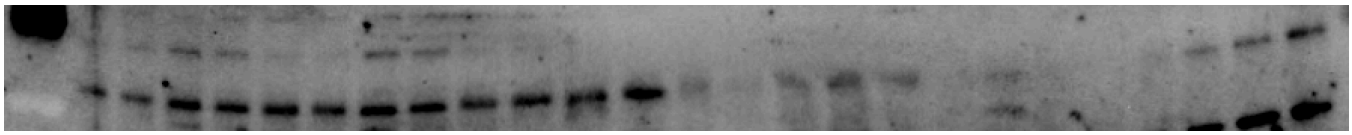
ATGL Western Blot Images



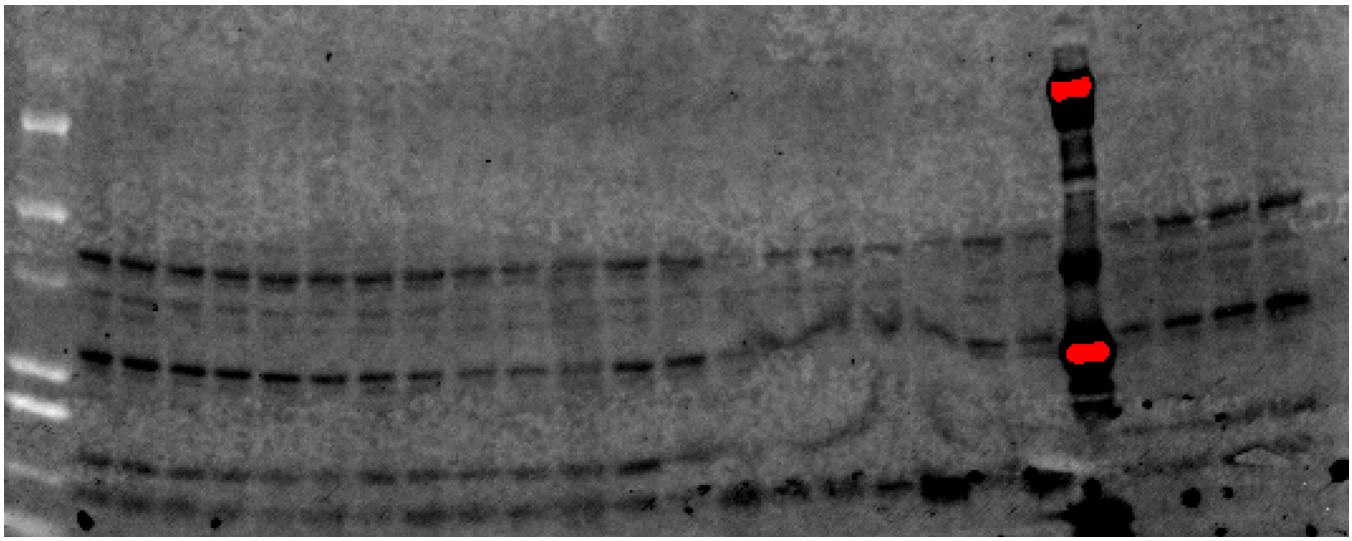
Supplementary Figure 1. Western blot image analysis for ATGL (top band). Bands follow the pattern of female (pre), skip one lane, then male (pre), for participants 1-9 and 11. The last four bands are pooled sample standards.



Supplementary Figure 2. Total protein image of the PVDF membrane used for ATGL content analysis for participants 1-9 and 11 post-transfer. The last four bands are pooled sample standards.

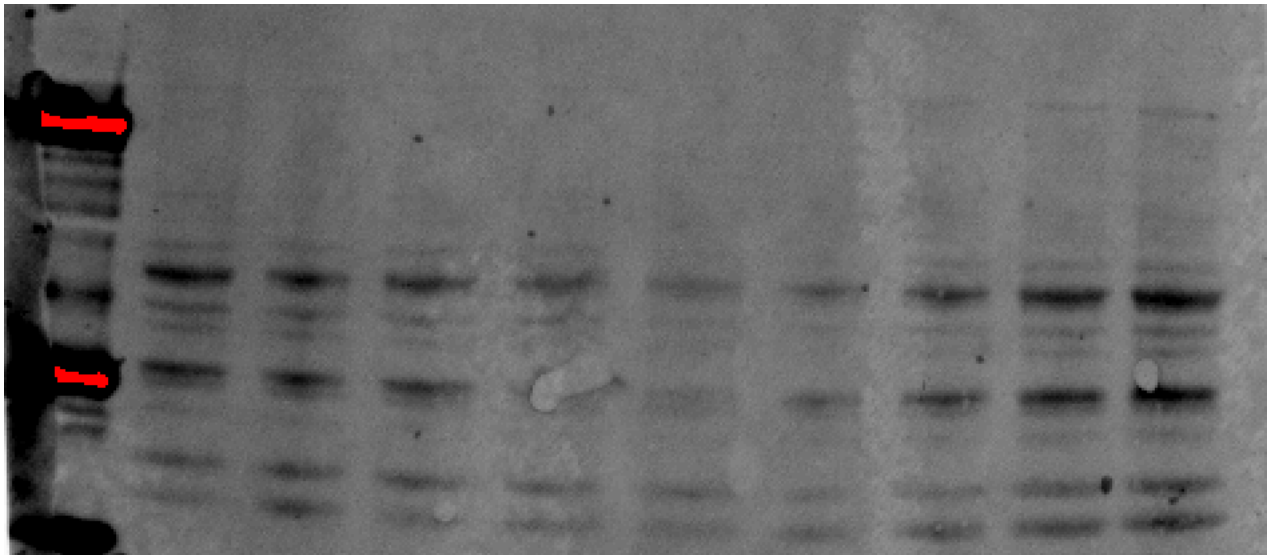


Supplementary Figure 3. Western blot image analysis for ATGL (top band). Bands follow the pattern of female (pre), skip one lane, then male (pre), for participants 10 and 12-20. The last four bands are pooled sample standards.



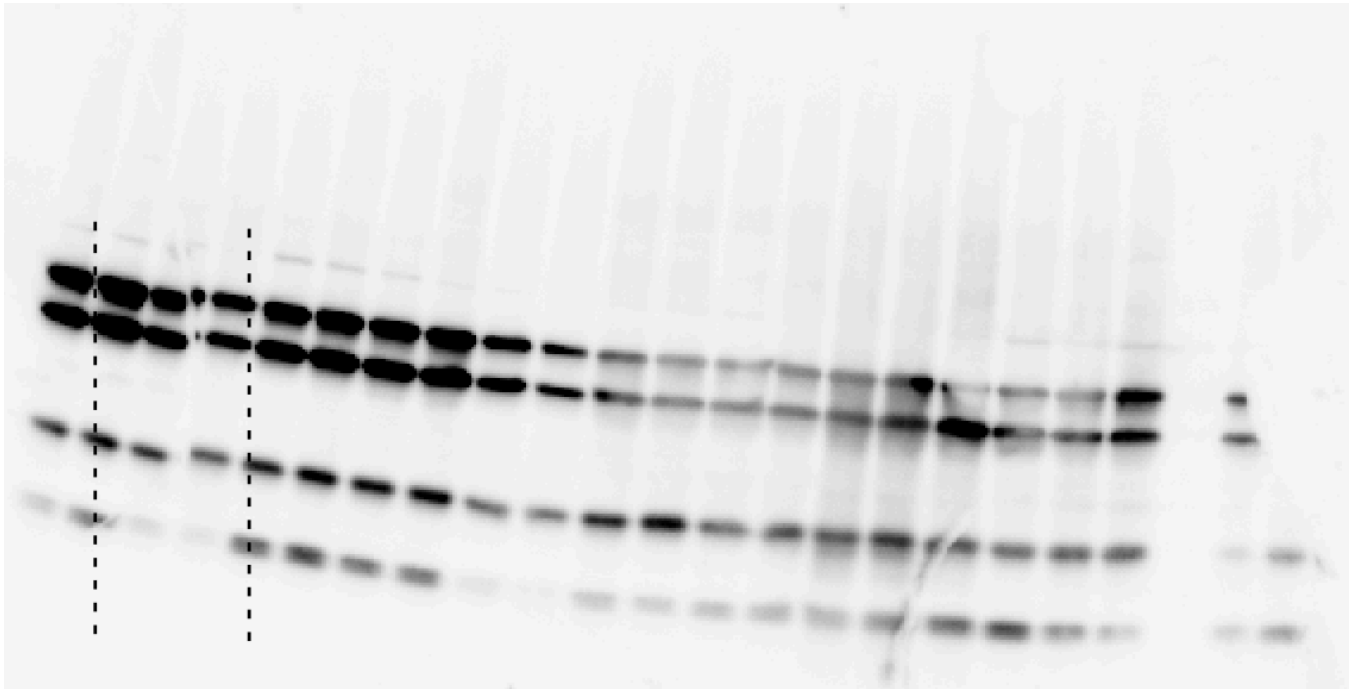
Supplementary Figure 4. Total protein image of the PVDF membrane used for ATGL content analysis for participants 10 and 12-20 post-transfer. The last four bands are pooled sample standards.

Supplementary Figure 5. Western blot image analysis for ATGL. Bands follow the pattern of female (pre) then male (pre), for participants 21-25. The last four bands are pooled sample standards.

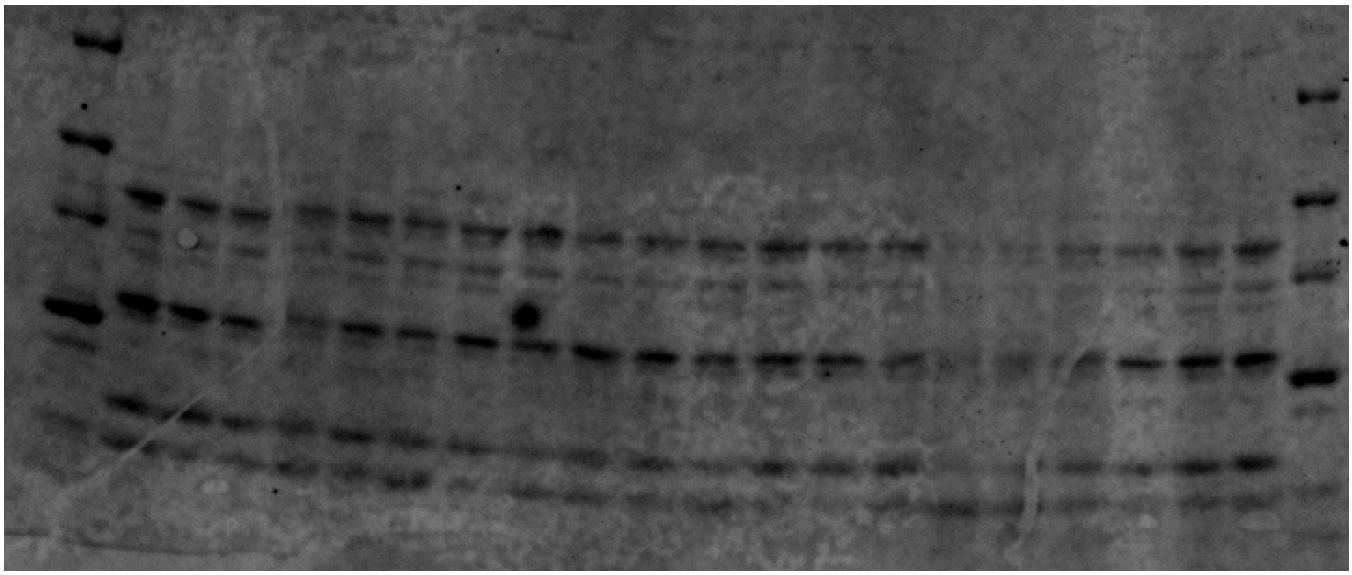


Supplementary Figure 6. Total protein image of the PVDF membrane used for ATGL content analysis for participants 21-25 post-transfer. The last four bands are pooled sample standards.

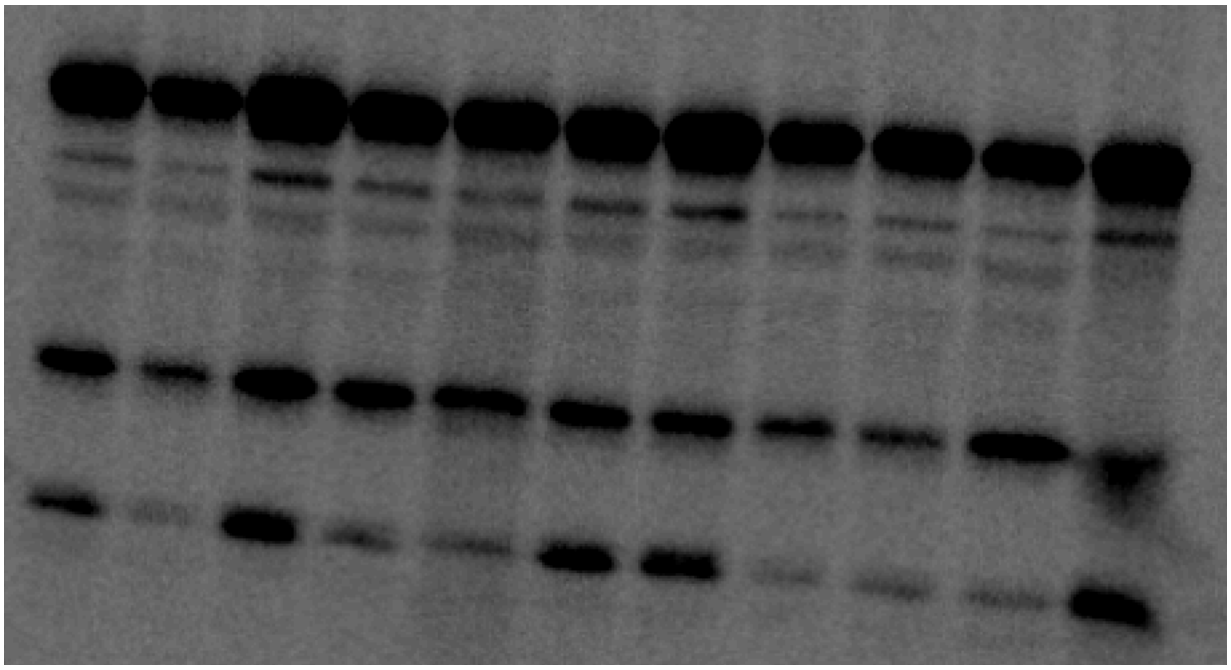
COX II-V Western Blot Images



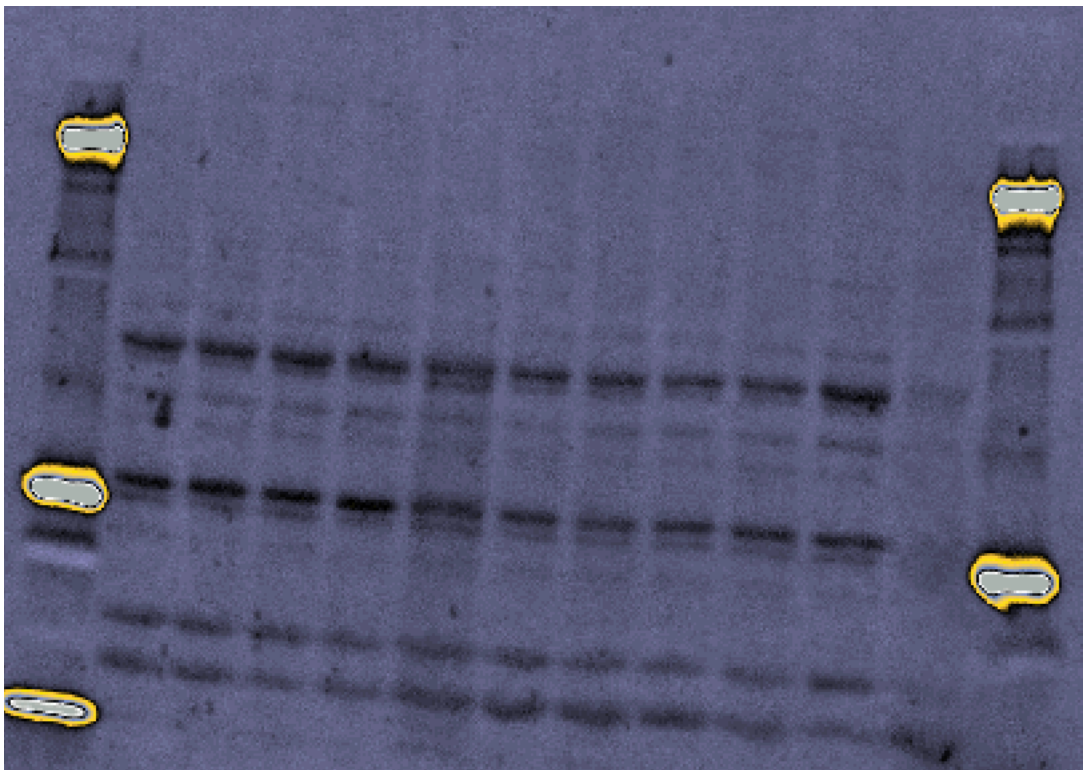
Supplementary Figure 7. Western blot image analysis for COX II (second from bottom band), III (second from top band), IV (bottom band), V (top band). Bands follow the pattern of female (pre), skip one lane, then male (pre), for participants 1-9 and 11. All bands within the dotted lines were not used in analysis and all other bands following the aforementioned pattern were used in analysis.



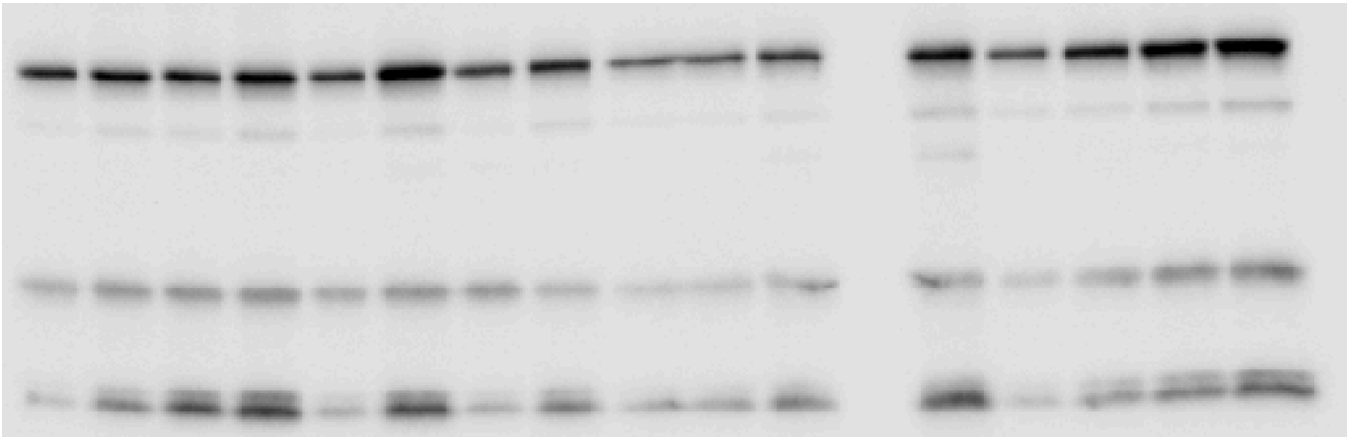
Supplementary Figure 8. Total protein image of the PVDF membrane used for COX II-V content analysis for participants 1-9 and 11 post-transfer.



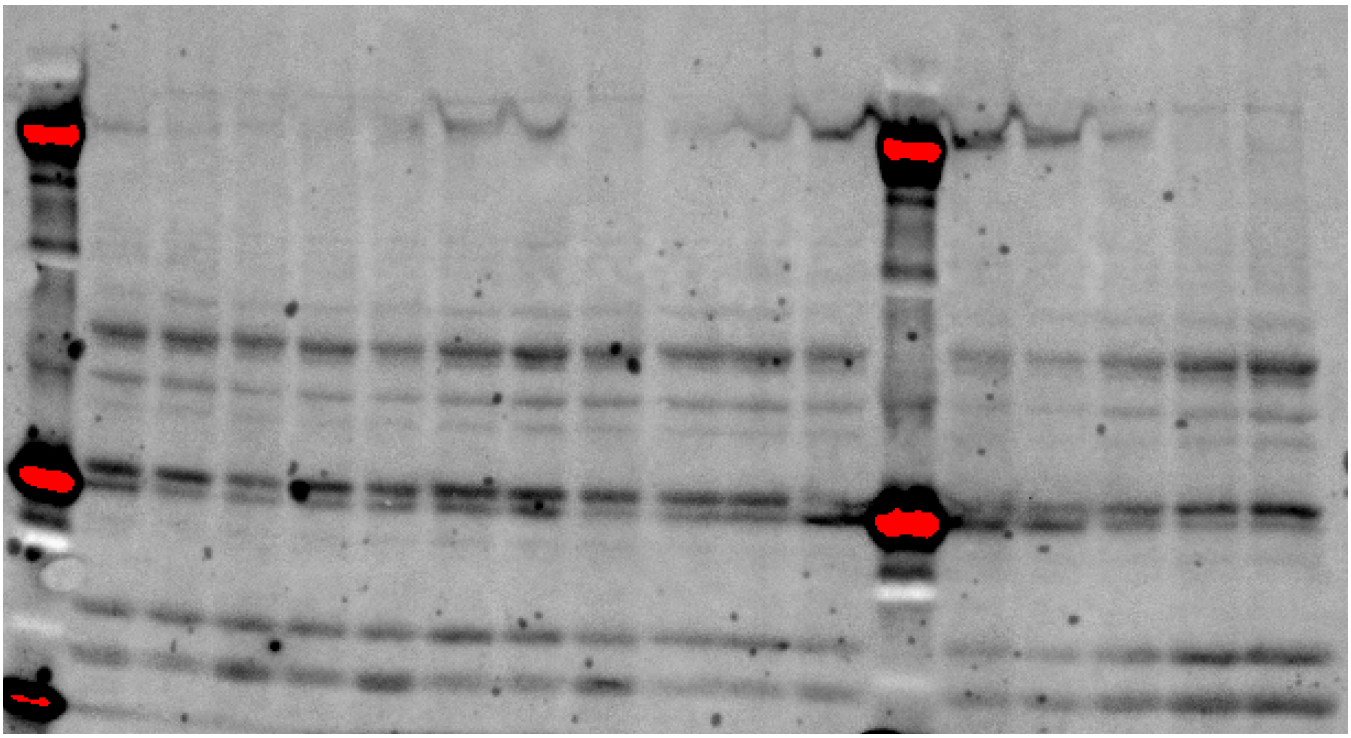
Supplementary Figure 9. Re-run Western blot image analysis for COX II (second from bottom band), III (second from top band), IV (bottom band), V (top band). Bands follow the pattern of male (pre) then female (pre), for participants 4, 9, 10, 12, 13, 15, 17-21.



Supplementary Figure 10. Total protein image of the PVDF membrane used for COX II-V content analysis for participants 4, 9, 10, 12, 13, 15, 17-21 post-transfer.

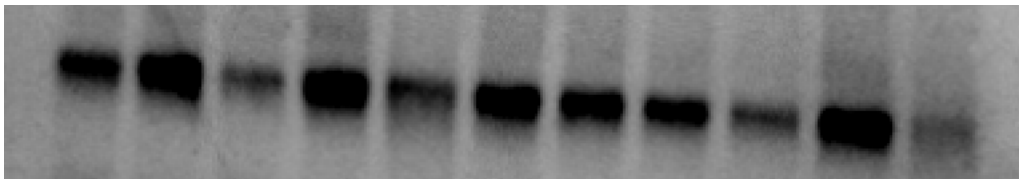


Supplementary Figure 11. Re-run Western blot image analysis for COX II (second from bottom band), III (second from top band), IV (bottom band), V (top band). Bands follow the pattern of female (pre) then male (pre), for participants 10, 12-15, 17, 21-25. The last four bands are pooled sample standards.

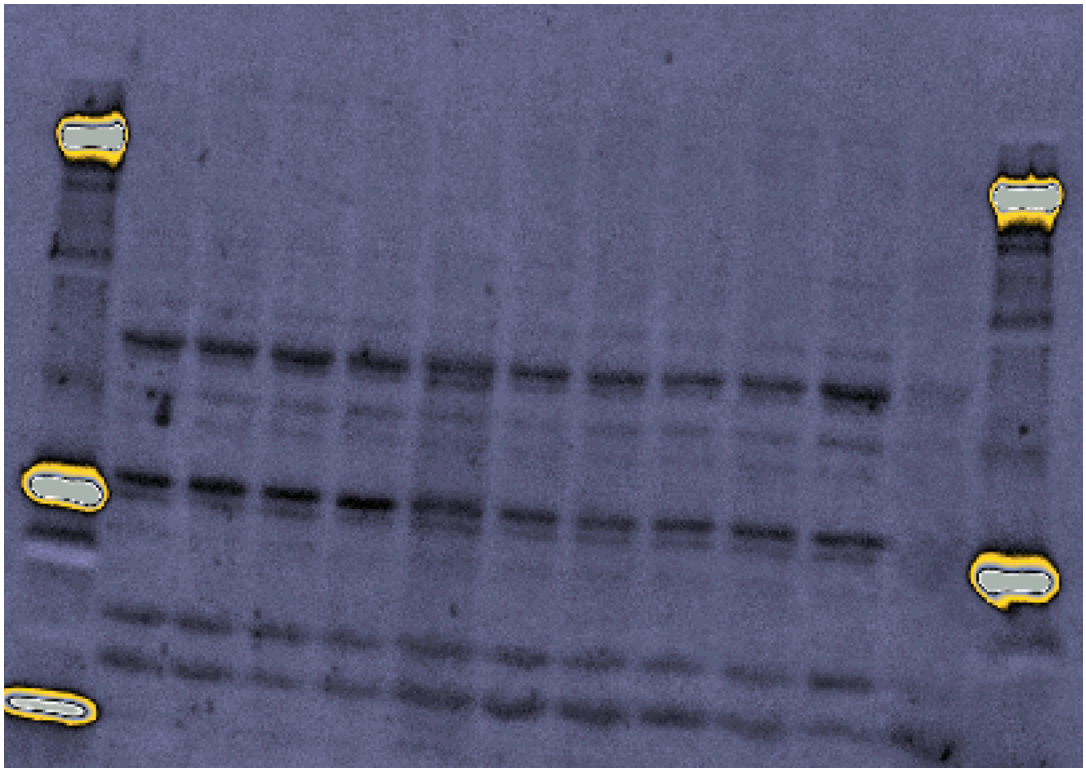


Supplementary Figure 12. Total protein image of the PVDF membrane used for COX II-V content analysis for participants 10, 12-15, 17, 21-25. The last four bands are pooled sample standards.

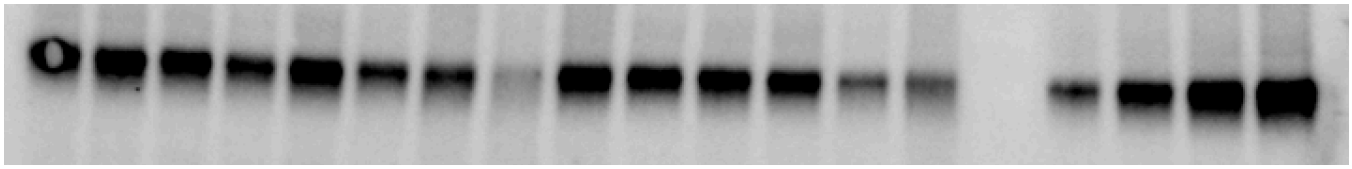
CPT1 Western Blot Images



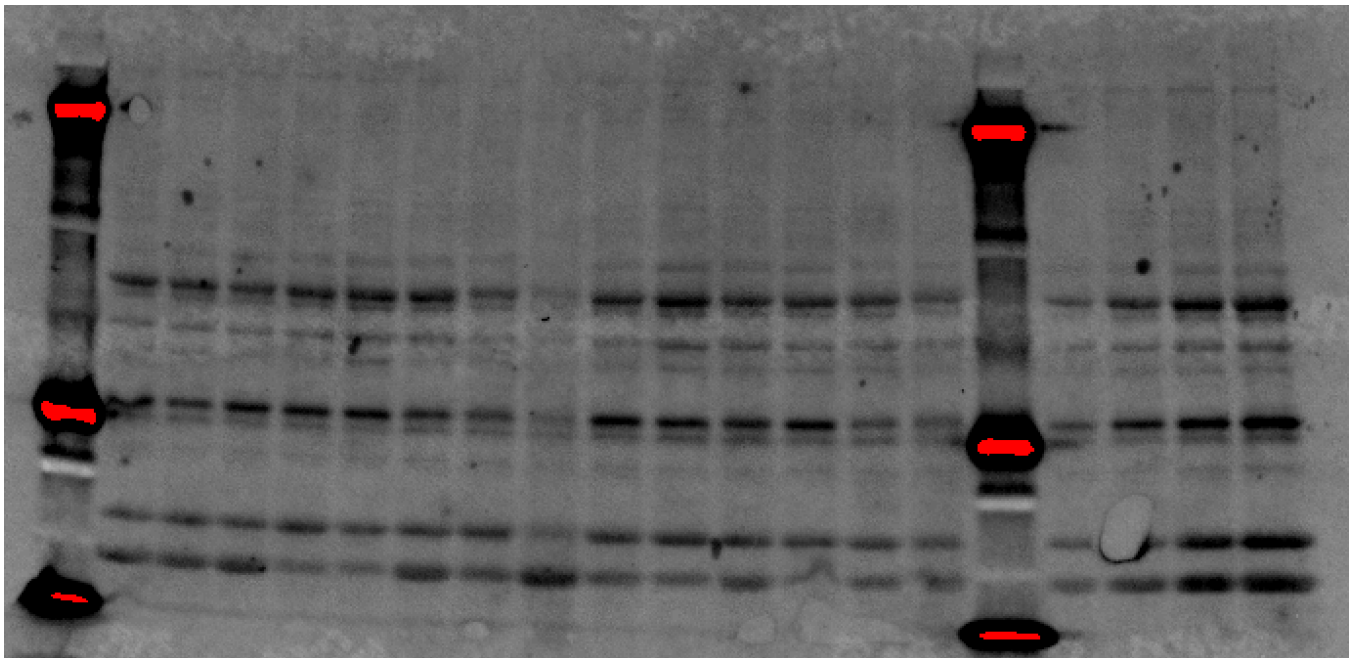
Supplementary Figure 13. Western blot image analysis for CPT1. Bands follow the pattern of female (pre) then male (pre), for participants 4, 9, 10, 12, 13, 15, 17-21.



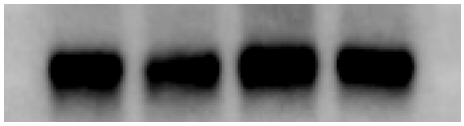
Supplementary Figure 14. Total protein image of the PVDF membrane used for CPT1 content analysis for participants 4, 9, 10, 12, 13, 15, 17-21 post-transfer. The last four bands are pooled sample standards.



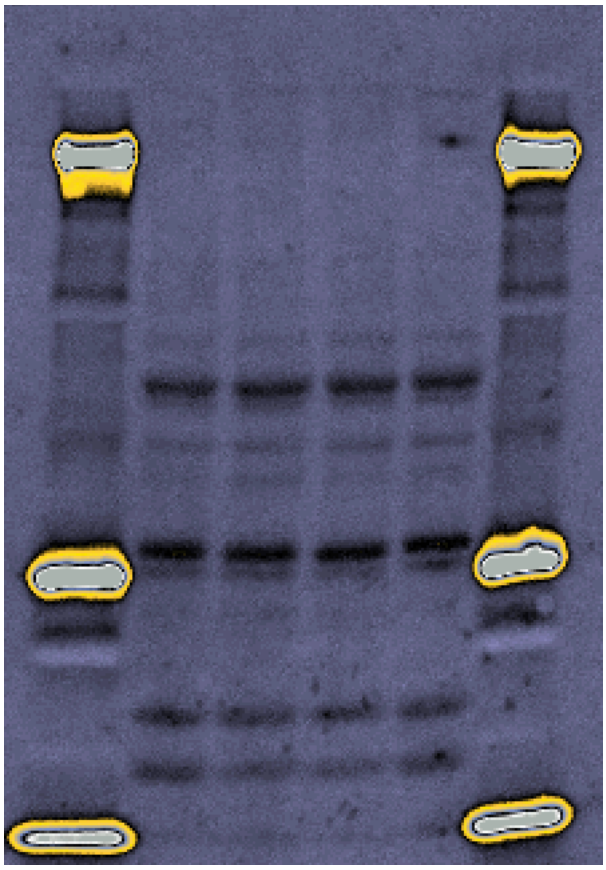
Supplementary Figure 15. Western blot image analysis for CPT1. Bands follow the pattern of female (pre) then male (pre), for participants 1-3, 5, 6-8, 14, 21-25. The last four bands are pooled sample standards.



Supplementary Figure 16. Total protein image of the PVDF membrane used for CPT1 content analysis for participants 1-3, 5, 6-8, 14, 21-25 post-transfer. The last four bands are pooled sample standards.

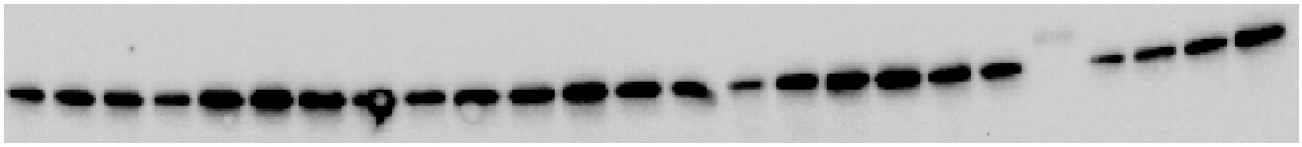


Supplementary Figure 17. Re-run Western blot image analysis for CPT1. Bands follow the pattern of participants 10, 11, 12, 16.

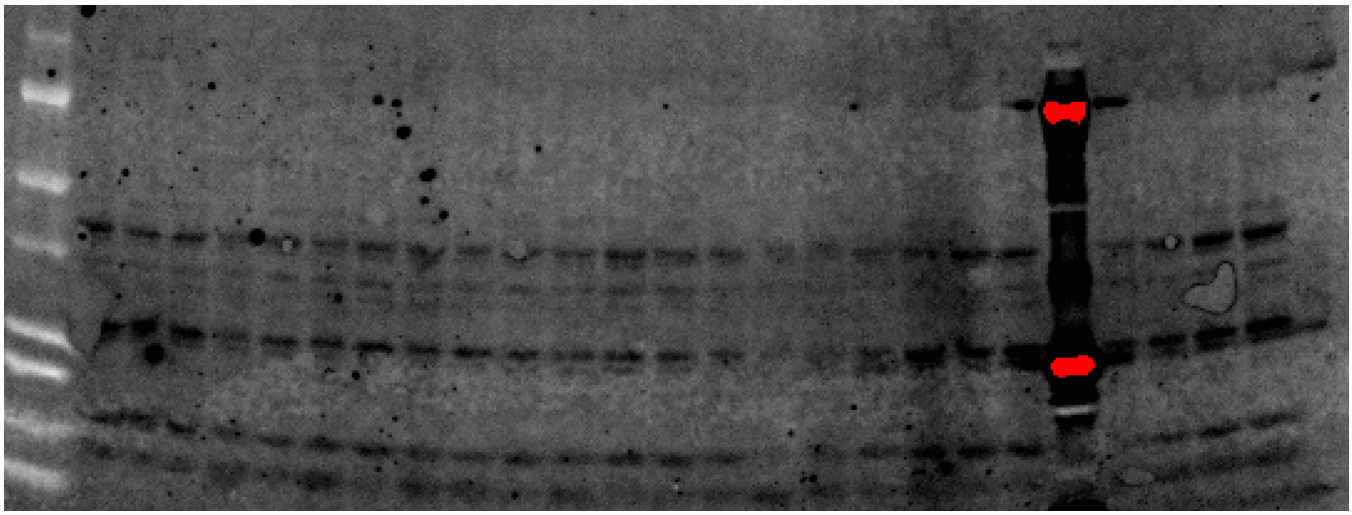


Supplementary Figure 18. Total protein image of the PVDF membrane used for CPT1 content analysis for participants 10, 11, 12, 16.

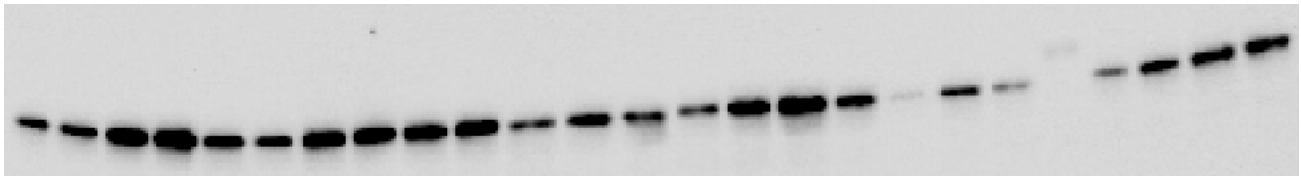
CS Western Blot Images



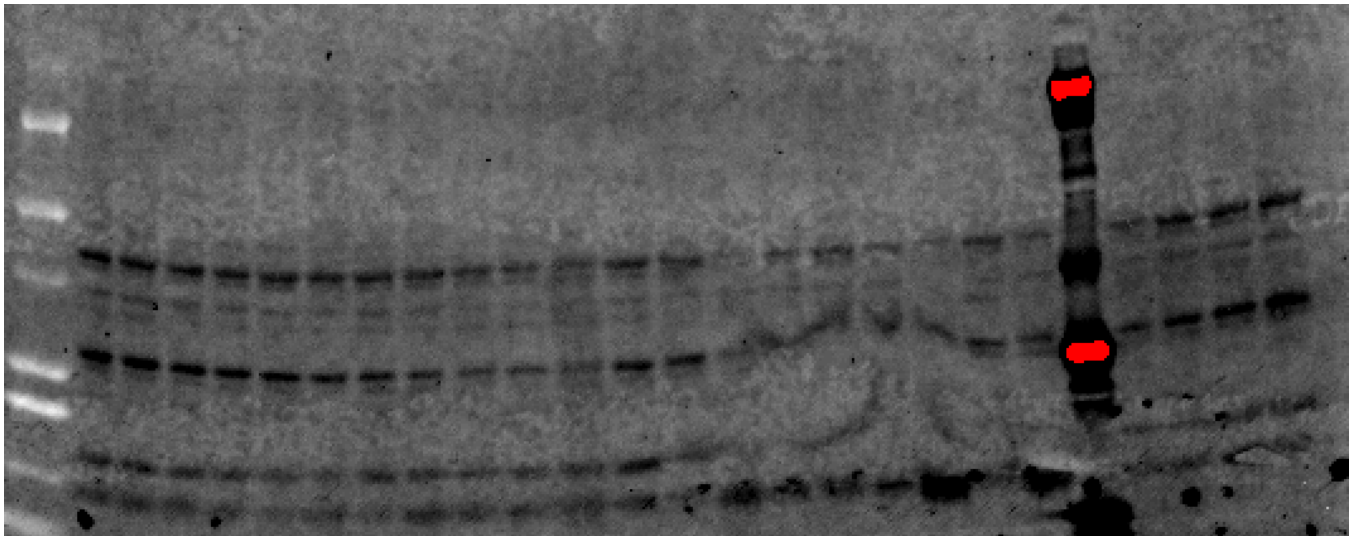
Supplementary Figure 19. Western blot image analysis for CS. Bands follow the pattern of female (pre), skip one lane, then male (pre), for participants 1-9 and 11. The last four bands are pooled sample standards.



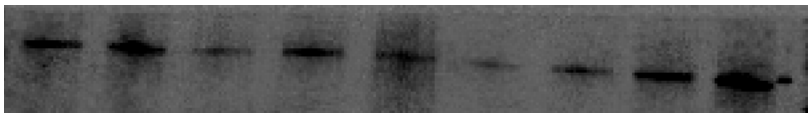
Supplementary Figure 20. Total protein image of the PVDF membrane used for CS content analysis for participants 1-9 and 11 post-transfer. The last four bands are pooled sample standards.



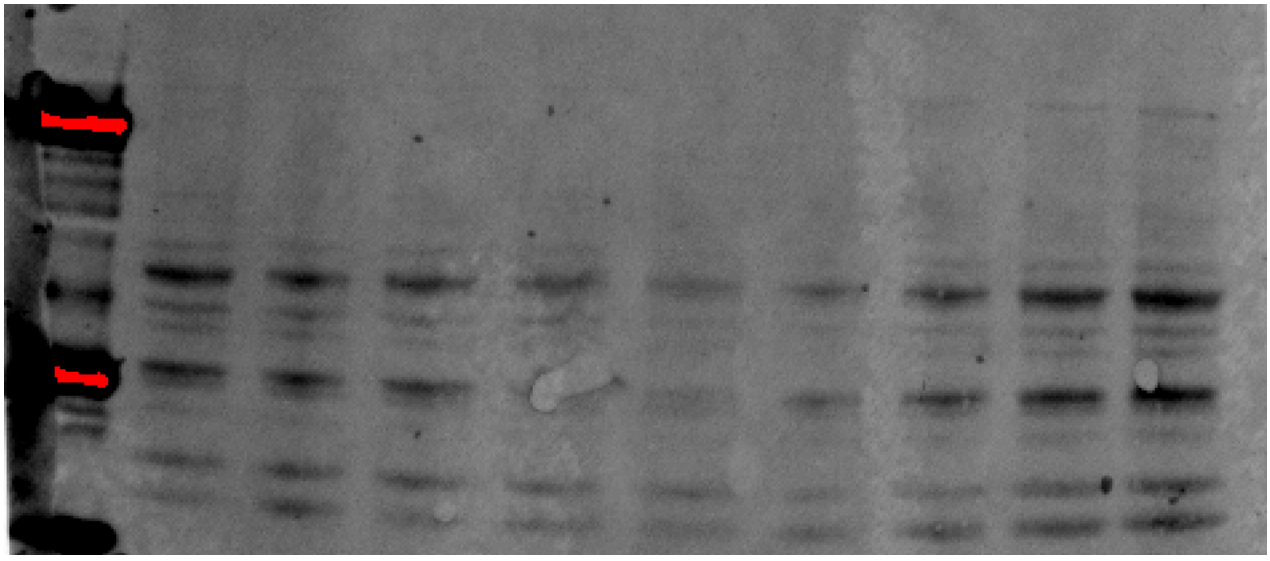
Supplementary Figure 21. Western blot image analysis for CS. Bands follow the pattern of female (pre), skip one lane, then male (pre), for participants 10 and 12-20. The last four bands are pooled sample standards.



Supplementary Figure 22. Total protein image of the PVDF membrane used for CS content analysis for participants 10 and 12-20 post-transfer. The last four bands are pooled sample standards.

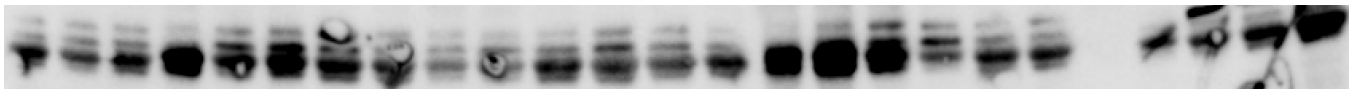


Supplementary Figure 23. Western blot image analysis for CS. Bands follow the pattern of female (pre) then male (pre), for participants 21-25. The last four bands are pooled sample standards.

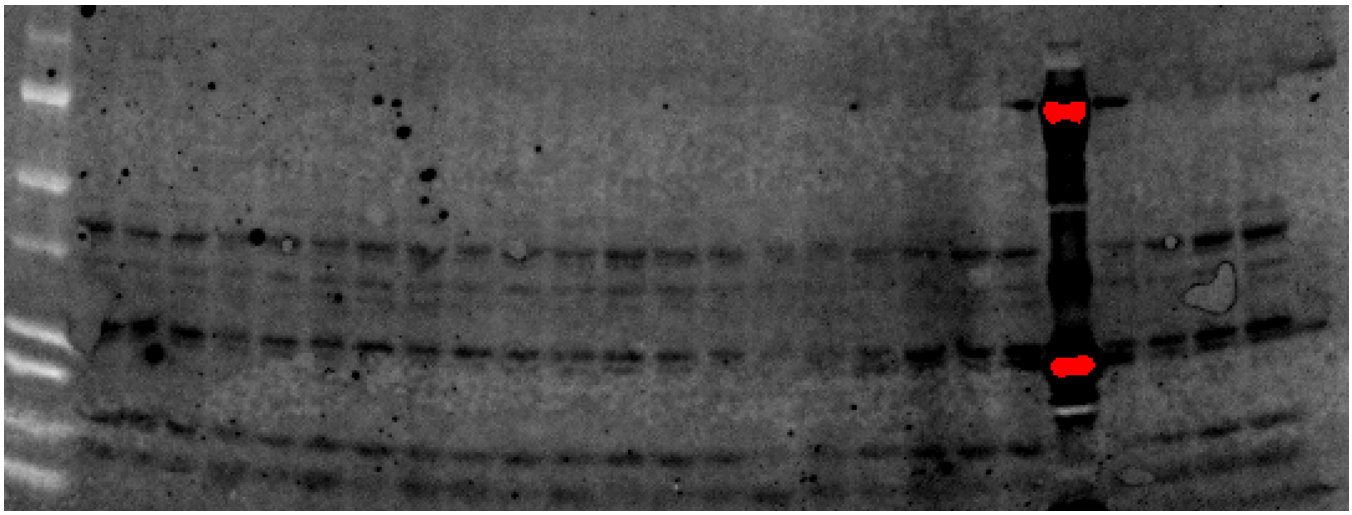


Supplementary Figure 24. Total protein image of the PVDF membrane used for CS content analysis for participants 21-25 post-transfer. The last four bands are pooled sample standards.

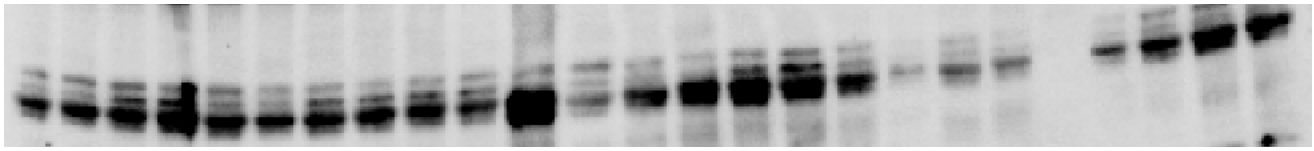
PLIN3 Western Blot Images



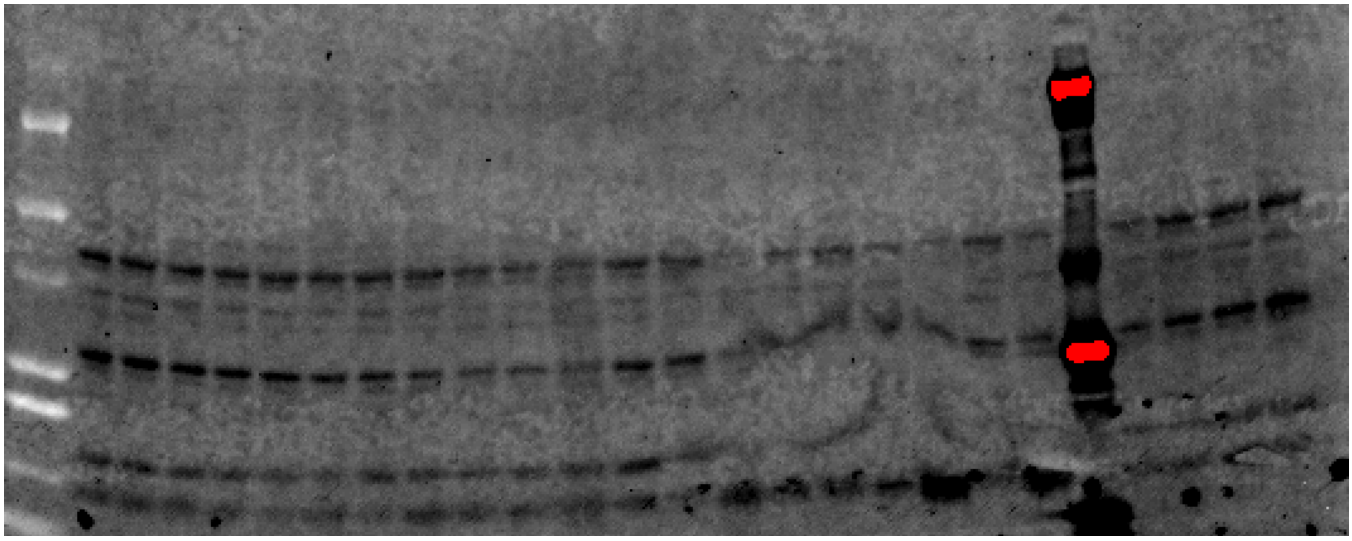
Supplementary Figure 25. Western blot image analysis for PLIN3 (bottom band). Bands follow the pattern of female (pre), skip one lane, then male (pre), for participants 1-9 and 11. The last four bands are pooled sample standards.



Supplementary Figure 26. Total protein image of the PVDF membrane used for PLIN3 content analysis for participants 1-9 and 11 post-transfer. The last four bands are pooled sample standards.

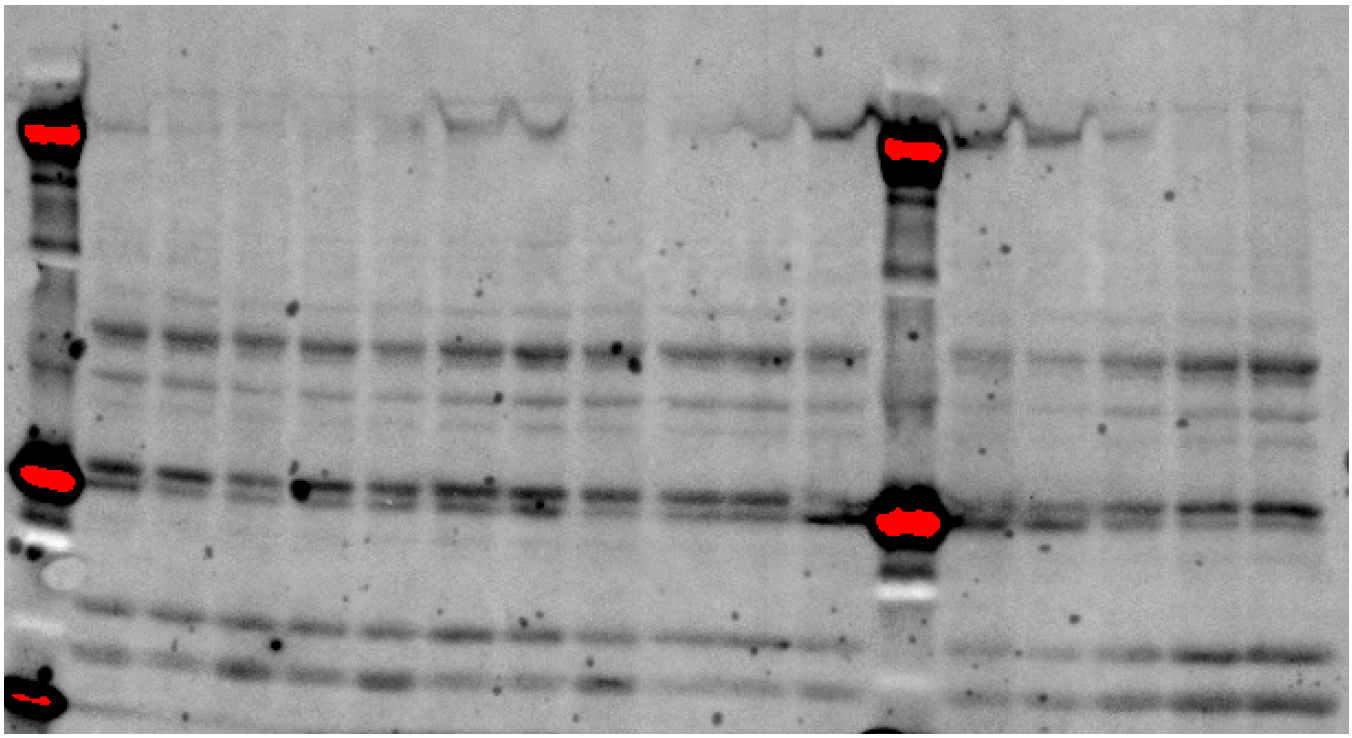


Supplementary Figure 27. Western blot image analysis for PLIN3 (bottom band). Bands follow the pattern of female (pre), skip one lane, then male (pre), for participants 10 and 12-20. The last four bands are pooled sample standards.



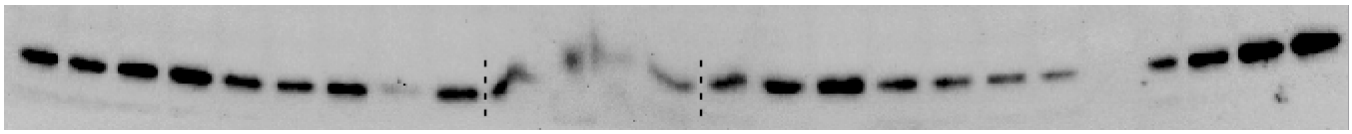
Supplementary Figure 28. Total protein image of the PVDF membrane used for PLIN3 content analysis for participants 10 and 12-20 post-transfer. The last four bands are pooled sample standards.

Supplementary Figure 29. Re-run Western blot image analysis for PLIN3 (bottom band). Bands follow the pattern of female (pre) then male (pre), for participants 10, 12-15, 17, 21-25. The last four bands are pooled sample standards.

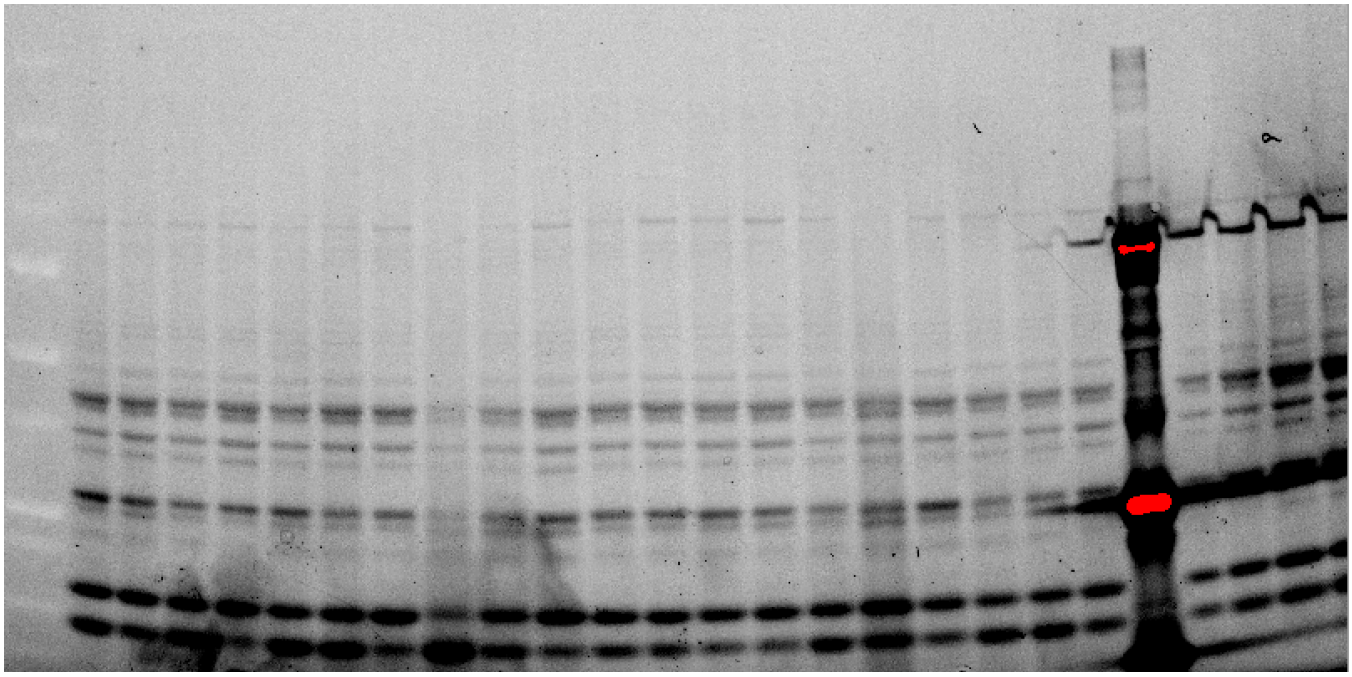


Supplementary Figure 30. Total protein image of the PVDF membrane used for PLIN3 content analysis for participants 10, 12-15, 17, 21-25. The last four bands are pooled sample standards.

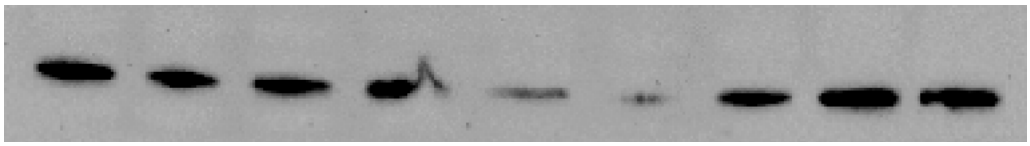
<u>β-HAD Western Blot Images</u>



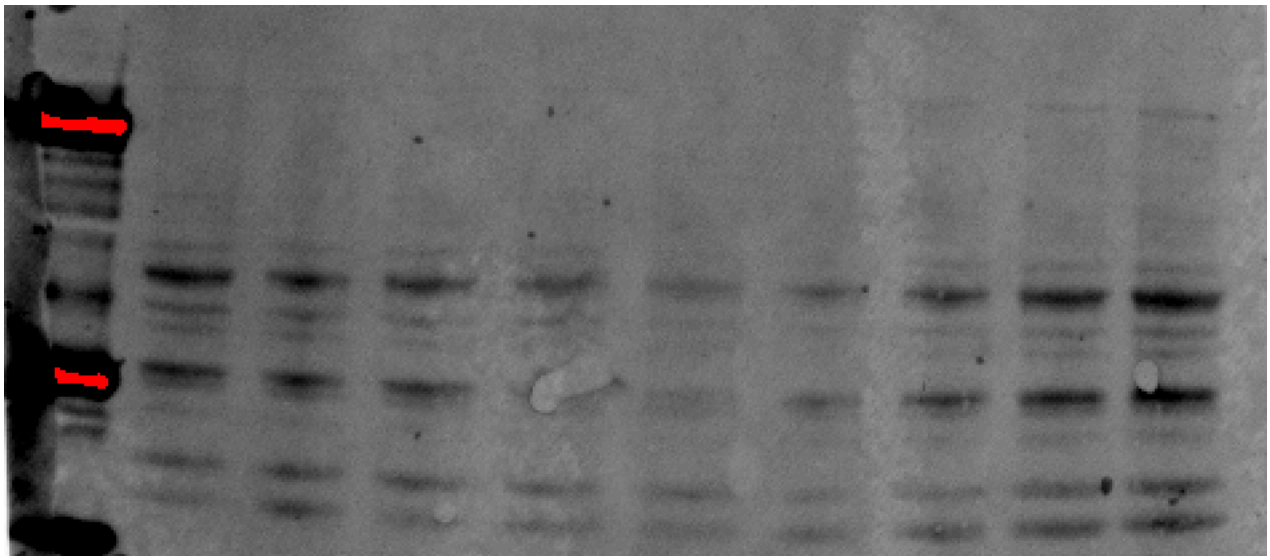
Supplementary Figure 31. Western blot image analysis for β-HAD. Bands follow the pattern of female (pre) then male (pre), for participants 1-20. The last four bands are pooled sample standards. All bands within the dotted lines were not included in analysis (participants 10-12 and 16).



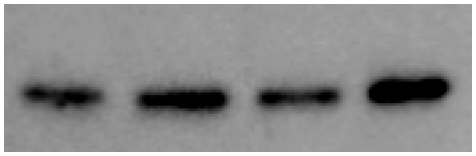
Supplementary Figure 32. Total protein image of the stain-free gel used for β-HAD content analysis for participants 1-20. The last four bands are pooled sample standards.



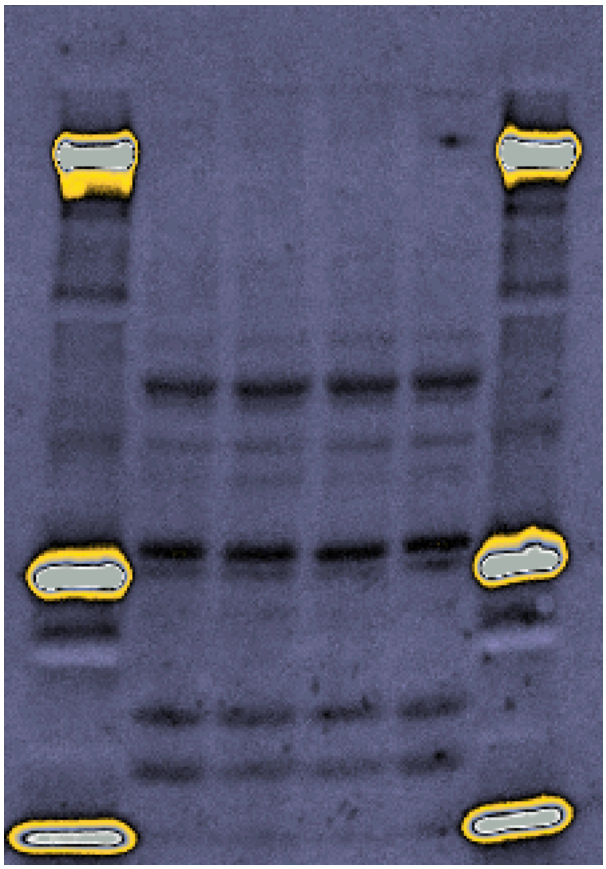
Supplementary Figure 33. Western blot image analysis for β-HAD. Bands follow the pattern of female (pre) then male (pre), for participants 21-25. The last four bands are pooled sample standards.



Supplementary Figure 34. Total protein image of the PVDF membrane used for β-HAD content analysis for participants 21-25 post-transfer. The last four bands are pooled sample standards.



Supplementary Figure 35. Western blot image analysis for β-HAD. Bands follow the pattern of participants 10, 11, 12, 16.



Supplementary Figure 36. Total protein image of the PVDF membrane used for β-HAD content analysis for participants 10, 11, 12, 16.