Impact of environmental and genetic regulation of skeletal muscle metabolism on metabolic response in women with overweight or obesity:
Molecular and cellular analyses and genetic association studies

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ABSTRACT

The following doctoral thesis focuses on genetic and environmental factors that influence skeletal muscle metabolism in women with overweight or obesity. The effects of Acyl-CoA Synthetase Long-Chain 5 (ACSL5) genotype on fatty acid metabolism was studied in vitro, ex vivo and in vivo. The effects of the environmental toxicant mono-(2ethylhexyl) phthalate (MEHP) was also studied in vitro and in vivo.

Statistical analyses illustrated how ACSL5 rs2419621 [T] allele carriers, with overweight or obesity had a greater reduction in their fat mass and visceral fat and greater increases in their percentage lean mass post diet/exercise intervention vs. non-carriers. This was paralleled with increased in vitro and in vivo fatty acid oxidation and ex vivo mitochondrial respiration within [T] allele carriers vs. non-carriers. Interestingly, it was noted that carriers of the polymorphism had increased levels of the ACSL5 683aa isoform in skeletal muscle, which was found to be localized in the mitochondria to a greater extent, playing a greater role in fatty acid oxidation vs. 739aa isoform. This explains in part why carriers of the polymorphism are more responsive to lifestyle interventions vs. non-carriers.

Studies conducted in women with obesity who participated in the National Health and Nutrition Examination Survey (NHANES) demonstrated an association between increased urinary MEHP and increased plasma fatty acid levels. In vitro work in C2C12 myotubes exposed to MEHP displayed a reduction in fatty acid oxidation and mitochondrial respiration. An increase in basal glycolysis was paralleled with increased levels of hexokinase II protein expression in C2C12 myotubes exposed to increasing levels of MEHP. Thus, these results suggest that increased exposure to MEHP as well as urinary MEHP contributes towards dysfunction in glucose and fatty acid utilization at both the muscle and whole-body level.
Hence, women with obesity may be more susceptible to the metabolic effects of MEHP, increasing their chances of metabolic dysfunction.

The following thesis, provides a more comprehensive view on the effects of both genetics and environmental factors on metabolic response within women with overweight and obesity. This provides insights into factors that should be considered for personalized medicine, to improve treatment options for combatting this disease.
ACKNOWLEDGEMENTS

The following thesis requires the utmost thanks to a number of lovely people.

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I would also like to thank my thesis advisory committee Dr. Céline Aguer and Dr. Denis Prud’homme for guiding me through out my doctoral research and providing me suggestions on how I could better my project. Your positive criticism and research suggestions have helped me in completing my project. Thank you for all your help.

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Hannah, you have become one of my best friends. Your huge heart and welcoming personality made my experience in the lab super comfortable! We have shared many years of happiness, sadness, stress and while our time in the lab together is coming to an end, we both know this is only the beginning of a long-lasting friendship. Thanks for all your advice throughout the years. Whether it was research-related or life-related, it helped me grow as both a scientist and person! You are a very determined, rational and kind individual. I only wish the best for you and your future endeavors. Stephen! While you are no longer in the lab with us, you definitely deserve a huge thank you. The three years spent together as lab mates grew into an amazing friendship which I won’t forget! From late nights at the lab working on statistics to all the goofy memories we have had together, thanks for being a great friend and lab mate. To my ladies at the Harper lab: Ava, Rajaa and Chantal. Thanks for the laughter, kindness and friendship. From helping each other with trouble-shooting experiments to just destressing about life, we have definitely made some great memories. You are all highly intelligent, determined, kind and not to mention, pretty ladies! I know all of you will be successful in your career paths. Dave and Georges, the two awesome men in our lab- thanks for all the dancing, singing and laughter- both of you have such a positive aura around you! Good luck on your future endeavors! I would also like to thank both Pierrette Bolongo from Dr. Tesson’s lab and Jian Xuan from Dr. Harper’s lab for managing and organizing the lab. For all the previous students in Dr. Tesson’s lab who helped with the ACSL5 and MEHP project: Jessica Chan, Katyanna Menard and Suzanne Simba, thank you. You have definitely helped in propagating these projects forward.

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<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>ACSBG</td>
<td>Acyl-CoA Synthetase Bubblegum</td>
</tr>
<tr>
<td>ACSL</td>
<td>Acyl-CoA Synthetase Long Chain</td>
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<tr>
<td>ACSM</td>
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<td>ACS</td>
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<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<td>Acid Soluble Products</td>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>BGS</td>
<td>Bovine Growth Serum</td>
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<td>BMI</td>
<td>Body Mass Index</td>
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<td>BPA</td>
<td>Bisphenol A</td>
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<td>CAO</td>
<td>Complications Associated with Obesity</td>
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<td>CHMS</td>
<td>Canadian Health Measures Survey</td>
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<td>COX</td>
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<td>Citrate Synthase</td>
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<td>CT</td>
<td>Computerized Tomography</td>
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<td>DEHP</td>
<td>Di-(2-ethylhexyl) phthalate</td>
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<td>DHA</td>
<td>Docosahexaenoic Acid</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
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<td>DXA/DEXA</td>
<td>Dual-energy X-ray Absorptiometry</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>ECAR</td>
<td>Extracellular Acidification Rate</td>
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<td>EECD</td>
<td>Electronic Entertainment and Communication Devices</td>
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<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FCCP</td>
<td>Carbonyl Cyanide p-trifluoromethoxyphenylhydrazone</td>
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<td>FTO</td>
<td>Fat Mass and Obesity-Associated</td>
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<td>GLA</td>
<td>Gamma-Linolenic Acid</td>
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<td>GLP-1</td>
<td>Glucagon-like Peptide-1</td>
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<td>GWAS</td>
<td>Genome Wide Association Studies</td>
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<tr>
<td>HDL-C</td>
<td>High-Density Lipoprotein- Cholesterol</td>
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<td>HOMA-IR</td>
<td>Homeostatic Model Assessment- Insulin Resistance</td>
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<td>HPLC-ESI-MS/MS</td>
<td>High Performance Liquid Chromatograph-Electrospray Ionization-Tandem Mass Spectrometry</td>
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<td>IRES</td>
<td>Internal Ribosome Entry Site</td>
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<tr>
<td>LDL-C</td>
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<td>Leptin</td>
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<td>LEPR</td>
<td>Leptin Receptor</td>
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<td>MAM</td>
<td>Mitochondrial Associated Membranes</td>
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<td>MBzP</td>
<td>Methylbenzylpiperazine</td>
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<td>MEHHP</td>
<td>Mono-(2-ethyl-5-hydroxyhexyl) Phthalate</td>
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<td>MEHP</td>
<td>Mono-(2-ethylhexyl) Phthalate</td>
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<td>MFN-1/2</td>
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<td>MHC</td>
<td>Myosin Heavy Chain</td>
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<td>Cyclohexane-1,2-dicarboxylic Acid-Mono(hydroxy-isononyl) ester</td>
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<td>MONET</td>
<td>Montréal-Ottawa New Emerging Team</td>
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<td>MRI</td>
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<td>NB</td>
<td>Naltrexone/Bupropion</td>
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<td>NCHS</td>
<td>National Center for Health Statistics</td>
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<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
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<td>NHES</td>
<td>National Health Examination Survey</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>OCR</td>
<td>Oxygen Consumption Rate</td>
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<td>OPA-1</td>
<td>Optic Atrophy-1</td>
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<tr>
<td>PAE</td>
<td>Phthalate Ester</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic Ovary Syndrome</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>POP</td>
<td>Persistent Organic Pollutant</td>
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<tr>
<td>PPARG</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma</td>
</tr>
<tr>
<td>PT</td>
<td>Phentermine/Topiramate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RQ</td>
<td>Respiratory Quotient</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<td>SLC27a</td>
<td>Solute Carrier Family 27 Member 1</td>
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<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid Cycle</td>
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<tr>
<td>TIM</td>
<td>Translocase of the Inner Membrane</td>
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<td>N,N,N’,N’-Tetramethyl-p-phenylenediamine Dihydrochloride</td>
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<tr>
<td>UCP</td>
<td>Uncoupling Protein</td>
</tr>
<tr>
<td>VO2</td>
<td>Volume of Oxygen</td>
</tr>
<tr>
<td>VCO2</td>
<td>Volume of Carbon Dioxide</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER ONE: GENERAL INTRODUCTION

1.1 Obesity, the disease

What defines a disease? Such a term is characterized as a pathological condition in a living system, resulting in the impairment of standard functions and the manifestation of particular signs and symptoms. While certain conditions such as diabetes and cancer can be readily characterized as diseases, an intricate condition such as obesity, which contributes to the development of a plethora of comorbidities, was only recently pronounced as a disease by both the Canadian and American medical associations as well as the World Health Organization. Obesity is a consequence of disrupted energy balance, specifically when the scales have been tipped towards an increase in dietary energy intake vs energy expenditure. Thus, this disease is defined as the accumulation of excess abnormal fat within the human body, with a body mass index (BMI) ≥ 30kg/m², while overweight is characterized by a BMI of 25-29.9 kg/m². While BMI measurement is the most common approach to establishing whether an individual suffers from obesity, other modes of determination include waist circumference measurements where over 35 inches (88.9 cm) in Caucasian women, and over 40 inches (101.6 cm) in Caucasian men were deemed to be risk factors for obesity-related metabolic complications. Furthermore, modes of measurement including waist circumference, waist-to-hip ratio, Dual Energy X-ray Absorptiometry (DEXA), Computerized Tomography (CT) and Magnetic Resonance Imaging (MRI), allow for the accurate measurements of fat deposition within the human body, facilitating precise measurement of abdominal/central obesity, known to contribute towards the onset of diseases such as type 2 diabetes (T2D) and cardiovascular disease.
1.2 Population Statistics on Obesity

A 2015 study published in the *New England Journal of Medicine*, reported that a total of 107.7 million children and 603.7 million adults globally were affected by obesity, while the prevalence of the disease was shown to have doubled since the 1980s in 73 countries\(^9\). Furthermore, while the frequency of obesity was 5% in children and 12% in adults, a higher percentage of obesity was noted amongst women in comparison to men across all ages\(^9\). Specifically, women between the ages of 60 to 64 and men 50-54 had the highest prevalence of obesity\(^9\). These observations parallel previous findings in 2014 by the World Health Organization, where 1.9 billion adults were determined to be overweight world-wide, while 600 million adults were suffering from obesity\(^5\).

The Canadian Health Measures Survey (CHMS), a Canadian nation-wide survey conducted to study the health status of the general population, observed that 62% of Canadian adults were either obese or overweight between 2012-2013, while the prevalence of obesity had doubled since 1978/79\(^10\). The National Health and Nutrition Examination Survey (NHANES), the American equivalent nation-wide survey, illustrated that 36.5% of U.S adults and 17% of youth suffered from obesity between 2011-2014\(^11\). While the prevalence of obesity was roughly the same between sexes in Canadian adults (males= 27% vs females= 26% ), similar to world-wide population statistics, a higher prevalence of obesity was observed amongst U.S women (38.3%) in comparison to men (34.3%)\(^10,11\). Nevertheless, a higher percentage of middle-aged and older adults suffered from obesity in comparison to younger adults in both Canada and United States\(^10,11\). Regarding ethnicity, a higher percentage of obesity was noted within adults and youth of Caucasians, African-Americans and Hispanic descent, in comparison to Asians within United States\(^11\).
A disease once classified as a “high-income country problem”, has become a global concern in low and middle-income countries as well. Population statistics documented by the World Health Organization has documented 10.6 million individuals in 2014 with obesity in Africa alone. Furthermore, it has been noted that overweight and obesity accounts for a higher percentage of deaths worldwide in comparison to underweight.

1.3 Co-morbidities of Obesity

Obesity has been shown to promote an increased risk in developing chronic diseases. As a result, a considerable increase in medical care costs have been reported in North America, with the United states estimated to contribute roughly $147 billion dollars/year towards medical care expenses. Obesity has been shown to increase mortality through metabolic impairment and co-morbidities, specifically through increased susceptibility to cardiovascular disease. Studies have reported obesity to be positively associated with mortality, with increases as high as two- to three-folds. Recent global studies on the effects of obesity on mortality have displayed an association between overweight and obesity with an increased all-cause mortality in Asia, Europe, Australia and North America.

Obesity increases the risk for disrupted glucose uptake and metabolism, specifically insulin resistance, which precedes and predicts type 2 diabetes mellitus. Other co-morbidities of obesity include dyslipidemia and cardiovascular diseases (i.e., hypertension and stroke). Sleep apnea, osteoarthritis, hyperuricemia and gall bladder disease have been shown to be influenced by increased weight gain/obesity, while infertility, polycystic ovary syndrome and reproductive hormonal imbalances are associated with the disease as well. Cancer has been associated with obesity, comprising of colorectal, prostate, endometrial, breast and gall bladder cancers. Finally, increased obesity has been shown to be associated with...
psychological problems including depression and as a result promote a binge-eating disorder.\textsuperscript{13,28}

1.4 Multi-Factorial Influences on Obesity

The etiology of obesity is linked with behavioral, environmental, genetic and epigenetic factors. The subsequent section elaborates on these factors in the development and progression of this disease.

1.4.1 Obesity and Behavioural Factors

Behavioral risk factors contributing to the pathology of diseases include lack of physical activity and poor diet. Thus, the following subsection focuses on the effects of physical activity and diet on weight gain/obesity.

1.4.1.1 Obesity and Food Intake

Energy balance, characterized by the relationship between energy intake and output, can lead to obesity, when the scales are tipped towards energy intake. Thus, one of the major contributors to weight gain and obesity, is the nutritional diet, specifically the amount of calories consumed by drinking and eating. Cross-sectional and prospective studies focused on examining the effects of junk food intake on weight gain in both younger and older adults, illustrated a significant positive association between the frequency of fast food consumption/restaurant visits with BMI.\textsuperscript{29–32} An experimental trial conducted on 891 women between the ages of 20-45, where intake of fast food was measured via questionnaire, revealed its increased association with total energy intake and body weight gain.\textsuperscript{33} However, while increased consumption of food or overfeeding has been associated with weight gain, undernutrition \textit{in utero} has previously been shown to predispose offspring to obesity at a later
stage in life. Specifically, the 1990 Barker theory, also known as the “foetal origins hypothesis”, initially based on observations during the Dutch famine 1944-1945, postulates that the fetal adaptation to undernutrition in utero, can permanently alter its metabolism and physiology\textsuperscript{34,35}. The results is an increased risk of disease development later in life, including obesity\textsuperscript{36}. Work conducted in our lab, has provided more insight into the effects of in utero undernutrition, where we have observed altered metabolic physiology through skeletal muscle energetics\textsuperscript{37}. This has been shown to hinder the response to adulthood hypocaloric diets and has been postulated to be an effect of mitochondrial dysfunction\textsuperscript{37}. Therefore, the following concept of “double-burden of malnutrition” addressed, is defined as co-existence of undernutrition along with overweight and obesity\textsuperscript{38}.

While, undernutrition in utero has been shown to play a role in post-natal obesity development, overnutrition in utero which is more prominent within Western societies has been shown to contribute to obesity as well. Specifically in rodent studies, offspring from dams fed a high-fat diet during pregnancy, have reported increased body fat accumulation\textsuperscript{39–42}.

1.4.1.2 Obesity and Physical Inactivity

While, overfeeding and in utero undernutrition have both been illustrated to play a role in obesity, physical inactivity can contribute to weight gain. A population-based 7 year prospective study conducted between 1985/86-1992/93 studying American men and women between the ages of 18-30 yrs., displayed a significant association between decreased physical fitness and increased weight gain in both sexes\textsuperscript{43}. This observation was also noted within a prospective follow-up study conducted on a Finnish population, illustrating a significant increased association between becoming inactive during the adolescence to adulthood transition with overweight in males, obesity in males and females, and severe abdominal
obesity within females\textsuperscript{44}. Studies on youth also paralleled previous observations noted on adults, where a decline in physical activity was associated with increase in BMI and skinfold thickness in girls\textsuperscript{45}.

Studies focused on observing the effects of television and video games on childhood obesity, have concluded that both sedentary behaviours result in increased weight gain. Specifically, a 1985 study on the National Health Examination Survey (NHES) Cycles II and III focused on examining children within the United States, observed a significant association between the time spent watching television and prevalence of obesity\textsuperscript{46}. The following observations were also noted within American adults, where men who viewed television more than 3 hours a day had a greater susceptibility to developing obesity in comparison to men who viewed television 1 hour/day\textsuperscript{47}. Interestingly, recent findings on the effects of bedroom electronic entertainment and communication devices (EECD), have reported an improvement in weight status in children who have no access to EECDs within their bedrooms\textsuperscript{48}. No access to EECDs including TV and video games, presented improved sleep duration, quality and efficiency, all previously shown to influence weight gain\textsuperscript{49,48}. Thus, findings confirm the effects of video game/TV access on sleep quality and obesity.

1.4.2 Obesity and Environmental Factors

Environmental factors, such as socio-demographics, environmental pollutants and gut microbiome have been shown to contribute towards weight gain/obesity. The following subsection focuses on these following factors.
1.4.2.1 Obesity and Socio-Demographics

The following section will focus on the effects of age, sex, ethnicity and economic status on obesity.

1.4.2.1.1 Age and Sex Effects on Obesity

Global population statistics conducted by the *New England Journal of Medicine* have reported an increased frequency of obesity within adults (12%) vs children (5%), while the highest prevalence of obesity was noted with women between the ages of 60 to 64 and men 50-54, clearly demonstrating an age-effect with obesity. Cross-sectional studies conducted on men and women have shown that both BMI and waist-to-hip ratio is affected with increasing age. Sex-effects have been previously observed in global population studies, where a higher percentage of obesity was noted amongst women in comparison to men across all ages. However, this global trend has not been observed when studying the Canadian population solely, as women and men were equally shown to be affected by obesity. Furthermore, results remain inconsistent when studying the effects of sex on obesity in childhood and adolescence. Specifically, while males were demonstrated to have increased prevalence of obesity within East Asian countries, females had greater prevalence for obesity within the Middle East. Thus, while further elucidation is required, it is evident that sex plays a role in obesity.

1.4.2.1.2 Obesity, Ethnicity and Economic Status

Obesity has been shown to be influenced by ethnicity. Literature overviews have revealed a greater total body fat within individuals of Asian background, in comparison to Caucasian Europeans displaying the same BMI. Specifically, south Asian background were observed
to have a higher chance of developing abdominal obesity, thus increasing the risk for co-
comorbidities such as type 2 diabetes and cardiovascular disease\textsuperscript{54,55}. Studies conducted on the
National Health and Examination Survey between the years 1999-2010, illustrated an
increased rate of obesity within non-Hispanic black women in comparison to Mexican
American, Hispanic and Non-Hispanic Caucasian women\textsuperscript{56}.

Interestingly, while an individual’s ethnic background could contribute towards weight gain/
obesity via genetics, cultural perception on what is subjectively deemed as obesity, has shown
to influence weight gain within society as well. While obesity has been acknowledged by the
World Health Organization and the Canadian and American Medical Associations as a
disease, an individual with increased weight may also be perceived as more healthy, beautiful,
stronger and fertile in different parts of the world\textsuperscript{57–59}. Previous research conducted on children
in the U.S reported more acceptance with weight gain and larger body proportions within
African-American children in comparison to Caucasians\textsuperscript{60,61}. Furthermore, a cross-sectional
survey conducted on rural women from South Africa reported a lack of concern with weight,
while individuals with overweight and obesity also did not want to lose weight\textsuperscript{62}.

While, the effects of ethnicity on obesity are evident, weight gain/obesity is highly influenced
by the economic status of the region of interest. In particular, higher rates of obesity have been
observed within low income populations in developed countries, while higher income
populations are more susceptible to obesity within developing countries\textsuperscript{63,64}. The previous
observation alludes to the increased globalization observed with higher income classes within
developing countries, providing individuals with increased accessibility of cheap high caloric
food\textsuperscript{65}. This has been validated by cross-sectional studies on Moroccan adults, where a positive
association was observed between risk of overweight and obesity and average family income in men\textsuperscript{51}.

1.4.2.2 Obesity and Environmental Pollutants

Environmental pollutants are defined as chemicals that result in adverse ecological and health consequences due to their introduction into the surrounding air, water, soil and food\textsuperscript{66}. Environmental pollutants encompass a wide array of chemicals, including but not limited to persistent organic pollutants (POP), phthalates (PAE), bisphenol A (BPA) and pesticides\textsuperscript{66}.

The concept of an “obesogen”, emphasizes the effects of certain chemicals in promoting increased fat accumulation and obesity by altering lipid metabolism and adipogenesis within the body\textsuperscript{67}. While chemicals such as BPA have consistently shown a positive correlation between increased exposure and obesity, the obesogenic effects of chemicals such as PAEs have been shown to be affected by factors including age\textsuperscript{66,68–71}. Due to increased exposure of PAEs through our diet, the following section will focus on the effects of PAEs on weight gain/obesity.

1.4.2.2.1 Obesity and Phthalate (PAE) Esters

Phthalate esters are esterified phthalic acids, predominantly used as a plasticizer to allow increased flexibility and durability of plastics\textsuperscript{72}. The most common source of human exposure to phthalates is through the diet\textsuperscript{72,73}. Furthermore, phthalates have a decreased propensity to covalently bind onto consumer product matrices, resulting in humans having increased susceptibility to the adverse health effects caused by phthalate exposure\textsuperscript{72,73}. Previous research on U.S women illustrated increase in weight gain with increase in urinary phthalate metabolites phthalic acid, methylbenzylpiperazine (MBzP) and monobutyl phthalate, while
elderly women also experienced an increase in waist circumference, total fat mass and subcutaneous adipose tissue with increased circulating concentrations of mono-isobutyl phthalate. While conflicting results have been observed on the effects of phthalate metabolite exposure on children, a dose-response relationship between monoethyl phthalate and increased BMI/waist circumference was observed in overweight children.

One of the primary phthalate plasticizers used in Canada is di (2-ethylhexyl) phthalate (DEHP). A common chemical compound found in food packaging, medical devices and cosmetics, DEHP’s mode of human exposure consists of dermal layer, inhalation and ingestion. DEHP is broken down to its preferentially absorbed monoester derivative form, mono-(2ethylhexyl) phthalate (MEHP). On average, Canadians have been documented to have daily exposure intakes ranging from 5.8 to 19.0 µg/kg, with medical environments increasing daily intake up to 167.9mg/day.

Previous studies on the effects of DEHP, MEHP and an oxidative DEHP metabolite mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), have shown a positive association between anthropometric indices related with abdominal obesity (including waist circumference and waist-to-hip ratio) as well as BMI, and increased urinary DEHP metabolites in adults and children. Interestingly, studies on women have also revealed an increase in MEHP to MEHHP (product of MEHP oxidation) ratio with increasing BMI and waist circumference, thus exhibiting a disrupted or slower rate of oxidative metabolism of the toxicant with increasing adiposity. Whether the following disruption is a consequence of decreased P450 enzymatic activity responsible for MEHP oxidation, or an overtly decreased metabolic rate requires further elucidation. Regardless, such a decrease in MEHP oxidation, may result in increased endocrine disrupting effects, dysregulation of the overall energy balance and thus
alter fatty acid utilization within these individuals caused by increased exposure to MEHP\textsuperscript{80}. 

\textit{In vitro} work focused on understanding the cellular effects of MEHP, have illustrated the toxicant as a potential endocrine disruptor by behaving as a potential PPARγ- agonist\textsuperscript{85}. Specifically, Feige et al. 2007 reported a dose-dependent cellular activation of a PPARγ receptor element (PPRE)-firefly luciferase reporter, in the presence of the adipogenic transcription factor PPARγ\textsuperscript{85}. This was observed when studying COS7, Hela and muscle C2C12 cells when exposed to MEHP\textsuperscript{85}. Furthermore, models predicting the potential interaction of MEHP with PPARγ, displayed MEHP to bind with the ligand binding domain of PPARγ similarly to rosiglitazone, a known PPARγ agonist\textsuperscript{85}.

\subsection*{1.4.2.3 Obesity and Gut Microbiome}

The human body is known to consist of over 2000 species of bacteria, with the majority found within our gut\textsuperscript{86}. One of the primary and crucial research findings illustrating the importance of the gut microbiota in the development of adiposity, was by Bäckhed \textit{et al.} 2004, where germ-free mice in the absence or presence of a higher caloric intake, had a 40\% reduction in total body fat than mice raised conventionally in the presence of microorganisms\textsuperscript{87,88}. However, following the introduction of a microbial environment into the gut of the germ-free mice, total body fat and hepatic triglycerides significantly increased\textsuperscript{87}. To affirm that the gut microbiota composition’s effect on obesity was causal and not a consequence, transplantation of gut microbiota of lean and obese mice into a germ-free host, illustrated an increase in adiposity with "obese microbiota" vs "lean microbiota"\textsuperscript{89}. Previous research on the distal gut microbiota composition between lean vs obese adults, have displayed a lower percentage of \textit{Bacteroidetes} and an increased amount of \textit{Firmicutes} with obesity\textsuperscript{90}. Furthermore, the composition of the diet was shown to influence the gut microbiota, where introducing a low-
caloric diet inducing weight loss, appeared to increase the \textit{Bacteroidetes} to \textit{Firmicutes} ratio within individuals\textsuperscript{90}. However, conflicting results have been also observed in other studies, where no differences or an increase in \textit{Bacteroidetes} was noted in individuals with obesity\textsuperscript{91–93}. Regardless, changes in gut microbiome composition has been shown to influence adiposity.

In order to further study the effects gut microbiome composition and increased adiposity, a randomized-control trial was conducted on pregnant women, to study the effects of gut microbiota manipulation on adiposity and weight gain in children\textsuperscript{94}. The study illustrated that perinatal probiotic intervention controlled the initial phase of weight gain in the first 24-48 months of age, but did not effect weight gain following 48 months of age\textsuperscript{94}. Furthermore, probiotic introduction in pregnant women appeared to result in trends towards a decreased birth-weight adjusted mean BMI in 4 years old children, illustrating the importance of early gut microbiota in a child’s growth rate\textsuperscript{94}.

Currently, crowd-funded citizen science projects such as the American Gut Project exist to enable participants to learn more about their gut microbiota\textsuperscript{95}. Such projects provide the ability to learn about the association between the human gut microbiome and health by studying the effects of factors such as diet, alcohol consumption and disease\textsuperscript{95}.

\textbf{1.4.3 Obesity and Endocrine Medical Conditions}

Endocrine-related conditions, that disrupt the energy balance of an individual due to altered hormonal levels can influence weight gain/obesity\textsuperscript{96}. Thus, endocrine disorders such as hypothyroidism, Cushing’s syndrome, polycystic ovarian syndrome and certain cancers such as craniopharyngioma play a role in weight gain\textsuperscript{97–100}. Thyroid hormones are responsible for controlling the body’s metabolism via pathways such as fatty acid oxidation and glycolysis, while also regulating food intake\textsuperscript{97}. Thyroid hormones have previously been associated with
thermogenesis and metabolic rate, while also contributing towards an alteration in BMI and obesity\textsuperscript{101}. Cushing’s syndrome, a consequence of high levels of cortisol exposure, has been shown to be associated with weight gain/adiposity by increased levels of adipocyte differentiation and adipogenesis\textsuperscript{98}. While obesity has been shown to affect polycystic ovarian syndrome (PCOS) due to increased insulin resistance in PCOS patients with obesity, the excess androgen levels found within individuals with PCOS, has been shown to be a key factor in the development of central obesity as well\textsuperscript{99}. Finally, cancers such as craniopharingioma, where tumours arise within the hypothalamic-pituitary region, have been shown to contribute towards hypothalamic obesity, a condition irreplaceable to exercise and diet, while still being associated with abnormal food seeking behaviors\textsuperscript{100}.

1.4.4 Obesity and Genetics

The following section will focus on the effects of genetics on obesity, specifically studying the effects of mutations and single nucleotide polymorphisms (SNPs) on the disease.

1.4.4.1 Obesity and Genetic Conditions

Genetic factors have been shown to contribute towards the development of obesity. Some of the genetic conditions including Prader-Willi syndrome, Bardet-Biedl syndrome, Alström syndrome and Cohen syndrome have been shown to lead towards increased weight gain/obesity\textsuperscript{102–105}.

1.4.4.2 Obesity and Heritability

Obesity has been characterised as a highly heritable disease, with studies illustrating an increased susceptibility to the disease when a family member suffers from obesity\textsuperscript{106}. Previous research conducted on twin adoption and family studies have reported varying ranges of heritability estimates for BMI (16-85\%) and body fat percentage (35-63\%)\textsuperscript{106–115}. Monogenic
obesity, representing a small percentage of obesity cases, is a condition that severely affects individuals as early as childhood, has been reported in 176 human obesity cases and is influenced by mutations found in 11 genes including but not limited to leptin (LEP) and leptin receptor (LEPR). Additionally, SNPs are one of the genetic aspects known to factor into the development of obesity. SNPs are single position nucleotide changes commonly found in the DNA within a population. Genome-wide association studies (GWAS), have been shown to be successful in determining genome-wide set of genetic variants/SNPs in individuals to study genetically-associated traits including diseases. Obesity has been shown to be influenced by SNPs found within genes known to regulate metabolic processes as well as adipocyte differentiation, triglyceride metabolism and leptin regulation. Specifically, a review published by Yang et al. 2007, has illustrated that in 426 studies, 127 candidate genes presented positive associations between SNPs and obesity-related phenotypes, with 22 of these genes exhibiting a positive association with obesity in at least five studies. These genes include, peroxisome proliferator-activated receptor gamma (PPARG), LEPR, and uncoupling proteins UCP1, 2 and 3.

Peroxisome proliferator-activated receptor gamma, is a nuclear receptor responsible for regulating genes that play a role in lipid and glucose metabolism and is a master regulator of adipogenesis. One of these response genes speculated to be regulated by PPARG is acyl-CoA synthetase long chain 5 (ACSL5), known to esterify intracellular free fatty acids into fatty acyl-CoA molecules. A polymorphism found on this gene (rs2419621), characterised by a C to T transition in the promoter region of the gene, has been shown to be associated with increased rate of weight loss within post-menopausal women with obesity. With an allele frequency of roughly 22%, rs2419621 has been shown to produce an additional EBOX site on
top of the two commonly found sites, as well as increasing the expression of the downstream gene *in vitro*\(^1\)\(^2\). A section of the following Ph.D thesis will be focusing on the effects of the rs2419621 polymorphism on obesity-related phenotypes, as well as its effect on fatty acid metabolism both *in vitro* and *ex vivo*.

### 1.4.5 Obesity and Epigenetics

Epigenetics, are heritable changes that influence the expression and function of genes without altering the DNA sequence of an individual\(^1\)\(^2\). Epigenetic changes including DNA methylation (i.e., promoter region CpG methylation) and histone modifications such as acetylation and methylation, can be influenced by external factors including environmental and behavioral modifications, resulting in consequences such as altered gene expression levels and genomic imprinting, where alleles can be silenced/activated due to parental origin\(^1\)\(^2\)\(^5\)–\(^1\)\(^7\). Epigenetics can be affected by both the pre-natal and post-natal environment of an individual. Recent studies have illustrated a positive association between maternal pre-pregnancy obesity and excessive neonatal adiposity/growth\(^1\)\(^8\). Furthermore, maternal methyl-donor dietary intake alters foetal development as well as DNA methylation status of metastable epialleles\(^1\)\(^9\). Additionally, studying human fetal DNA methylation analyses, have illustrated the effects of overnutrition during pregnancy as well as high pre-pregnancy maternal BMI on altered DNA methylation in metabolic genes in offspring cord blood DNA\(^1\)\(^3\)\(^0\),\(^1\)\(^3\)\(^1\). Interestingly, pregnant women with obesity who underwent bariatric surgery to increase weight loss were shown to give birth to children with decreased susceptibility to obesity as well as altered DNA methylation profile of metabolism-related genes\(^1\)\(^3\)\(^2\),\(^1\)\(^3\)\(^3\). Furthermore, the Barker hypothesis has also noted how starvation *in utero* can also lead to overweight in adulthood as well as diseases associated with obesity including cardiovascular problems and diabetes\(^3\)\(^4\)–\(^3\)\(^6\). These findings
have been shown to be in part due to epigenetic changes *in utero*, illustrating how maternal nutritional imbalances can alter fetal development and susceptibility to disease such as obesity\textsuperscript{134,135}.

Human and rodent studies have revealed how epigenetic changes can be influenced by age, toxicity exposure and diet\textsuperscript{136}. Consumption of a high-fat diet has been shown to alter the DNA methylation status of genes, in adipose tissue and skeletal muscle of both humans and mice\textsuperscript{126,137,138}. Furthermore, dietary omega-3 supplementation as well as caloric restriction have also presented epigenetic changes in both blood leukocytes and subcutaneous adipose tissue in humans respectively\textsuperscript{139,140}. Studies on exercise examining individuals with obesity and T2D, have presented altered DNA methylation in genes responsible for fatty acid metabolism and glucose transport, following 16 weeks of chronic exercise training\textsuperscript{141}.

Previous work studying epigenotoxic effects, the study of toxicants or “obesogen” influence on epigenetics, have illustrated how BPA exposure through the diet, results in altered expression of metastable epialleles such as *Agouti* in mice\textsuperscript{142}. Studies conducted in zebrafish, have also reported an alteration in DNA methylation status and consequently an increased expression in genes involved in adipogenesis, with increased exposure to the obesogen, MEHP\textsuperscript{143}. Thus, factors such as behaviour and environment have shown to regulate pre- and post-natal epigenetic changes, influencing weight and adiposity.

**1.5 Management and Treatment Options**

The treatment modality implemented for obesity, is reliant on not only BMI and body fat distribution, but also requires the consideration of co-morbidities, age, sex, ethnicity, genetics and psychosocial etiologies\textsuperscript{144}. Thus, overweight and obesity treatment guidelines typically
rely on a two-step process of 1) assessing the degree of obesity and exposure to risk factors and co-morbidities, followed by 2) management consisting of reduction, control and maintenance of both weight loss and associated risk factors following the treatment\textsuperscript{144,145}. Previous guidelines on the treatment and management of overweight and obesity, have illustrated how, while a weight reduction by 3-5\% may improve cardiovascular risk factors, a 10\% reduction could significantly ameliorate complications associated with the obesity\textsuperscript{145}. Details on assessing the degree of obesity and co-morbidities can be found in sections 1.1 and 1.3. The following section will focus on obesity treatment/management.

1.5.1 Obesity and Lifestyle Changes

Lifestyle changes to manage and treat obesity consist of low-caloric diets, increased physical activity and behavioral modifications to shift from a positive to a negative energy balance\textsuperscript{145}. Thus, this shift requires an energy deficit via reduced diet energy intake. However, due to the possible presence of co-morbidities and altered health status, various modes of reducing dietary energy intake exist. These include recommendation of a specific systematic energy intake target (i.e., 1200-1500 kcal/day for women, and 1500-1800 kcal/day for men), estimated energy deficit goals for an individual, such as a 30\% energy deficit or methods through which diet composition is altered to eliminate certain food groups\textsuperscript{145}. Dietary approaches that promote weight loss include, but are not limited to, high protein diets (calorie composition: 25\% protein; 30\% fat; 45\% carbohydrate), low carbohydrate diets (<30g/d of carbohydrates) and low fat diets (20\% of calories from fat)\textsuperscript{145}. The Mediterranean diet, initially observed and documented by Ancel Keys in the 1960s, consists of meals rich in fruits, vegetables and legumes, low in meat, while olive oil represents the main source of fat\textsuperscript{146}. Previous epidemiological studies on the effects of the Mediterranean diet on obesity, have
illustrated a reduction in BMI with increased adherence and consumption of the diet within cohort studies\textsuperscript{147}. While diets and meal replacements are easily accessible at home, commercially available diets are also available through companies including Weight Watchers\textsuperscript{145}. Furthermore, other modes of energy deficit also include clinically supervised meal replacement plans provided through weight management clinics (i.e., Optifast-900 used in the 900kcal/day diet and behavioral program at the Ottawa Hospital Weight Management Clinic) and other very low calorie diet approaches\textsuperscript{145}.

Exercise, characterized by repetitive physical activity consisting of either resistance or aerobic training, has been shown to be a treatment option for overweight and obesity\textsuperscript{148}. The practice guidelines published by the Obesity Society and American College of Cardiology/American Heart Association Task Force prescribe increased aerobic training of 150min/week, while 200-300min/week are suggested for weight maintenance and to prevent regain following weight loss\textsuperscript{145}. The ability of physical activity to assist in weight reduction has been illustrated in multiple large population studies including both the Canada Fitness Survey and the American “First National Health Nutrition and Examination Survey”\textsuperscript{148}. However, previous research has illustrated how exercise alone results in marginal improvement in weight loss\textsuperscript{148}. Thus, interventions consisting of both a diet and exercise regimen have been shown to improve weight loss considerably\textsuperscript{149,150}. Furthermore, while studies illustrate no difference in the degree of weight loss between varying intensities of exercise training in the presence of a diet, selected studies illustrate vigorous activity to be more efficient in weight reduction in comparison to moderate or light intensity exercise training, in the absence of dietary alterations\textsuperscript{148}. However, apart from contributing towards weight loss, exercise has been shown to participate in improving the overall health of an individual by the reduction of systolic blood
pressure, serum triglyceride levels and fasting serum glucose, with increased improvement with high intensity vs low intensity exercise\textsuperscript{148}.

While both exercise and diet ameliorate the negative health effects of obesity, as well as aiding in weight loss, behavioral modifications are required to minimize the potential weight gain that occurs following a lifestyle intervention\textsuperscript{145}. Generally, lifestyle interventions consist of a program in which patients are taught to monitor food intake, physical activity and their body weight to promote weight maintenance and prevent weight gain\textsuperscript{145}.

\subsection*{1.5.2 Obesity and Pharmacotherapy}

Pharmacotherapy, the use of drugs for the therapy of a disease, has been shown to provide beneficial results for long-term weight management in adults with obesity and overweight individuals with co-morbidities, such as type 2 diabetes and hypertension\textsuperscript{151}. Currently, only two of these drugs are available in Canada, Orlistat (Xenical\textsuperscript{®}) and Liraglutide 3.0mg (Saxenda\textsuperscript{®}).

Orlistat, available since the late 1990s has been shown to reduce dietary fat absorption through the gut with studies showing roughly a 2-3\% reduction in weight loss following drug administration\textsuperscript{151}. However, side effects for this drug include severe gastrointestinal effects, consequently resulting in only 10\% Canadian individuals prolonging prescription one year later\textsuperscript{152,153}.

Liraglutide, an agonist that targets the glucagon-like-peptide-1 (GLP-1), was initially approved for treating T2D, through its positive insulin stimulation and secretory glucagon suppression\textsuperscript{151}. However, due to the role of GLP-1 in energy homeostasis, via its ability to suppress appetite in T2D patients, the drug was marketed as an anti-obesity drug as well\textsuperscript{154,155}. 
Phase 3 trials illustrated an 8% weight loss in individuals with obesity with prediabetes at baseline\textsuperscript{156}. Furthermore, a phase 3 trial focused on studying the effects of the drug in weight maintenance and weight loss in the presence of a low-caloric diet, illustrated an average weight loss of 6.2% in individuals with obesity or overweight\textsuperscript{157}. Common side effects of the drug include nausea, vomiting and diarrhea\textsuperscript{151}.

Other anti-obesity drugs that have been approved by the FDA are: Phentermine/topiramate (PT) (Qsymia), lorcaserin (Belviq) and naltreoxone/bupropion (NB)\textsuperscript{151}. However, both NB as well as PT have been shown to require dose titration, while also contributing towards severe health effects including neuropsychiatric and teratogenicity for PT\textsuperscript{151}.

1.5.3 Obesity and Bariatric Surgery

Bariatric surgery, is an invasive surgical procedure conducted on the stomach and small intestine, to restrict food capacity within the stomach and decrease food intake/absorption of nutrients, while also causing alterations in hormonal levels\textsuperscript{158}. As the most successful treatment modality for combatting severe obesity (individuals with a BMI>40kg/m\textsuperscript{2} or suffering from co-morbidities with a BMI>35 kg/m\textsuperscript{2}), common bariatric surgery procedures include adjustable gastric band, Roux-en-Y gastric bypass, biliopancreatic diversion with duodenal switch and laparoscopic sleeve gastrectomy\textsuperscript{158,159}. With Roux-en-Y gastric bypass being the most frequently conducted bariatric surgery procedure (46.6%), a 2011 study reported both the United States and Canada to perform the highest number of operations worldwide (101 645 operations)\textsuperscript{159,160}.

Roux-en-Y procedures are conducted by ligating a small portion of the sectioned upper stomach to the bottom end of the divided small intestine\textsuperscript{158}. The top portion of the divided
small intestine is also ligated onto the bottom portion of the small intestine, to facilitate the mixing of stomach acids and digestive enzymes from the bypassed stomach into the newly constructed pouch.\(^{158}\)

While the key outcome of bariatric surgery is considerable weight loss, operations have also been shown to contribute towards the remission of T2D.\(^{159}\) Randomized control trials studying the effects of bariatric procedures in individuals suffering from severe obesity, provided evidence of increased short-term weight loss and remission of T2D, regardless of the procedure implemented in comparison to non-surgical treatments.\(^{159,161–166}\). Furthermore, improvements in serum triglyceride and high-density lipoproteins occur following bariatric surgery, while obesity associated co-morbidities including cancer and death were also decreased.\(^{161,167,168}\) However, bariatric procedures are not without risks, with side effects including, psychosocial, hormonal disturbances, nutritional deficiency including iron deficiency anemia and pulmonary complications.\(^{159,169}\) There is always the risk of reoperation due to postoperative complications and/or insufficient weight loss.\(^{159}\)

All *rectus abdominis* tissue and derived myocytes used within this Ph.D thesis were obtained during bariatric surgery procedures on women who had previously participated in the Ottawa Hospital Weight Management Clinic.

**1.6 Skeletal Muscle, Fatty Acid Metabolism and Obesity**

The following section will focus on skeletal muscle, as well as the interactions between the tissue, fatty acid metabolism and obesity.
1.6.1 Skeletal Muscle: Structure and Function

Skeletal muscle is one of the major organs of the human body, comprising roughly 40% of an average lean adult’s body weight\(^\text{170}\). As one of the three major muscle types (other two being smooth muscle and cardiac muscle), skeletal muscle’s main function is the conversion of chemical energy into mechanical energy to aid physical movement\(^\text{170}\). However, regarding whole-body metabolism, skeletal muscle is one of the key tissues that utilizes free fatty acids as a source of energy while contributing towards basal energy metabolism\(^\text{170}\). Other direct functions of skeletal muscle include storage site for various substrates including amino acids and carbohydrates for both itself and other tissues, as well as body core temperature maintenance via heat production\(^\text{170,171}\). Skeletal muscle can also indirectly impact the maintenance of blood glucose levels during starvation as well as regulate response to stress factors and illness\(^\text{170}\).

Skeletal muscle is a form of striated muscle tissue that is composed of muscle fibers, myofibril bundles surrounded by connective tissue called fasciae\(^\text{170}\). Skeletal muscle is attached to the bone via collagen fibers known as tendons, which allow the assisted movement of bones via skeletal muscle contraction\(^\text{170}\). Muscle fibers are characterized as being multinucleated, post-mitotic and are formed through myogenesis, the process where myoblast cells fuse into myotubes. The basic unit of the skeletal muscle responsible for contraction and the striated appearance of the tissue, are the sarcomeres, composed of long fibrous and filamentous proteins such as actin and myosin that help to make up the myofibrils within the muscle fibers\(^\text{170}\). Satellite cells, found between the sarcolemma and basal lamina are known to participate in muscle growth and repair as well as play a role in new muscle fiber generation.
when stimulated by myogenic factors\textsuperscript{170}. Thus, in order to meet all the energy demands of the tissue, muscle cells and consequently skeletal muscle, can be enriched with mitochondria\textsuperscript{170}.

Muscle fibers contain a sarcoplasmic reticulum responsible for calcium storage and release responsible for muscle contraction, and a highly interconnected network of mitochondria, unique from the commonly described circular form of the organelle\textsuperscript{170}. However, the following cellular components are significantly regulated by factors including exercise training and aging. Specifically, studies have illustrated how endurance and aerobic exercise can increase mitochondrial biogenesis, augmenting both number and size of mitochondria, while aging muscle results in a dysfunction of calcium release and inactivation of muscle due to fragmented sarcoplasmic reticulum\textsuperscript{170,172,173}.

Due to the high metabolic heterogeneity amongst different muscle fibers, to facilitate participating in diverse metabolic and mechanical processes, fiber-type composition of skeletal muscle is also variable\textsuperscript{170}. Fiber type composition of muscle fibers can be determined by the presence of myosin heavy chain (MHC) isoforms. Fiber-types are therefore categorized into two main types: Type I consisting of slow twitch oxidative aerobic muscle fibers (MHC-I isoform expression), and Type II consisting of fast twitch muscle fibers that are subcategorized into Type IIA referring to fast twitch oxidative glycolytic (MHC-IIa isoform expression) and Type IIX referring to fast twitch glycolytic fibers (MHC-IIx isoform expression)\textsuperscript{170}. Type I and IIA fibers have also been shown to have high levels of mitochondria compared to other fiber types\textsuperscript{170}.

The following Ph.D thesis focuses on studying human \textit{rectus abdominis} and \textit{vastus lateralis} cells and tissue. Studies on \textit{Vastus lateralis} muscle has shown that sex influences the area of occupancy for specific fiber-types, where men had a larger area occupied by Type IIA fibers.
while women had a larger area of Type I muscle fibers\textsuperscript{174}. While \textit{Rectus abdominis} tissue from non-obese subjects have been reported to present equal levels of both Type I and Type II muscle fibers, fiber composition has been shown to be affected by adiposity (decrease in type I fibers noted with obesity) and \textit{in vitro} glucose transport rate within humans\textsuperscript{175,176}.

### 1.6.2 Skeletal Muscle and Obesity

While a healthy lean individual’s skeletal muscle contributes towards 20-30\% of total resting oxygen uptake, over 90\% of the whole-body oxygen uptake during maximal physical activity is attributed to muscle oxygen consumption\textsuperscript{177}. The effects of obesity on muscle strength has been previously assessed in both adult and adolescents\textsuperscript{178–180}. Studies have illustrated a reduction in muscle strength when adjusted for body mass in adolescents, young adults and elderly individuals with obesity\textsuperscript{178–180}. This decrease in strength was coupled with defects in the activation of agonist muscles in individuals, illustrating profound defects in skeletal muscle action with weight gain\textsuperscript{181}. Studies have also illustrated an impairment in skeletal muscle regeneration within obesity-related models\textsuperscript{182}.

Apart from the effects on muscle strength and function obesity can also result in increased lipid deposition within skeletal muscle\textsuperscript{183,184}. Lipotoxic species linked to ectopic fat accumulation can interfere with insulin signalling, and contribute towards obesity induced insulin resistance\textsuperscript{185,186}. It has been reported that diet-induced weight loss can improve insulin sensitivity, paralleled by a decrease in intramyocellular lipids (IMCL), while a high fat diet increased IMCL and impaired sensitivity to insulin\textsuperscript{187,188}. In contrary, opposite findings have also been reported in athletes, entitled the “athlete’s paradox”, where highly insulin-sensitive athletes have also been shown to have elevated intramuscular lipid content including increased triglyceride levels\textsuperscript{189}.
1.6.3 Mitochondria and Obesity

Mitochondria, commonly known as the “powerhouses” of the cells, are the organelles that contribute towards cellular and consequently whole-body energy expenditure, via the intermediary pathways involved in the production of adenosine triphosphate (ATP). These vital organelles, also play a major role in other metabolic and cell signalling pathways including amino acid breakdown, apoptosis, ketogenesis and steroidogenesis\(^{190}\).

As mitochondria are highly dynamic, the morphology of these organelles is also quite variable and dependent on cell type. While fibroblast mitochondria are characterized to be predominantly filamentous, hepatocyte mitochondria are more fragmented and oval in shape\(^{191–193}\). The structural components of the mitochondria consist of a double lipid bilayer (an outer and inner mitochondrial membrane), the matrix (space that is encapsulated by the inner membrane and holds both metabolic enzymes and mitochondrial DNA), intermembrane space (region between the inner and outer membranes) and cristae (folds produced by the inner membrane)\(^{194}\).

The outer and inner membranes of the mitochondria are key in regulating the entry of molecules. Specifically, while the outer membrane is more porous and allows diffusion of molecules as large as 5 kDa into the intermembrane space via an integral membrane protein called porin, the highly selectively impermeable inner membrane only allows the transport of proteins through the translocase of the inner membrane (TIM)\(^{194}\).

The mitochondrial inner membrane contains enzymes that are responsible for translocating free fatty acids into the matrix, for fatty acid break down and energy transduction via β-oxidation and the Krebs’ cycle/tricarboxylic acid (TCA) cycle\(^{194}\). These are processes that
occur within the matrix, resulting in the final production of ATP through oxidative phosphorylation on the inner membrane\textsuperscript{194}. Section 1.6.4.2 will focus on describing the various mitochondrial pathways that play a role in fatty acid break down and energy production, as well as the effects of obesity on these pathways.

Mitochondria are commonly characterized as dynamic organelles that constantly undergo fusion and fission, processes that contribute towards mitochondrial structure and function maintenance\textsuperscript{195}. While the inner membrane is highly responsible for the various mitochondrial metabolic processes, it is also embedded with proteins responsible for mitochondrial dynamics such as DRP1, Mfn1/2 and Opa1\textsuperscript{196}. Fusion, the merging of mitochondrial structures, allow the mixing of mitochondrial contents and DNA via cross-complementation, mitigating the effects of environmental and genetic stressors that cause mitochondrial damage\textsuperscript{196}. Thus, a partially damaged mitochondrion can be rescued by fusing to one that is healthy and intact. Mammalian fusion proteins responsible for fusing the outer membranes are the membrane-anchored dynamin family members Mfn1 and 2, while inner membrane fusion is regulated by the single dynamin mammalian family member called Opa1\textsuperscript{197,198}. While fission, the division or splitting of mitochondria is a process that allows to produce new mitochondria, it also commonly functions as a facilitator of apoptosis and the removal of damaged mitochondria\textsuperscript{196,199}. Fission is mediated by the mammalian cytosolic protein dynamic family member (DRP1)\textsuperscript{198}.

Research studying the effects of obesity on mitochondrial dynamics have illustrated reduction in Mfn2 expression and mitochondrial size in Zucker rats suffering from obesity and insulin resistance\textsuperscript{200}. The reduction in Mfn2 levels, specifically within skeletal muscle of obese Zucker rats, was paralleled by a reduction in mitochondrial network\textsuperscript{201}. Human studies on
patients with obesity also displayed reduced expression levels of Mfn2\textsuperscript{200}. In contrast, the levels of proteins responsible for fission were shown to be elevated within the skeletal muscle of genetically and diet-induced obese mice\textsuperscript{202}. An association has also been observed between increased fission levels and mitochondrial dysfunction within liver and skeletal muscle in mice diet-induced obesity\textsuperscript{203}. Thus, levels of fission and fusion proteins, have been shown to be dysregulated by obesity, resulting in mitochondrial dynamic and metabolic dysfunction.

1.6.4 Cellular Metabolism and Obesity

Fatty acid metabolism is characterized by the combination of catabolic and anabolic processes that allow for the breaking down and storage of fatty acids, respectively. Fatty acids, chemically defined as a carboxylic acid with a long unsaturated or saturated aliphatic chain, are an important source of fuel for the human body. Processing fatty acids at the cellular level requires the conversion of fatty acids into esterified fatty acyl-CoA molecules via enzymes known as acyl-CoA synthetases (ACS)\textsuperscript{204}. Following esterification, the fatty acyl-CoA molecules can be shuttled with the assistance of other metabolic proteins into the endoplasmic reticulum, where they can play a role in lipid biosynthesis, or to the mitochondria, for fatty acid break down and energy production via β-oxidation, Krebs’ cycle and electron transport chain (ETC)\textsuperscript{204}. Energy can also be produced through glycolysis, the breaking down of glucose, to produce the necessary metabolites for energy production through the TCA cycle and ETC, via aerobic respiration\textsuperscript{204}. The following section will focus on explaining the different metabolic processes responsible for energy production, as well as how these pathways and associated-proteins are modified with obesity.
1.6.4.1 Acyl-CoA Synthetases and Obesity

Acyl-CoA Synthetases (ACS) are enzymes known to esterify intracellular free fatty acids into fatty acyl-CoA molecules. This process consists of two key steps where an acyl-AMP intermediate is generated from ATP, followed by AMP being replaced with CoA to produce the activated fatty acyl-CoA form\textsuperscript{121}. While the functional role of ACS enzymes is consistent, their substrate preference based on length of fatty acid carbon chains varies. Specifically, ACS can be separated into five sub-families based on their fatty acid chain length preference: short chain (ACSS) prefer C2-C4; medium chain (ACSM) prefer C4-C12; long chain (ACSL), which shall be the focus of this thesis prefer C12-C20; bubblegum (ACSBG) prefer C14-C24 and very long-chain also known as solute carrier family 27a (SLC27a) prefer C18-C26\textsuperscript{121}. It is important to note that while sub-families prefer a particular fatty acid chain length, this does not prevent ACS enzymes from recognizing and reacting to other fatty acids that are not within their preferred range. Specifically, ACSL6 has been shown to also metabolise very-long fatty acids such as Docosahexaenoic acid (DHA)\textsuperscript{205}. The following section will focus on the acyl-CoA synthetase long chain proteins (ACSLs).

ACSL proteins consists of five different members that differ by their substrate specificity, tissue and cellular localization: 1, 3, 4, 5 and 6\textsuperscript{117,204–223,224,225}. The cDNA of ACSL2 and ACSL1 were determined to be the same sequence\textsuperscript{121}. However, based on alternative splicing, each ACSL can also generate different spliced transcript variants that have differential cellular and tissue localization, thus contributing towards various metabolic processes. Specifically, while ACSL 3, 4, 5 and 6 mRNAs have been shown to produce long and short protein isoforms from upstream and downstream AUG start codons respectively, ACSL1 and ACSL6 also produce protein isoforms with different Gate-domains\textsuperscript{121,226–232}. The presence of two isoforms
has been reported for ACSL3 in rodent, ACSL5 in humans and ACSL4/6 in human and rodent\textsuperscript{121}. Previous \textit{in silico} analysis has also illustrated the possibility of varying subcellular localization for the different isoforms of the same ACSL protein\textsuperscript{121}.

ACSL proteins have been shown to have varying tissue distribution. Specifically, research conducted on studying ACSL mRNA levels observed the highest expression of rodent ACSL1 in liver, heart and adipose tissue, ACSL3 in brain and testis, ACSL4 in adrenal glands and liver, ACSL5 in brown adipose tissue, liver and duodenal mucosa and ACSL6 within brain, testis and gastrocnemius muscle\textsuperscript{233}. Human ACSL5, which is the key protein of interest for this Ph.D. thesis has also been shown to be one of the ACSLs expressed in brain, kidney and skeletal muscle\textsuperscript{234}.

Subcellular localization studies on ACSL proteins have shown varying cellular distribution of the enzymes, thus affecting their functional roles as well. ACSL1 and 5 have been shown to be present in the nucleus, on the endoplasmic reticulum, mitochondria and as well as the mitochondria-associated membrane (MAM), a region of the endoplasmic reticulum that links to the mitochondria, contributing towards processes such as lipid transport\textsuperscript{121,207,210–214,235}. ACSL6 has been shown to be present on the mitochondrial membrane as well\textsuperscript{121,212–214}. ACSL3 and ACSL4 have been shown to be present within lipid droplets, while ACSL4 is also present on the endoplasmic reticulum and MAM\textsuperscript{121,206,208,210}.

Rodent studies conducted on studying the functional roles of the different ACSL proteins have illustrated an upregulation of ACSL 1 and 4 mRNA following fasting and a reduction following refeeding of rats, while ACSL3 and 5 appeared to be reduced following fasting and increased after refeeding\textsuperscript{233}. However, it is important to note that when the same studies focused on ACSL1 and 4 protein expression levels, the opposite effects were noticed, thus
illustrating that the differences between mRNA and protein expression may be a result of altered translational or post translational regulation of the ACSLs\textsuperscript{233}. Furthermore, similar rodent studies conducted on isolated mitochondria from rats fasted for 48 hours illustrated an increase in ACSL5 protein levels and a reduction in ACSL4 protein levels, validating the possibility of translational and post-translational modifications of ACSL proteins\textsuperscript{210}. The remainder of this section will focus on the ACSL5 protein, the key protein of interest in this Ph.D. thesis. Furthermore, previous research conducted on ACSL5 function and association with obesity and other diseases, in both humans and rodents will be discussed as well.

1.6.4.1 ACSL5 and Obesity

Alternative splicing of the human ACSL5’s encoding exons results in three transcript variants, which has been shown to generate two major protein isoforms\textsuperscript{121}. Specifically, the upstream in-frame AUG-encoding exon produces a 739 aa long protein, while the downstream AUG produces a 683 aa short protein\textsuperscript{121}. The production of an uncommon human isoform of 659 aa (ACSL5Δ20) has also been reported and may contribute towards the regulation of TRAIL-induced apoptosis\textsuperscript{236}. Specifically, an increase in ACSL5/ACSL5Δ20 at the crypt-villus axis has been shown to result is death ligand TRAIL sensitization\textsuperscript{236}. However, while human ACSL5 has multiple protein isoforms, rodent ACSL5 only produces a single protein of 683aa, 81\% identical to the human short protein isoform\textsuperscript{121}.

Studies focused on understanding the functional role of ACSL5 and its association with obesity have generated controversial evidence. Specifically, rodent ACSL5 overexpression studies conducted in McArdle-RH7777 liver cell line have illustrated the proteins subcellular colocalization to be within mitochondria and endoplasmic reticulum\textsuperscript{237}. Furthermore, incubating these cells with 1\textsuperscript{14}C oleic acid resulted in a 30\% greater rate of fatty acid uptake
in comparison to control cells. Studies conducted on knockout mice models of ACSL5 (−/−) have illustrated not only a reduction in fat mass and adipose fat pad weights, but also an increase in energy expenditure and delayed fat absorption. However, studies focused on studying mitochondrial ACSL5 protein expression have illustrated that it is increased following a 48 hour fast in rats. It is important to note that such discrepancies may be a result of different experimental models (organelle studies vs cell culture and whole-body), while compensation by other ACSLs (increase in ACSL4 mRNA observed by Bowman et al. 2016) in rodent ACSL5 KO models may contribute to the in vivo observations. Regardless, while the functional role of rodent ACSL5 has been studied extensively, the separate functional roles of the two major human ACSL5 protein isoforms remains to be elucidated.

Previous research conducted in our lab focused on studying the genetic effects of an ACSL5 SNP, rs2419621, and the ability to lose weight, illustrated an association between the rare T allele with an increase in the rate of weight loss in women with obesity. Rs2419621, characterised by a cytosine (C) to thymine (T) transition in the promoter region of the ACSL5 gene, is located 12 nucleotides upstream of the second AUG transcription start site. Our lab has also previously shown that the presence of the T allele results in the generation of an additional cis-regulatory Ebox (CANNTG) site at the promoter region, increasing MyoD recruitment to the promoter, and thus increasing the expression of the downstream gene in vitro. Furthermore, skeletal muscle biopsies taken from women with obesity and who underwent a caloric restriction, illustrated a significant association between the presence of the rs2419621 [T] allele and increased ACSL5 mRNA levels. A section of the following thesis will focus on further establishing the genetic implications of carrying the rs2419621 T
allele on whole-body fat deposition, fatty acid oxidation and the *in vitro* and *ex vivo* effects of the polymorphism on fatty acid metabolism.

While ACSL5 has been shown to contribute towards fatty acid metabolism and obesity, a surplus of research has been conducted on the effects of ACSL5 expression on cancer progression and *vice versa*. Studies conducted on human glioblastoma cell lines, have illustrated an overproduction of ACSL5 mRNA in comparison to normal brain cells, while adenoviral introduction of ACSL5 human cDNA into glioma cell lines, also resulted in an increase in cell growth in the presence of palmitate\textsuperscript{234}. Research conducted on human colorectal cancer tissues illustrated contradictory results where studies have illustrated both a decrease and increase in ACSL5 mRNA within cancerous tissue\textsuperscript{239,240}. Furthermore, studies conducted on the human small intestine to study adenocarcinomas, adenomas of the small intestine and Crohn’s disease illustrated a reduction in ACSL5 mRNA within these conditions in comparison to control samples\textsuperscript{241–243}. Thus, while discrepancies in ACSL5 mRNA expression and regulation do exist when observing different cancerous tissues, research does imply the importance of ACSL5 regulation in both obesity and cancer. Furthermore, while studies continue to observe the effects of human ACSL5 protein on metabolism, the separate metabolic functional roles of the two common human isoforms remains to be elucidated.

**1.6.4.2 Fatty Acid and Glucose Oxidation/Mitochondrial Bioenergetics**

Following the esterification of free fatty acids, the fatty acyl CoA molecules are transported into the mitochondria via carnitine palmitoyl transferase 1 and 2 (CPT1 and CPT2 respectively)\textsuperscript{204}. Following entry into the mitochondrial matrix, fatty acyl-CoA molecules are broken down through the β-oxidation pathway, a catabolic process that produces acetyl-CoA metabolites for the downstream TCA cycle, as well as reducing agents NADH and FADH\textsubscript{2} for
the ETC\textsuperscript{204}. β-oxidation, characterized by the oxidation of the beta carbon of the fatty acyl-CoA molecule, consists of four key enzymes: acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and thiolase\textsuperscript{204}.

Acetyl-CoA undergoes further breakdown through the TCA cycle to produce carbon dioxide and additional reducing agents for the final step of energy production via the ETC\textsuperscript{204}. Specifically, isocitrate to α-ketoglutarate production produces carbon dioxide as well as NADH\textsuperscript{204}. NADH is also produced during conversions of α-ketoglutarate to succinyl-CoA and malate to oxaloacetate, while FADH\textsubscript{2} is produced during the conversion of succinate to fumarate\textsuperscript{204}.

Fatty acid oxidation results in the production of both acid soluble products (ASP) and carbon dioxide, indicative of incomplete and complete fatty acid oxidation respectively\textsuperscript{244,245}. ASP, are intermediate metabolites produced during both β oxidation as well as TCA cycle, that are not completely metabolized to produced carbon dioxide\textsuperscript{244,245}.

While acetyl-CoA can be provided through fatty acid breakdown, glycolysis, the catabolic process resulting in glucose breakdown, also produces acetyl-CoA via pyruvate metabolism. Specifically, glycolysis, results in the production of pyruvate and two molecules of ATP, while also producing reducing agents for the electron transport chain\textsuperscript{204}. Pyruvate, the final product of glycolysis can undergo either aerobic respiration via TCA cycle or anaerobic respiration, when there isn’t sufficient oxygen for aerobic respiration to occur\textsuperscript{204}.

The ETC, the site of oxidative phosphorylation which is found on the mitochondrial inner membrane, consists of four key protein complexes (Complex I-IV) responsible for transferring electrons from electron donors to electron acceptors\textsuperscript{204}. While participating in these series of
redox reactions (coupled reduction and oxidation reactions), oxygen is also consumed during this process. Reducing agents formed through β oxidation, TCA cycle and glycolysis, drive the following reactions\textsuperscript{204}. The energy produced from the electron transfer, provides the complexes adequate energy to pump protons across the inner membrane into the intermembrane space, contributing towards the formation of an electrochemical proton gradient\textsuperscript{204}. This proton gradient drives the synthesis of ATP by shuttling back protons into the matrix via the ATP synthase (Complex V)\textsuperscript{204}. This chemical energy moiety ATP, is created through combining adenosine diphosphate and a phosphate molecule to produce a high-energy bond\textsuperscript{246}. The complete oxidation of palmitate has been shown to produce a maximum of 106 ATP molecules\textsuperscript{204}.

While protons can be shuttled into the matrix through the ATP synthase, uncoupling proteins (UCP1-5), have been shown to contribute towards proton leak, defined as the travelling of protons through the membrane into the matrix, independent of ATP synthase\textsuperscript{246}. Energy in this process, is released as heat. In human skeletal muscle, UCP3 has been shown to contribute towards proton leak, while being predominantly expressed in this tissue\textsuperscript{247,248}. Furthermore, proton leak has been shown to contribute towards roughly 50% of basal respiration within resting skeletal muscle tissue; the rate is much lower in contracting muscle\textsuperscript{249}.

Reactive oxygen species (ROS), are formed during oxidative phosphorylation in the ETC, where incomplete reduction of oxygen produces superoxide radicals, known to be the precursor molecule to ROS. Specifically, ROS formation via complex I and III, is in part due to the lack of protons present for the efficient reduction of oxygen to water molecules, increasing the formation of superoxide radicals. Excess ROS has been shown to contribute towards oxidative damage and can be regulated by uncoupling proteins via increased proton
Recent research conducted in studying the concept of supercomplex formation have illustrated its role in regulating ROS production. A concept initially proposed in 1955, the formation of groups of ETC complexes on the mitochondrial inner membrane, involving complexes I, III and IV have been shown to play a role in increasing oxygen consumption, electron flux and consequently mitochondrial respiration, by reducing the distance that electrons are carried across complexes.

1.6.5 Skeletal Muscle, Fatty Acid Metabolism and Obesity

Skeletal muscle is one of the primary tissues to utilize free fatty acids as a source of energy. Studies have shown that subjects with obesity have decreased fatty acid oxidation resulting in increased accumulation of intra-myocellular lipids within skeletal muscle. Furthermore, studies conducted on the effects of overfeeding rats with increased fat and sucrose within their diet, illustrated a reduction in muscle mitochondrial oxidative phosphorylation activity. Additional observations regarding a decrease in ETC protein expression in skeletal muscle, have been also associated with obesity. Specifically, individuals with obesity presented reduced levels of cytochrome C oxidase (COX), citrate synthase (CS) and CPT1 in comparison to lean subjects. Conversely, research findings on humans with obesity who underwent an exercise training have illustrated an increase in CPT1 expression and activity, which could result in the improved muscle mitochondrial fatty acid oxidation levels that has been observed.

However, while increase in fatty acid oxidation may increase fatty acid breakdown and reduce lipid accumulation, excessive fatty acid oxidation without the upregulation of TCA cycle and ETC, could also result in high rate of incomplete fatty acid oxidation and oxidative stress due to mitochondrial overload, contributing towards insulin resistance. Studies on mice and cell
models illustrated how obesity-related insulin resistance during exposure to a high fat diet/environment, resulted in an increased expression of genes related to fatty acid oxidation as well as ASP, resulting in increased incomplete fatty acid oxidation and accumulation of intermediary metabolites such as excessive acylcarnitines\textsuperscript{263}.

While studies have shown a decrease in skeletal muscle mitochondrial number to be implicated in insulin resistance, via IMCL accumulation due to reduced fatty acid oxidation, some rodent high-fat studies have observed increased mitochondrial biogenesis via PPAR\(\delta\), resulting in a post-transcriptional increase of PGC-1\(\alpha\)\textsuperscript{245,264–267,268}. Interestingly, work conducted in our lab have shown an impairment in \textit{rectus abdominis} mitochondrial oxidative phosphorylation and ETC supercomplex assembly within diabetic obese individuals\textsuperscript{269}.

\textbf{1.7 Research Objectives and Hypotheses}

While treatment modalities for obesity include bariatric surgery, lifestyle modifications and pharmacotherapy, a recently discovered treatment option is the concept of “personalized medicine” in the context of obesity. Personalized treatment caters towards an individual's genotype in order determine the appropriate treatment options available for an individual with obesity\textsuperscript{270}. This includes diet alteration and behavioral changes. However, while current studies on personalized medicine focuses on genotype, there has been an increased necessity to consider gene-environmental interactions because environment has been proven to play a pivotal role in obesity. Thus, the overall goal of this Ph.D. thesis was to study the interactions between environmental and genetic factors on the metabolic response in women with overweight and obesity with a focus on skeletal muscle metabolism.
This was achieved by the testing the following hypotheses:

**General Hypothesis 1:** The ACSL5 genotype plays a key role in response to diet and exercise intervention in women with overweight and obesity by influencing fatty acid metabolism.

**Objective #1 (Genetic Association Study Approach):** Test the association between ACSL5 rs2419621 genotype and response of women with obesity and overweight to lifestyle interventions.

**Objective #2: (Molecular Mechanistic Approach):** Determine the cellular and molecular mechanisms by which rs2419621 ACSL5 polymorphism exerts its effect on fatty acid metabolism.

**General Hypothesis 2:** The MEHP toxicant plays a key role in the dysregulation of fatty acid and glucose metabolism *in vitro* and *in vivo*.

**Objective #3 (Environmental Association Study Approach):** Test the association between MEHP exposure and fatty acid utilization within women with obesity who participated in observational studies.

**Objective #4 (Molecular Mechanistic Approach):** To determine the cellular and molecular mechanisms by which MEHP exerts its effect in skeletal muscle.
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CHAPTER TWO: THE ASSOCIATION BETWEEN ACSL5 RS2419621 GENOTYPE AND RESPONSE OF WOMEN WITH OBESITY AND OVERWEIGHT TO LIFESTYLE INTERVENTIONS.

ACYL-COA SYNTHETASE LONG-CHAIN 5 GENOTYPE IS ASSOCIATED WITH BODY COMPOSITION CHANGES IN RESPONSE TO LIFESTYLE INTERVENTIONS IN POSTMENOPAUSAL WOMEN WITH OVERWEIGHT AND OBESITY: A GENETIC ASSOCIATION STUDY ON COHORTS MONTRÉAL-OTTAWA NEW EMERGING TEAM, AND COMPLICATIONS ASSOCIATED WITH OBESITY

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2.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

2.1.1 STATEMENT OF MANUSCRIPT STATUS

The manuscript “Acyl-CoA synthetase long-chain 5 genotype is associated with body composition changes in response to lifestyle interventions in postmenopausal women with overweight and obesity: a genetic association study on cohorts Montréal-Ottawa New Emerging Team, and Complications Associated with Obesity” has been published in the journal of *BMC Medical Genetics*.

2.1.2 CONTRIBUTION STATEMENT

AR participated in genotyping DNA samples from patients who participated in the studies, performed the statistical analyses, interpreting the data, participated in the design of the study, drafted the manuscript. GL participated in the statistical analysis by revising the statistical methodology critically. He also revised the entire manuscript. PB participated in the genotyping studies and revised the article critically. MH participated in interpreting the data and revised the article critically. KA participated in interpreting the data and revising the article critically. ED participated in recruitment of patients and established the database. He also helped in revising the article critically. RRL participated in recruitment of patients and established the database. He also helped in revising the article critically. DP participated in recruitment of patients and established the database. He also helped in study design and revising the article critically. FT performed majority of the study design, interpreted the data and revised the article critically. All authors read and approved the final manuscript.

2.1.3 COMPETING INTEREST STATEMENT

The authors declare that they have no competing interests.
2.2 ABSTRACT

Background: Genetic studies on Acyl-CoA Synthetase Long-Chain 5 (ACSL5) demonstrate an association between rs2419621 genotype and rate of weight loss in women with obesity in response to caloric restriction. Our objectives were to (1) confirm results in two different populations of women with overweight and obesity (2) study rs2419621’s influence on body composition parameters of women with overweight and obesity following lifestyle interventions. Methods: rs2419621 genotype was determined in women with overweight and obesity who participated in the Montréal-Ottawa New Emerging Team (MONET n = 137) and Complications Associated with Obesity (CAO n = 37) studies. Genotyping was done using TaqMan MGB probe-based assay. Multiple linear regression analyses were used to test for associations. Results: When studying women with overweight and obesity, rs2419621 [T] allele carriers had a significantly greater decrease in visceral fat, absolute and percent fat mass and a greater increase in percent lean mass in response to lifestyle intervention in comparison to non-carriers. Studying only individuals with obesity showed similar results with rs2419621 [T] allele carriers also displaying a significantly greater decrease in body mass index following the lifestyle intervention in comparison to non-carriers. Conclusion: Women with overweight and obesity carrying the ACSL5 rs2419621 [T] allele are more responsive to lifestyle interventions in comparison to non-carriers. Conducting such genetic association studies can aid in individualized treatments/interventions catered towards an individual’s genotype.

2.3 INTRODUCTION

Obesity, recently defined as a disease by the American Medical Association, and overweight are major risk factors for a variety of chronic diseases including cardiovascular diseases, type 2 diabetes and cancer [1]. Genetic and epigenetic mechanisms, mediated by lifestyle and
environmental exposures have been implicated in the development of obesity and these chronic diseases [2, 3].

As a critical component of metabolic pathways, fatty acyl-CoA molecules are known to be implicated in energy production by β-oxidation, energy storage through lipid biosynthesis and as lipid components of the cell. The acyl-CoA synthetases long-chain (ACSL) stimulates intracellular free long-chain fatty acids by converting them to fatty acyl-CoA molecules. Members of the ACSL family, ACSL 1, 3, 4, 5 and 6 are characterized by varying subcellular localization, fatty acid substrate and tissue specificity [4]. ACSL5 is present in various species including humans and rodents, while also being distributed in a wide range of tissues including skeletal muscle, liver, and brain [5]. ACSL5 has been detected in rat liver cytosol, endoplasmic reticulum and mitochondrial outer membrane [6]. Increased ACSL5 protein levels have been observed during food deprivation in rats [6]. Furthermore, ACSL5 plays a role in facilitating fatty acid channelling between anabolic lipid synthesis and catabolic β-oxidation pathway [6, 7]. Previous research conducted in our laboratory demonstrated that among 8 polymorphisms along the ACSL5 gene, only the common single nucleotide polymorphism (SNP) rs2419621, found in the promoter region, displayed a significant association with rate of weight loss response in women with obesity participating in a weight management program (which included an initial 6-week period of 900 kcal meal replacement) [8]. Characterised by a cytosine to thymine transition, rs2419621 is located 12 nucleotides upstream of the second transcription start site of ACSL5. The presence of the [T] allele produces a new cis-regulating E-box site (DNA binding sites for E-proteins and myogenic regulatory factors such as MyoD) at the promoter region of ACSL5 in addition to the two wildtype E-box elements [9]. The presence of this extra E-box, has been shown to increase the amount of MyoD recruited to the
ACSL5 promoter *in vitro* and to increase the expression of the downstream gene [9]. Furthermore, a 2.2-fold increase of ACSL5 transcript level was observed in skeletal muscle biopsies from individuals that are homozygous for the rs2419621 [T] allele when compared to homozygous wildtype individuals [8].

The objective of the present study was (1) to validate the influence of rs2419621 ACSL5 polymorphism on an independent population of women with obesity (2) To study the influence of rs2419621 on weight loss and body composition changes in response to lifestyle interventions on women with overweight and obesity. This article reports an increased response in women with overweight and obesity carrying the ACSL5 rs2419621 [T] allele to lifestyle interventions in comparison to non-carriers.

2.4 METHODS

*Subjects*

Women categorized as being overweight or obese (n = 174) who participated in two hypocaloric lifestyle intervention studies Montréal Ottawa New Emerging Team study (MONET study) and Complication Associated with Obesity study (CAO study) were examined.

*Ethics, consent and permissions*

All participants provided informed consent to engage in the lifestyle intervention as well as to the genetics components of these studies. Both cohort studies were approved by Université de Montréal ethics committee with agreement to the Declaration of Helsinki.
**MONET and CAO intervention studies**

The MONET study (n = 137) and the CAO study (n = 37) recruited women between 2003 and 2007. The MONET study included postmenopausal women with overweight or obesity, while the CAO study population was composed of 35 women with obesity and two women with overweight. Both the MONET and the CAO cohorts and lifestyle intervention have been previously described and share major similarities [10]. Briefly, women were eligible to participate if they met the required criteria including: (1) Body mass index (BMI) ≥ 27 kg/m², (2) menstruation ceased >1 year and plasma follicle-stimulating hormone levels ≥ 30 U l⁻¹, (3) non-diabetic, (4) non-smokers, (5) no hormone replacement therapy, and (6) <2 h/week of structured exercise. The aim of MONET and CAO studies lifestyle intervention was a 10 % body weight reduction over 6 months. Caloric restriction targets for both studies were determined by subtracting 500 to 800 kcal from participants’ daily energy needs. The daily energy needs were calculated by multiplying resting metabolic rate of each participant (determined by indirect calorimetry [11]) to a physical activity factor of 1.4 as women were not exercising regularly at the onset of the trial. Dietary prescriptions ranged from 1100 to 1800 kcal/day. The diet macronutrient composition was 55, 30, and 15 % of energy intake from carbohydrates, fat, and protein, respectively [11]. A third of the MONET participants were enrolled in a resistance-training program whereas the CAO participants were not enrolled in resistance training. Subjects from both MONET and CAO studies were combined for statistical analyses after verifying that there were no significant differences between participants’ weight loss and body composition changes in response to the different lifestyle interventions.
**Studied variables**

Analyses of these variables have been previously described [10]. Briefly, body weight measurement was conducted using a calibrated balance (Balance Industrielle Montréal, Québec, Canada) while other body composition variables, including fat mass and lean mass, were measured by Dual-energy X-ray absorptiometry (DXA) scans using the LUNAR Prodigy system (software version 6.10.019; General Electric Lunar Corporation, Madison, WI, USA). Height was measured using a wall stadiometer (Perspective Enterprises, Portage, MI, USA) while BMI was calculated as body weight (kg)/height(m)^2. Visceral fat and thigh muscle attenuation were measured by computed tomography scan (General Electric Medical Systems, Milwaukee, WI, USA). Adjusting variables were measured using the COBAS INTEGRA 400 analyzer (Roche Diagnostic, Montréal Canada) for high-density lipoprotein cholesterol (HDL-C), triglycerides and glucose. The Friedwald formula was utilised to calculate cholesterol (LDL-C) concentration. C-reactive protein (CRP), haptoglobin, transferrin and orosomucoid levels were measured by immunonephelometry on an IMMAGE analyzer (Beckman Coulter, Villepinte, France).

**Isolation of DNA and rs2419621 genotyping using TaqMan MGB probe-based assay chemistry**

Isolation of genomic DNA from the blood samples of individuals who participated in both MONET and CAO studies, was performed using a Qiagen Flexigene DNA kit. The ACSL5 rs2419621 SNP (CC, CT, TT) was genotyped using the TaqMan MGB probe-based assay chemistry (Life Technologies- Applied Biosystems). The PCR cycle (Biorad’s CFX-96 Real Time PCR) was as follows: enzyme activation at 95 °C for 10 min., denaturation at 92 °C for 15 s., 60 °C for 1 min-annealing/extension followed by 39 more repeats from the denaturation
step. A positive control was used in order to ensure reliability of results. Biorad’s CFX96 real-
time system was used for allelic discrimination while analysis of the data from end point
fluorescence measurements were used to determine genotypes. Random samples were
 sequenced (Applied Biosystem) in order to confirm the genotype.

**Statistical analysis**

Departure from Hardy-Weinberg proportions was tested on combined MONET and CAO
participants using a Pearson’s chi-squared test.

Multiple linear regression analyses were conducted on subjects whose blood samples and
biological parameter measurements (for dependent and adjusting variables) were available.
Multiple linear regression analyses were conducted on: 1) All MONET and CAO participants
and, 2) MONET and CAO women with obesity only (BMI ≥30).

Percentage changes in variables following intervention were calculated by subtracting post-
intervention from baseline values divided by baseline values multiplied by 100. For this study
we only had access to the pre and post-intervention values. Variables studied included BMI,
fat mass, lean mass, visceral fat and thigh muscle attenuation. Both the absolute values and
percentage values were studied for fat mass and lean mass [percent fat mass = (fat mass / total
body mass) x 100,  with total body mass = fat mass + lean mass; percent lean mass = (lean
mass / total body mass) x 100].

Regression analyses on the data sets for BMI, fat mass, lean mass, visceral fat and thigh muscle
attenuation were conducted. Best subset regression analysis was conducted to determine which
covariables should be included and adjusted for in the multiple linear regression analysis.
Specifically we considered the following independent variables 1) based on their causative
effect on weight loss: age, baseline weight, height, changes in transferrin, glucose, CRP, orosomucoid and haptoglobin 2) based on their correlation with weight loss: HDL-C, LDL-C, triglyceride [8]. The inclusion of a variable that is correlated with the response can reduce the error variance and increase the power of the regression analysis. The selection criteria to determine the best subset of independent variables was based on the adjusted R-squared and Mallows Cp. Specifically, we conducted a best subset regression analysis on data from populations consisting of women with overweight or obesity (Table 1) and women with obesity (Table 2). However prior to conducting any detailed statistical analyses, any extreme outliers found within the data sets of dependent and independent variables (if present) were excluded. Extreme outliers were determined based on observed values with a large standardized residual and large leverage. Specifically, one value from haptoglobin, transferrin and CRP was excluded, while two values were excluded for orosomucoid.

In order to determine whether MONET & CAO populations could be pooled together for statistical analysis, unpaired t-tests were conducted to test for study effect. Both pre-intervention and post-intervention parameter values were compared between lifestyle intervention studies. Multiple linear regression analysis was also conducted in order to observe study effect through the use of multiple dummy variables on data for women with overweight or obesity combined. Studies were conducted comparing CC carriers from the MONET study to CC carriers from the CAO study. Specifically independent variables included three dummy variables representing the subgroups (CAO-CC carriers, CAO-CT/TT carriers and MONET CT/TT carriers) with the exception of CC carriers from the MONET study and the covariables that were determined to be appropriate for adjustment. Covariables were determined through best subset regression models for each dependent variable (Table 1). Similar analyses were
conducted comparing CT/TT carriers from the MONET study and CT/TT carriers from the CAO study. Analysis was also conducted on women with obesity alone using the appropriate covariables. Covariables were determined by best subset regression (Table 2).

A multiple linear regression analysis was used to evaluate the association between ACSL5 rs2419621 genotype and changes in anthropometric and metabolic characteristics following the lifestyle intervention. A dominant genetic model (CT/TT vs. CC) was utilized due to the lack of individuals homozygous for the [T] allele. The multiple linear regression models included the genotypes as an independent variable. The multiple linear regression models were conducted on data sets of dependent variables. Covariables that were included were only specific to the best subset regression conducted for each dependent variable (Tables 1 and 2). P-values <0.05 were considered statistically significant. Data analysis was performed with Minitab software 17.

2.5 RESULTS

Comparison of pre- and post-intervention anthropometric variables’ values in each studied cohort and test for intervention effects

Pre- and post-intervention anthropometric variables from the MONET and CAO cohorts were compared using unpaired t-tests (Table 3). No significant differences in age, height, percent fat and lean mass were found between the two cohorts pre-intervention. Furthermore, no significant differences were observed for pre- and post-intervention absolute values for lean mass, visceral fat, and thigh muscle attenuation. However, significant differences were observed between pre- and post-intervention values for BMI and absolute value of fat mass
between the two cohorts, as well as post-intervention values for percent fat mass and percent lean mass.

When analysing study effects through the use of multiple linear regression analysis using a general linear model, no significant difference was observed between the changes in variables following lifestyle intervention, between CC carriers in MONET and CAO studies. The same results were observed between CT/TT carriers in MONET and CAO studies, illustrating no study effect between the two cohorts of interest (Tables 4 and 5).

Association analysis between ACSL5 rs2419621 genotype and changes in anthropometric variables following the lifestyle intervention

The ACSL5 rs2419621 genotype frequencies were in Hardy-Weinberg equilibrium (HWE). Data sets were analyzed using multiple linear regression analysis adjusted for confounding variables specific to each dependent variable, to determine the association between rs2419621 genotype and changes in anthropometric variables following the lifestyle intervention (Table 6). [T] allele carriers showed a statistically significant greater decrease in their visceral fat as well as their absolute and percent fat mass values and a statistically significant greater increase in their percent lean mass in comparison to non-carriers following the interventions.

Comparison of pre- and post-intervention anthropometric variables' values in women with obesity from each cohort studied and test for lifestyle intervention effect

The pre- and post-intervention anthropometric variables' values from the MONET and CAO cohorts consisting of only women with obesity were compared using unpaired t-tests (Table 7). No significant differences were observed between the different cohorts’ pre- and post-intervention values for any of the biological dependent variables studied.
A multiple linear regression analysis using a general linear model was also utilised to test whether body composition parameter changes were influenced by lifestyle interventions. No significant difference was observed between the changes in outcome variables (specifically change in BMI, absolute and percent fat mass, absolute and percent lean mass, visceral fat and muscle attenuation) following the lifestyle intervention, between CC carriers with obesity in MONET and CC carriers with obesity in CAO cohorts. Similar results were observed when studying CT/TT carriers between the two cohorts illustrating no significant difference between both study lifestyle interventions (data not shown).

Association analysis between ACSL5 rs2419621 genotype and changes in anthropometric variables following the lifestyle intervention in MONET and CAO women with obesity

Dependent variable data sets were analyzed using multiple linear regression analysis adjusted for co-variates, to determine the association between rs2419621 genotype and changes in anthropometric variables following the lifestyle intervention in women with obesity (Table 8). A statistically significant greater decrease in BMI, absolute and percent of fat mass values, as well as visceral fat was noticed following the lifestyle intervention in women with obesity carrying the [T] allele vs. non-carriers. A statistically significant greater increase in percent lean mass values was observed in [T] allele carriers vs. non-carriers women with obesity following the lifestyle intervention.

2.6 DISCUSSION

In 2014, according to the World Health Organization (WHO), obesity was shown to be present in about 13 % of the world’s adult population, while 39 % of adults 18+ were overweight, with 40 % of women being overweight [12]. The objective of this study was to evaluate the
influence of ACSL5 rs2419621 genotype on the changes in body composition parameters in response to lifestyle intervention in women with overweight or obesity. In order to do so, the body composition changes in women participating in two lifestyle weight-reducing interventions, the MONET (diet and/or resistance training) and CAO (diet) studies were analyzed according to their genotype.

Prior to conducting analysis to determine the influence of rs2419621 genotype, lifestyle intervention-related changes in body composition parameter differences were compared between the two cohorts in order to determine whether they could be combined for statistical analyses. The MONET and CAO study population and designs shared a lot of similarities [10]. Specifically, both studies used the same hypocaloric diet interventions. Both studies also included non-diabetic postmenopausal women, with the MONET study including women with overweight or obesity, while CAO focused on women with obesity. A portion (one third) of the MONET study participants were assigned to resistance training, in addition to the diet intervention. Furthermore, criteria for exclusion were similar between studies. Significant differences were observed between pre and post intervention BMI and absolute fat mass values between the two cohorts, as well as for percentage of fat mass and percentage of lean mass in post intervention. These observations were expected as CAO cohort was nearly exclusively composed of women with obesity. No significant differences in age, height, pre percentage of fat/lean mass, pre and post values for visceral fat, absolute lean mass and thigh muscle attenuation were identified. The significant differences seen in pre and post intervention values between the cohorts for various outcome variables studied when women with overweight or obesity were pooled together, were abolished once the population was narrowed down to women with obesity only. This indicates that rather than observing a study effect, significant
differences observed in the pre and post-intervention values between MONET and CAO cohorts consisting of both women with overweight or obesity, was a result of difference in BMI between the two cohorts, with a much larger proportion of women being overweight in the MONET cohort.

Association analyses confirmed that women with overweight or obesity carrying the [T] allele were more responsive to the MONET and CAO lifestyle interventions in comparison to non-carriers. More specifically, carriers of the [T] allele had a greater decrease in fat mass, while also displaying a greater increase in lean mass in comparison to non-carriers. [T] allele carriers also displayed a greater decrease in their visceral fat in comparison to non-carriers. Furthermore, when considering just women with obesity, in addition to observing similar results as the study with individuals with overweight and obesity, [T] allele carriers also illustrated a greater decrease in their BMI in comparison to CC individuals.

The results of the present study demonstrate a greater decrease in visceral fat in carriers of the ACSL5 rs2419621 [T] allele, in comparison to non-carriers when subjected to lifestyle intervention aimed at weight reduction. Excess accumulation of visceral fat, characterised as fat packed between inner organs, is associated with an increased risk of metabolic syndrome [13]. Various proposed mechanisms, such as the “portal theory” have been suggested to explain the relationship between visceral adiposity and common cardiometabolic diseases. Specifically, the “portal theory” states a rise in lipolytic activity within visceral adipocytes, contributing to an increased delivery of metabolic by-products such as free fatty acids into the liver, eventually resulting in insulin resistance [14, 15]. Furthermore, elevated serum leptin levels are correlated with subcutaneous fat but not visceral fat deposition [16]. This decrease
in serum leptin levels observed with omental fat tissue deposition contributes to a decreased regulation of appetite and impacts energy balance regulation [16].

Individuals with the rs2419621 [T] allele appeared to have a greater decrease in fat mass and a greater increase in their lean mass following the intervention, in comparison to non-carriers. While fat mass encompasses the overall fat composition of an individual, lean mass represents non-fat containing tissue mass including bone connective tissue, skin, organs and the muscle mass of an individual. Thus carriers of the [T] allele have a greater loss in fat and increase in lean body mass, in comparison to non-carriers.

Our results obtained from studying the effects of the rs2419621 polymorphism on subjects who participated in the MONET and CAO lifestyle intervention studies, validate our previous results from the 900-cal/day meal replacement study [8]. While our previous work focused on studying the effects of rs2419621 [T] allele carriers on rate of weight change, the present paper has shown the effects of the polymorphism on changes in body composition indices. Furthermore, the results from our current study illustrate that the rs2419621 polymorphism has a strong effect on subjects’ response to diet/ exercise intervention, resulting in moderate weight and fat loss, as statistically significant findings were observed even with interventions that did not have a strict meal replacement plan implemented.

At this point, the molecular mechanism by which the rs2419621 [T] allele exerts its effect is still unknown. However, the [T] allele has been shown to be associated with increased ACSL5 expression in *rectus femoris* muscle [8]. Furthermore, we showed, *in vitro*, that the [T] allele generates a cis-regulatory E-box element recognized by MyoD, a myogenic regulatory factor [9]. The [T] allele promotes MyoD-dependent activation of the ACSL5 promoter, suggesting a direct link between level of expression of ACSL5 and rs2419621 genotype [9]. Since ACSL5
is known to be mitochondrially localized, [T] allele carriers might present higher fat oxidation levels due to increased level of ACSL5.

2.7 CONCLUSION

Based on the observed statistical analyses, carriers of the ACSL5 rs2419621 [T] allele have been shown to be more responsive to the MONET and CAO lifestyle interventions in comparison to non-carriers. The strong association observed between rs2419621 [T] allele and response to diet/exercise intervention, has led to the hypothesis of individuals with the [T] allele having increased fat oxidation in comparison to individuals with the wild type. Our study confirms and provides additional insights on the influence of the rs2419621 polymorphism in response to lifestyle interventions as it is an expansion of the previous work from Adamo et al. 2007 [8]. However, it should be noted that population stratification was not controlled for in our study. As our current study focused on the effect of rs2419621 on postmenopausal women, future directions include replicating these findings in a large male population or male and female population. Furthermore future work with in vitro/ in vivo models studying the regulation of fatty acid β-oxidation by the rs2419621 [T] polymorphism are needed. Such genetic association studies can aid in designing individualized treatments/interventions for weight loss catered towards an individual’s genotype specifically by modifying both diet and exercise.

2.8 REFERENCES


### 2.9 TABLES

**Table 1:** Best subset regression analysis conducted on MONET and CAO women with overweight or obesity

<table>
<thead>
<tr>
<th>Models of dependent variable studied</th>
<th>R-Sq (%)</th>
<th>R-Sq (adj)</th>
<th>Covariables associated with mentioned R value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in BMI</td>
<td>13.1</td>
<td>8.5</td>
<td>Height, change in glucose, change in CRP, change in transferrin and change in triglyceride</td>
</tr>
<tr>
<td>Change in Lean Mass</td>
<td>17.8</td>
<td>12.7</td>
<td>Height, initial weight, change in HDL cholesterol, change in LDL cholesterol, change in triglyceride, change in glucose</td>
</tr>
<tr>
<td>Change in Fat Mass</td>
<td>21.3</td>
<td>16.4</td>
<td>Initial weight, change in glucose, change in CRP, change in transferrin, change in LDL cholesterol, change in triglyceride</td>
</tr>
<tr>
<td>Change in % Fat mass</td>
<td>28.7</td>
<td>23.6</td>
<td>Initial weight, change in HDL cholesterol, change in glucose, change in CRP, change in transferrin, change in LDL cholesterol, change in triglyceride</td>
</tr>
<tr>
<td>Change in % Lean mass</td>
<td>25.0</td>
<td>20.3</td>
<td>Initial weight, change in HDL cholesterol, change in orosomucoid, change in CRP, change in triglyceride, change in transferrin</td>
</tr>
<tr>
<td>Change in Visceral fat</td>
<td>15.9</td>
<td>11.4</td>
<td>Age, height, change in CRP, change in LDL cholesterol, change in triglyceride</td>
</tr>
<tr>
<td>Change in Muscle attenuation</td>
<td>14.0</td>
<td>11.0</td>
<td>Height, change in HDL, change in orosomucoid</td>
</tr>
</tbody>
</table>

*Results used to determine covariables for the multiple linear regression analysis. Data sets that were used for dependent variables were data from the MONET and CAO studies*
Table 2: Best subset regression analysis conducted on MONET and CAO women with obesity

<table>
<thead>
<tr>
<th>Dependent variable studied</th>
<th>R-Sq (%)</th>
<th>R-Sq (%) (adj)</th>
<th>Covariables associated with mentioned R value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in BMI</td>
<td>13.2</td>
<td>8.8</td>
<td>Change in transferrin, change in triglyceride and change in orosomucoid</td>
</tr>
<tr>
<td>Change in Lean Mass</td>
<td>13.8</td>
<td>8.2</td>
<td>Initial weight, change in HDL cholesterol, change in triglyceride, change in transferrin and change in CRP</td>
</tr>
<tr>
<td>Change in Fat Mass</td>
<td>21.6</td>
<td>15.4</td>
<td>Height, initial weight, change in CRP, change in transferrin, change in triglyceride</td>
</tr>
<tr>
<td>Change in % Fat mass</td>
<td>25.4</td>
<td>19.5</td>
<td>Height, initial weight, change in CRP, change in transferrin, change in triglyceride</td>
</tr>
<tr>
<td>Change in % Lean mass</td>
<td>25.7</td>
<td>18.7</td>
<td>Height, initial weight, change in HDL cholesterol, change in CRP, Change in transferrin, change in triglyceride</td>
</tr>
<tr>
<td>Change in Visceral fat</td>
<td>21.0</td>
<td>15.9</td>
<td>Age, initial weight, change in CRP and change in triglyceride</td>
</tr>
<tr>
<td>Change in Muscle attenuation</td>
<td>17.6</td>
<td>12.3</td>
<td>Change in HDL cholesterol, change in transferrin, change in orosomucoid and change in haptoglobin</td>
</tr>
</tbody>
</table>

* Results used to determine covariables for the multiple linear regression analysis. Data sets that were used for dependent variables were data from the MONET and CAO studies.
Table 3: Pre- and post-intervention anthropometric variable values from MONET and CAO women with overweight or obesity

<table>
<thead>
<tr>
<th>Biological factor observed</th>
<th>MONET study</th>
<th>CAO study</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std. Dev.</td>
<td>Size (n)</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>57.9</td>
<td>4.87</td>
<td>106</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.61</td>
<td>0.06</td>
<td>106</td>
</tr>
<tr>
<td>Pre-Intervention</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.4</td>
<td>4.74</td>
<td>106</td>
</tr>
<tr>
<td>Lean Mass (kg)</td>
<td>42.8</td>
<td>6.66</td>
<td>106</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>38.5</td>
<td>9.57</td>
<td>106</td>
</tr>
<tr>
<td>Lean Mass (%)</td>
<td>51.5</td>
<td>4.59</td>
<td>106</td>
</tr>
<tr>
<td>Fat Mass (%)</td>
<td>45.6</td>
<td>4.70</td>
<td>106</td>
</tr>
<tr>
<td>Visceral Fat (cm²)</td>
<td>185</td>
<td>54.7</td>
<td>105</td>
</tr>
<tr>
<td>Thigh Muscle Attenuation (HU)</td>
<td>48.8</td>
<td>3.34</td>
<td>106</td>
</tr>
<tr>
<td>Post-Intervention</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.3</td>
<td>4.76</td>
<td>105</td>
</tr>
<tr>
<td>Lean Mass (kg)</td>
<td>41.9</td>
<td>5.70</td>
<td>104</td>
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<tr>
<td>Fat Mass (kg)</td>
<td>33.9</td>
<td>10.1</td>
<td>104</td>
</tr>
<tr>
<td>Lean Mass (%)</td>
<td>54.2</td>
<td>5.36</td>
<td>104</td>
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<tr>
<td>Fat Mass (%)</td>
<td>42.6</td>
<td>5.52</td>
<td>104</td>
</tr>
<tr>
<td>Visceral Fat (cm²)</td>
<td>162</td>
<td>57.7</td>
<td>102</td>
</tr>
<tr>
<td>Thigh Muscle Attenuation (HU)</td>
<td>48.6</td>
<td>3.56</td>
<td>105</td>
</tr>
</tbody>
</table>

* Results are from unpaired t-tests. Test was conducted on data from all subjects whose blood samples were available for study.
Table 4: Regression analysis comparing CC carriers of MONET to CAO in women with overweight or obesity

<table>
<thead>
<tr>
<th>Multiple regression model</th>
<th>Dependent variable</th>
<th>Number of subjects (n)</th>
<th>Parameter estimate</th>
<th>Standard error</th>
<th>Variable p-value</th>
<th>R² (adjusted)</th>
<th>Model p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Δ BMI</td>
<td>123</td>
<td>0.15</td>
<td>1.55</td>
<td>0.923</td>
<td>0.130</td>
<td>0.069</td>
</tr>
<tr>
<td>2</td>
<td>Δ Lean Mass</td>
<td>124</td>
<td>-2.46</td>
<td>1.51</td>
<td>0.105</td>
<td>0.212</td>
<td>0.150</td>
</tr>
<tr>
<td>3</td>
<td>Δ Fat Mass</td>
<td>121</td>
<td>4.73</td>
<td>3.04</td>
<td>0.123</td>
<td>0.233</td>
<td>0.170</td>
</tr>
<tr>
<td>4</td>
<td>Δ % Lean Mass</td>
<td>126</td>
<td>-2.98</td>
<td>1.72</td>
<td>0.085</td>
<td>0.268</td>
<td>0.212</td>
</tr>
<tr>
<td>5</td>
<td>Δ % Fat Mass</td>
<td>121</td>
<td>3.98</td>
<td>2.04</td>
<td>0.053</td>
<td>0.315</td>
<td>0.252</td>
</tr>
<tr>
<td>6</td>
<td>Δ Visceral Fat</td>
<td>124</td>
<td>2.40</td>
<td>5.41</td>
<td>0.658</td>
<td>0.155</td>
<td>0.096</td>
</tr>
<tr>
<td>7</td>
<td>Δ Muscle attenuation</td>
<td>128</td>
<td>0.10</td>
<td>1.02</td>
<td>0.921</td>
<td>0.134</td>
<td>0.091</td>
</tr>
</tbody>
</table>

* Multiple linear regression analysis was conducted. Independent variables included three dummy variables (CAO CC Carriers, CAO CT/TT carriers and MONET CT/TT carriers) with the exception of MONET CC carriers while adjusting for covariables determined by best subset multiple linear regression, independent for each model (Table 1). The following results illustrate parameter estimates, standard error, p-values corresponding to CAO CC carriers independent variable.
Table 5: Regression analysis comparing CT/TT carriers of MONET to CAO in women with overweight or obesity

<table>
<thead>
<tr>
<th>Multiple regression model</th>
<th>Dependent variable</th>
<th>Number of subjects (n)</th>
<th>Parameter estimate</th>
<th>Standard error</th>
<th>Variable p-value</th>
<th>R²</th>
<th>R²(adjusted)</th>
<th>Model p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>∆ BMI</td>
<td>123</td>
<td>0.36</td>
<td>1.50</td>
<td>0.812</td>
<td>0.130</td>
<td>0.069</td>
<td>0.038</td>
</tr>
<tr>
<td>2</td>
<td>∆ Lean Mass</td>
<td>124</td>
<td>−0.35</td>
<td>1.39</td>
<td>0.802</td>
<td>0.212</td>
<td>0.150</td>
<td>0.001</td>
</tr>
<tr>
<td>3</td>
<td>∆ Fat Mass</td>
<td>121</td>
<td>2.15</td>
<td>2.92</td>
<td>0.462</td>
<td>0.233</td>
<td>0.170</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>∆ % Lean Mass</td>
<td>126</td>
<td>−1.10</td>
<td>1.68</td>
<td>0.513</td>
<td>0.268</td>
<td>0.212</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>∆ % Fat Mass</td>
<td>121</td>
<td>1.73</td>
<td>1.89</td>
<td>0.361</td>
<td>0.315</td>
<td>0.252</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6</td>
<td>∆ Visceral Fat</td>
<td>124</td>
<td>1.28</td>
<td>4.97</td>
<td>0.798</td>
<td>0.155</td>
<td>0.096</td>
<td>0.011</td>
</tr>
<tr>
<td>7</td>
<td>∆ Muscle attenuation</td>
<td>128</td>
<td>0.58</td>
<td>0.95</td>
<td>0.543</td>
<td>0.134</td>
<td>0.091</td>
<td>0.007</td>
</tr>
</tbody>
</table>

* Multiple linear regression analysis was conducted. Independent variables included three dummy variables to identify the polymorphism group (CC CAO, CC MONET and CT/TT CAO) with the exception of CT/TT MONET while adjusting for covariables determined by best subset multiple linear regression, independent for each model (Table 1). Following results illustrate parameter estimates, standard error, p-values corresponding to CT/TT CAO independent variable.
Table 6: Regression analysis studying lifestyle intervention effect in CT/TT vs CC women with overweight or obesity

<table>
<thead>
<tr>
<th>Multiple regression model</th>
<th>Dependent variable</th>
<th>Number of subjects (n)</th>
<th>Parameter estimate</th>
<th>Standard error</th>
<th>Variable p-value</th>
<th>r² model</th>
<th>r² adjusted model</th>
<th>Model p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>∆ BMI</td>
<td>123</td>
<td>-1.36</td>
<td>0.87</td>
<td>0.121</td>
<td>0.130</td>
<td>0.085</td>
<td>0.012</td>
</tr>
<tr>
<td>2</td>
<td>∆ Lean Mass</td>
<td>124</td>
<td>0.93</td>
<td>0.81</td>
<td>0.254</td>
<td>0.193</td>
<td>0.145</td>
<td>0.001</td>
</tr>
<tr>
<td>3</td>
<td>∆ Fat Mass</td>
<td>121</td>
<td>-3.82</td>
<td>1.66</td>
<td>0.023</td>
<td>0.213</td>
<td>0.164</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>∆ % Lean Mass</td>
<td>126</td>
<td>2.33</td>
<td>0.94</td>
<td>0.014</td>
<td>0.248</td>
<td>0.203</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>∆ % Fat Mass</td>
<td>121</td>
<td>-2.99</td>
<td>1.08</td>
<td>0.007</td>
<td>0.287</td>
<td>0.236</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6</td>
<td>∆ Visceral fat</td>
<td>124</td>
<td>-5.74</td>
<td>2.87</td>
<td>0.048</td>
<td>0.153</td>
<td>0.110</td>
<td>0.003</td>
</tr>
<tr>
<td>7</td>
<td>∆ Muscle attenuation</td>
<td>128</td>
<td>0.97</td>
<td>0.56</td>
<td>0.085</td>
<td>0.131</td>
<td>0.103</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* Multiple linear regression analysis was conducted. Parameter estimate of independent variable = \( X_t \) was studied. Physiological factors (dependent variables) were studied while adjusting for confounding factors independent for each model (Table 1). Analysis was conducted on MONET and CAO studies combined.
Table 7: Pre- and post-intervention anthropometric variable values from MONET and CAO studies in women with obesity

<table>
<thead>
<tr>
<th>Biological factor observed</th>
<th>MONET study</th>
<th>CAO study</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std. Dev.</td>
<td>Size (n)</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>58.1</td>
<td>4.91</td>
<td>62</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.61</td>
<td>0.06</td>
<td>62</td>
</tr>
<tr>
<td>Pre-Intervention</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>35.3</td>
<td>4.04</td>
<td>62</td>
</tr>
<tr>
<td>Lean Mass (kg)</td>
<td>45.5</td>
<td>6.82</td>
<td>62</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>43.9</td>
<td>8.64</td>
<td>62</td>
</tr>
<tr>
<td>Lean Mass (%)</td>
<td>49.6</td>
<td>4.43</td>
<td>62</td>
</tr>
<tr>
<td>Fat Mass (%)</td>
<td>47.6</td>
<td>4.48</td>
<td>62</td>
</tr>
<tr>
<td>Visceral Fat (cm²)</td>
<td>207</td>
<td>51.9</td>
<td>61</td>
</tr>
<tr>
<td>Muscle Attenuation (HU)</td>
<td>48.5</td>
<td>3.70</td>
<td>62</td>
</tr>
<tr>
<td>Post-Intervention</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>33.0</td>
<td>4.42</td>
<td>61</td>
</tr>
<tr>
<td>Lean Mass (kg)</td>
<td>44.1</td>
<td>5.69</td>
<td>60</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>39.1</td>
<td>9.90</td>
<td>60</td>
</tr>
<tr>
<td>Lean Mass (%)</td>
<td>51.9</td>
<td>5.28</td>
<td>60</td>
</tr>
<tr>
<td>Fat Mass (%)</td>
<td>45.1</td>
<td>5.41</td>
<td>60</td>
</tr>
<tr>
<td>Visceral Fat (cm²)</td>
<td>182</td>
<td>58.5</td>
<td>60</td>
</tr>
<tr>
<td>Muscle Attenuation (HU)</td>
<td>48.2</td>
<td>4.09</td>
<td>61</td>
</tr>
</tbody>
</table>

* Results are from unpaired t-tests. Test was conducted on data from all subjects whose blood samples were available for study
Table 8: Regression analysis studying lifestyle intervention effect in CT/TT vs CC women with obesity

<table>
<thead>
<tr>
<th>Multiple regression model</th>
<th>Dependent variable</th>
<th>Number of subjects (n)</th>
<th>Parameter estimate</th>
<th>Standard error</th>
<th>Variable p-value</th>
<th>r² model</th>
<th>r² adjusted model</th>
<th>Model p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Δ BMI</td>
<td>87</td>
<td>-2.14</td>
<td>1.05</td>
<td>0.045</td>
<td>0.129</td>
<td>0.087</td>
<td>0.022</td>
</tr>
<tr>
<td>2</td>
<td>Δ Lean Mass</td>
<td>85</td>
<td>0.49</td>
<td>1.07</td>
<td>0.645</td>
<td>0.151</td>
<td>0.086</td>
<td>0.041</td>
</tr>
<tr>
<td>3</td>
<td>Δ Fat Mass</td>
<td>85</td>
<td>-5.13</td>
<td>1.97</td>
<td>0.011</td>
<td>0.212</td>
<td>0.151</td>
<td>0.004</td>
</tr>
<tr>
<td>4</td>
<td>Δ % Lean Mass</td>
<td>85</td>
<td>3.12</td>
<td>1.19</td>
<td>0.010</td>
<td>0.257</td>
<td>0.189</td>
<td>0.001</td>
</tr>
<tr>
<td>5</td>
<td>Δ % Fat Mass</td>
<td>85</td>
<td>-3.30</td>
<td>1.30</td>
<td>0.013</td>
<td>0.252</td>
<td>0.194</td>
<td>0.001</td>
</tr>
<tr>
<td>6</td>
<td>Δ Visceral fat</td>
<td>85</td>
<td>-9.53</td>
<td>3.47</td>
<td>0.007</td>
<td>0.214</td>
<td>0.164</td>
<td>0.002</td>
</tr>
<tr>
<td>7</td>
<td>Δ Muscle attenuation</td>
<td>87</td>
<td>1.20</td>
<td>0.72</td>
<td>0.098</td>
<td>0.177</td>
<td>0.126</td>
<td>0.007</td>
</tr>
</tbody>
</table>

* Multiple linear regression analysis was conducted. Parameter estimate of independent variable = Xt was studied. Physiological factors (dependent variables) were studied while adjusting for confounding factors independent for each model (Table 2). Analysis was conducted on MONET and CAO studies combined.
CHAPTER THREE: DETERMINING THE CELLULAR AND MOLECULAR MECHANISMS BY WHICH RS2419621 ACSL5 POLYMORPHISM EXERTS ITS EFFECT.

ACSL5 GENOTYPE INFLUENCE ON FATTY ACID METABOLISM:
A CELLULAR, TISSUE, AND WHOLE-BODY STUDY

Abishankari Rajkumar\textsuperscript{1,2}, Awa Liaghati\textsuperscript{1}, Jessica Chan\textsuperscript{2}, Gilles Lamothe\textsuperscript{3}, Robert Dent\textsuperscript{4}, Éric Doucet\textsuperscript{5}, Remi Rabasa-Lhoret\textsuperscript{6,7}, Denis Prud’homme\textsuperscript{5,8}, *Mary-Ellen Harper\textsuperscript{1} and *Frédérique Tesson\textsuperscript{2}

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8-Institut de recherche de l’Hôpital Montfort, Hôpital Montfort, Ottawa, ON, K1K 0T1, Canada
3.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

3.1.1 STATEMENT OF MANUSCRIPT STATUS

The manuscript “ACSL5 Genotype Influence on Fatty Acid Metabolism: A Cellular, Tissue and Whole-body Study” has been accepted for publication by the journal of Metabolism: Clinical and Experimental.

3.1.2 CONTRIBUTION STATEMENT

AR conducted majority of the experimental procedures (apart from O2K ex vivo experiments), genotyping and statistical analyses, interpreted the data and wrote the manuscript. AL conducted the ex vivo experiments using the O2K system. JC constructed the vectors used in the in vitro C2C12 studies. GL revised the statistical analyses and methodology critically. RD participated in recruitment of patients for the Ottawa Hospital Weight Management Clinic and established the database. ED participated in recruitment of patients for Montréal Ottawa New Emerging Team and established the database. RRL participated in recruitment of patients for Montréal Ottawa New Emerging Team and established the database. DP participated in recruitment of patients for Montréal Ottawa New Emerging Team and established the database. MH performed the study design, interpreted the data and revised the article critically. FT performed the study design, interpreted the data and revised the article critically. All authors read and approved the final manuscript.

3.1.3 COMPETING INTEREST STATEMENT

The authors declare that they have no competing interests.
3.2 ABSTRACT

Background: Acyl-CoA Synthetase Long Chain 5 (ACSL5) gene’s rs2419621 T/C polymorphism was associated with ACSL5 mRNA expression and response to lifestyle interventions. However, the mechanistic understanding of the increased response in T allele carriers is lacking. Study objectives were to investigate the effect of rs2419621 genotype and ACSL5 human protein isoforms on fatty acid oxidation and respiration. Methods: Human ACSL5 overexpression in C2C12 mouse myoblasts was conducted to measure $^{14}$C palmitic acid oxidation and protein isoform localization in vitro. $^{14}$C palmitic acid oxidation studies and western blot analysis of ACSL5 proteins were carried out in rectus abdominis primary myotubes from 5 rs2419621 T allele carriers and 4 non-carriers. In addition, mitochondrial high-resolution respirometry was conducted on vastus lateralis muscle biopsies from 4 rs2419621 T allele carriers and 4 non-carriers. Multiple linear regression analysis was conducted to test the association between rs2419621 genotype and respiratory quotient related pre- and post-lifestyle intervention measurements in postmenopausal women with overweight or obesity. Results: In comparison to rs2419621 non-carriers, T allele carriers displayed higher levels of i) 683aa ACSL5 isoform, localized mainly in the mitochondria, playing a greater role in fatty acid oxidation in comparison to the 739aa protein isoform. ii) in vitro $\text{CO}_2$ production in rectus abdominis primary myotubes iii) in vivo fatty acid oxidation and lower carbohydrate oxidation post-intervention iv) ex vivo complex I and II tissue respiration in vastus lateralis muscle. Conclusions: These results support the conclusion that rs2419621 T allele carriers, are more responsive to lifestyle interventions partly due to an increase in the short ACSL5 protein isoform, increasing cellular, tissue and whole-body fatty acid utilization. With the increasing effort to develop personalized medicine to combat obesity, our findings provide additional
insight into genotypes that can significantly affect whole body metabolism and response to lifestyle interventions.

Keywords: Obesity, Fatty Acid Metabolism, ACSL5 Genotype

Highlights

- **ACSL5** genotype and fatty acid oxidation/mitochondrial bioenergetics were studied.
- rs2419621 [T] carriers had increased ACSL5 683aa protein levels vs non-carriers.
- rs2419621 [T] carriers had increased fatty acid oxidation vs non-carriers.
- rs2419621 [T] carriers have increased muscle tissue respiration.
- rs2419621 [T] carriers have reduced carbohydrate oxidation vs non-carriers.

3.3 BACKGROUND

According to a recent 2015 study published by the New England Journal of Medicine, prevalence of obesity has doubled since 1980 with over 600 million individuals worldwide suffering from obesity [1]. While treatments options include lifestyle interventions, medication and more invasive procedures such as bariatric surgery, treatment response differs between individuals[2,3]. Thus, there is a growing trend towards identifying predictors of weight loss response for personalized lifestyle interventions. Specifically, of interest is identifying high sensitive weight loss genotypes and understanding their impact on cellular and molecular mechanisms.

Skeletal muscle, one of the key tissues that utilizes free fatty acids as a source of energy has been shown to constitute roughly 40% of body mass in a healthy individual [4]. One key player of fatty acid metabolism in skeletal muscle are Acyl-CoA Synthetase Long-Chain (ACSL)
proteins, involved in the esterification of free fatty acids into fatty-acyl CoA molecules [5]. These ACSL proteins differ in their subcellular localization, substrate and tissue specificity [5]. ACSL5, one of the members of this protein family, has been widely studied for its role in fatty acid metabolism and has been shown to be present in various tissues, including brown adipose tissue, skeletal muscle, liver and brain [6,7].

The rat and mouse Acsl5 genes have been shown to produce a protein of 683 amino acids (Supplementary Figure 1). Mashek et al. demonstrated that rat ACSL5 overexpression in McArdle-RH7777 cells resulted in the presence of ACSL5 in both the mitochondria and endoplasmic reticulum, with a 30% higher rate of fatty acid uptake when incubated with 1-14C oleic acid [8]. Moreover, research conducted on Acsl5 (-/-) mice, illustrated an increase in whole-body energy expenditure and delayed fat absorption [9]. Lewin et al. documented an increase in liver mitochondrial ACSL5 activity following a 48-hour fast in rats [10]. While studies continue to dissect the function of rodent ACSL5, little is known about the subcellular localization and function of ACSL5 human protein isoforms.

While rodents produce one ACSL5 of 683aa, the human ACSL5 gene (ACSL5) produces three transcript variants encoding two major protein isoforms of ACSL5 [5]. The production of a long protein isoform of 739aa requires an upstream AUG, while the 683aa short protein isoform relies on an AUG downstream of the sequence [5]. Previous work conducted in our lab illustrated that the ACSL5 rs2419621 genotype (C to T transition) was associated with increased rate of weight and fat loss in women with overweight or obesity who underwent a hypocaloric diet [11,12]. Located 12 nucleotides upstream of the second AUG site of ACSL5, this polymorphism was shown to create an additional cis-regulatory E-box (CANNTG) in the promoter region, increasing MyoD recruitment, as well as the expression of the downstream
reporter gene in vitro [13]. Furthermore, the presence of the rs2419621 T allele was associated with an elevated level of ACSL5 mRNA in human skeletal muscle biopsies [11]. Thus, we hypothesize that individuals carrying the rs2419621 T allele are more responsive to lifestyle interventions due to an increase in the ACSL5 protein expression. In the current study we aimed to 1) study how ACSL5 protein levels influence fatty acid metabolism in vitro 2) confirm that the presence of the rs2419621 polymorphism is associated with increased ACSL5 protein levels and fatty acid metabolism in vitro, and 3) determine the effect of the ACSL5 polymorphism on overall metabolic respiration rate ex vivo and in vivo.

3.4 MATERIALS AND METHODS

Individuals participating in the study

Individuals participating in our studies were enrolled in two weight management interventions. Characteristics, including the rs2419621 genotype of individuals enrolled in the Ottawa Hospital Weight Management Program, who gave their informed written consent (Human Research Ethics Board of the Ottawa Hospital) are presented in Table 1[2,14]. Biopsies were obtained from non-diabetic individuals (ages 30-66) during Roux-en-Y gastric bypass surgery for rectus abdominis and by Bergstrom needle biopsy for vastus lateralis tissue. These tissues were used for cellular fatty acid oxidation and tissue mitochondrial respiration as described below. Individuals with overweight or obesity participating in the Montréal Ottawa New Emerging Team (MONET) lifestyle intervention, provided informed written consent (Université de Montréal ethics committee). They were enrolled in a caloric restriction intervention while a third of the subjects also underwent resistance exercise training [12,15,16]. In this population, we tested for association between rs2419621 genotype and respiratory quotient related parameters.
Isolation of rectus abdominis human primary myocytes and vastus lateralis tissue

Primary myocytes from rectus abdominis and tissue from vastus lateralis muscle biopsies were isolated and processed as described previously[14,17].

Cell Cultures

C2C12 mouse muscle myoblasts (ATCC® CRL-1772™) were cultured in standard Dulbecco’s modification Eagle’s medium (DMEM; See Supplementary Materials for culture medium nutrient supplementation).

Human rectus abdominis primary myocytes were cultured using F10 nutrient mixture (Ham’s) medium (Gibco) with nutrient supplementation. Cells were differentiated into myotubes by incubation in low glucose DMEM with nutrient supplementation (See Supplementary Materials) for 7 days.

HepG2 liver carcinoma cell line was cultured in standard Eagle's minimum essential media with nutrient supplementation (See Supplementary Materials).

All cell types were grown at 37°C in a humidified 5% CO₂ atmosphere.

Construction of Vectors

cDNA of Homo sapiens acyl-CoA synthetase long-chain family member 5 (ACSL5), (NM_016234.3) was purchased within pOTB7 vectors (OpenBiosystems, GE Dharmacon). For transient overexpression of ACSL5 cDNA vectors in cells, cDNA of the human long (739aa) and short (683aa) protein isoform were amplified by PCR (See Supplementary Materials). PCR was conducted using manufacturer’s protocol for PfuUltra High-Fidelity DNA Polymerase (Agilent). PCR products were purified using the QIAquick Gel Extraction
kit (Qiagen). pEYFP-N1 (Clontech Laboratories) and pCAGIG (Addgene) were restriction digested with XhoI-BamHI and XhoI-NotI respectively, while ligation was conducted using T4 DNA ligase company protocol (New England Biolabs). The YFP was expressed at the C-termini of both ACSL5 isoforms while ACSL5 cDNA cloned into pCAGIG vectors were placed before the internal ribosome entry site (IRES), thus producing ACSL5 protein and GFP separately. The IRES-GFP was from pMX-IRES-GFP, while the other parts of the vector were from pCAGGS with modified multiple cloning sites (pCAGEN) [18,19]. The pCAGIG vector was constructed by Takahiko Matsuda [20]. The sequence of all plasmid constructs was checked by sequencing using the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Transfection of C2C12 myoblast with ACSL5 cDNA

Following growth of C2C12 mouse myoblasts to 80% visual confluency, cells were transfected with pCAGIG and pEYFP-N1 vectors containing ACSL5 cDNA of long and short protein isoforms using Metafectene® Pro (Biontex). pCAGIG was also transfected alone as control. As functionality studies on ACSL5 were solely conducted to observe localization and function of proteins, myoblasts were used due to increased transfection efficiency vs myotubes. Transfection studies were conducted in triplicates using company protocol for 24-well and 10mm plates.

Immunostaining

C2C12 cells were stained for endoplasmic reticulum using calreticulin antibody and mitochondria using Tom20 antibody. An anti-ACSL5 antibody was used to study endogenous mouse ACSL5 in C2C12 cells as well as to study untagged human ACSL5 when overexpressed in C2C12 cells (See Supplementary Material for antibodies used with catalogue numbers). Human cells from rectus abdominis were stained for endogenous ACSL5 using an
anti-ACSL5 antibody and mitochondria with Tom20 antibody. Briefly, cells were incubated in 4% paraformaldehyde fixative for 20 minutes, then permeabilized with 500µl of 0.1% PBS-Tween 20 solution for 30 minutes. Cells were incubated in 300µl primary antibody/well for 1 hour at room temperature and incubated with 300µl secondary antibody/well at room temperature for 1 hour in the dark.

Cell imaging was conducted with Zeiss LSM 510/AxioImager.M1 confocal microscope using ZEN2009 software (See Supplementary Material on microscope settings/image acquisition). Z-stack images were acquired. Subcellular colocalization of human ACSL5 isoforms with organelles of interest were measured using Mander’s coefficient (using Image J software). The average gray value and area of Tom20 staining was assessed in human muscle cell images to determine if there was a change in the number of mitochondria between rs2419621 T allele carriers vs non-carriers (using Image J software). Statistical analyses were conducted using unpaired t-tests to determine whether significant differences in subcellular localization existed between the two human ACSL5 protein isoforms (n=3 independent experiments performed in triplicates). Unpaired t-tests were also conducted to study the proportion of mouse ACSL5 localization in mitochondria vs endoplasmic reticulum (n=3 independent experiments performed in triplicates), as well as the proportion of human ACSL5 localization in mitochondria and the mitochondria quantity between rs2419621 carriers (n=5) vs non-carriers (n=4). P<0.05 was considered significant.

$^{14}$C palmitic acid oxidation studies

$^{14}$C palmitic acid oxidation studies were conducted in 12-well plates on 64-68 hours post pCAGIG-ACSL5 transfected C2C12 myoblasts or on human *rectus abdominis* primary myocytes. 24-48 hours post transfection, cell were sorted for GFP positive cells using the
Beckman Coulter MoFlo XDP/Beckman Coulter MoFlo Astrios. Palmitic acid oxidation assessment was conducted as described in Aguer et al. [21]. ASP (acid-soluble products) from growth medium and cells was extracted and analyzed [22–24]. The remainder of solution in the glass vials containing benzethonium hydroxide was used to measure carbon dioxide. Scintillation counting was conducted using the PERKIN ELMER Tri-Carb 2910 TR LSC. The protein concentration of samples was determined with the Protein Assay kit (Bio-Rad) using BSA as standard. Total fatty acid oxidation was calculated from the amount of radiolabelled $^{14}$C incorporated in both carbon dioxide and acid soluble products. Human ACSL5 driven palmitic acid oxidation was assessed by 1) \( \text{human ACSL5 driven } CO_2 \text{ production} = \text{Total } CO_2 \text{ measured} - \text{endogenous } CO_2 \) and 2) \( \text{human ACSL5 driven } ASP \text{ production} = \text{Total } ASP \text{ measured} - \text{endogenous } ASP \). Endogenous CO$_2$ or ASP were the results obtained from palmitic acid oxidation in cells transfected with an empty vector for control. One-way ANOVA and unpaired Student t-tests were utilized for statistical analysis between groups. Studies conducted on pCAGIG-ACSL5 transfected C2C12 myoblasts were conducted in triplicates with \( n=4 \) independent experiments. Studies conducted on human rectus abdominis cells consisted of 5 rs2419621 T allele carriers and 4 non-carriers. Experiments were performed in triplicates. \( P<0.05 \) was considered significant.

**Western blot analyses**

Western blot analyses were conducted on C2C12 myoblasts transfected by ACSL5 expression vectors, on HepG2 liver carcinoma cells as well as on human skeletal muscle cells differentiated for 7 days into myotubes. Whole cell protein was extracted using the Active Motif nuclear kit- “Preparation of Whole Cell Extract from Cells” protocol. Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) was used to determine whole cell protein
concentrations. 12% SDS-PAGE casted gels were run. PageRuler™ Prestained Protein Ladder (266166; ThermoFisher Scientific) was used. Following transfer, nitrocellulose membranes 0.2µm (Biorad) were incubated overnight with ACSL5 primary antibody at 4ºC and the following day with the secondary antibody at room temperature for 1 ½ hours. GAPDH was used as the loading control. (See Supplementary Materials for antibodies). Protein band detection was conducted with the VersaDoc MP 4000 system (Biorad) and analysed with the Quantity One 4.6.9 Software (Chemi Hi Sensitivity 0.5x Gain Application). Prior to using GAPDH as a loading control, an unpaired two-tail Student t-test analysis was conducted between GAPDH volume intensities between carriers vs non-carriers to ensure no difference was observed. An unpaired two-tail Student t-test analysis was conducted to compare ACSL5 volume intensity values adjusted for GAPDH volume intensity values between rs2419621 T allele carriers (n=5) vs non-carriers (n=4). Student t-test and one-way Anova analyses were also conducted to test for difference in ACSL5 expression level between C2C12 myoblasts transfected with pCAGIG-ACSL5 long or short isoform and pCAGIG (n=3). P<0.05 was considered significant.

*High Resolution Respirometry of Biopsied Muscle*

*In situ* mitochondrial function of permeabilized muscle fibers from *vastus lateralis* tissue was assessed by using high-resolution respirometry in an Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria), as previously described [17]. Specific electron transport chain complex-supported respiration was assessed in respiratory chambers maintained at 37ºC (See Supplementary Materials). Unpaired two-tailed Student t-test analysis was utilized to compare results between rs2419621 T allele carriers (n=4) vs non-carriers (n=4). P<0.05 was considered significant.
137 post-menopausal women with overweight or obesity were enrolled in the MONET (Montréal Ottawa New Emerging Team) lifestyle intervention study [12,15,16]. Pre- and post-lifestyle intervention measurements were done. Resting energy expenditure was determined by indirect calorimetry following a 12 hour overnight fast. 24 hours prior to testing, subjects refrained from physical activity. Both carbon dioxide and oxygen concentrations were measured with a ventilated hood technique using a SensorMedics Delta Track II (Datex-Ohmeda, Helsinki, Finland) [16]. The duration of measurement was 40 minutes, including a 10 minute period for acclimatization.

Genotyping of DNA from participants of the Montréal Ottawa New Emerging Team was conducted using TaqMan MGB probe-based assay as previously described [12]. A Pearson’s chi-squared test showed that the ACSL5 rs2419621 genotypes did not depart from the Hardy-Weinberg proportions in the studied MONET population. Percentage changes in metabolic parameters following the intervention were calculated by: (post intervention values – pre-intervention values)/ pre-intervention values x 100. Regression analyses were conducted on the following metabolic parameters as dependent variables: Respiratory quotient (Rq), volume of oxygen inhaled per minute (VO\textsubscript{2}) at rest, maximal carbon dioxide expired per minute (VCO\textsubscript{2}) at rest, fatty acid oxidation and carbohydrate oxidation. Prior to conducting multiple linear regression analyses, best subset regression analyses were performed to determine the appropriate covariates for adjustment in the multiple linear regression models (See Supplementary Materials for covariates considered; Supplemental Data Table S2). Multiple linear regression analyses were conducted to test the association of ACSL5 rs2419621 genotype with changes in metabolic parameters post-intervention. A dominant genetic model
was utilized for regression analyses (CT/TT vs CC). Genotypes were included as an independent variable in the regression models. Statistical significance was determined with a p-value < 0.05 and all data analysis was performed with Minitab software 17. The R project software was used to calculate statistical power for resting carbohydrate oxidation and resting fatty acid oxidation levels as both the regression models were statistically significant.

3.5 RESULTS

*Functional studies on the effect of human ACSL5 long and short protein isoform overexpression on fatty acid metabolism in vitro*

To assess the function of human ACSL5 long and short isoforms on fatty acid oxidation, the break-down of $^{14}$C-labelled palmitic acid into CO$_2$ and acid soluble products was measured (Figure 1a). We observed that C2C12 cells overexpressing the human ACSL5 short protein isoform contributed towards a significantly greater increase in CO$_2$ levels (0.133 nmol/hr/mg ± SEM 0.040) and ASP in culture medium (0.232 nmol/hr/mg ± SEM 0.071) in comparison to C2C12 cells overexpressing the human ACSL5 long isoform (CO$_2$ = 0.031 nmol/hr/mg ± SEM 0.015; ASP$_{medium}$ = 0.037 nmol/hr/mg ± SEM 0.025). No significant difference in ASP isolated from cells was observed between C2C12 cells overexpressing the short or long isoform of human ACSL5. It is important to note that no significant difference was observed between the amounts of ACSL5 isoform overexpressed within the different samples (Figure 1b). Furthermore, no significant difference was observed between the levels of endogenous mouse ACSL5 when cells were transfected with either the human ACSL5 long or human ACSL5 short isoforms (Figure 1b).
**Subcellular localization of transiently overexpressed human ACSL5 long and short isoforms in C2C12 cells**

Prior to studying the subcellular localization of overexpressed human ACSL5 within C2C12, we determined the subcellular localization of the endogenous mouse ACSL5. Analyses of confocal microscopy images showed that the mouse ACSL5 was localized in the endoplasmic reticulum to a greater extent than in the mitochondria (Supplementary Figure 2b: Mander’s Coefficient for mouse ACSL5 in endoplasmic reticulum = 0.809 SEM = 0.003 n= 47; Mander’s Coefficient for mouse ACSL5 in mitochondria = 0.297 SEM = 0.020 n = 45; p<0.001). To characterize the subcellular primary location of human ACSL5 isoforms, YFP-tagged human protein isoforms were transiently expressed in C2C12 myoblasts. Mitochondria and endoplasmic reticulum were stained for TOM20 (Translocase of Outer Membrane 20), a mitochondrial import receptor subunit, and calreticulin, a molecular calcium-binding chaperone, respectively (Figure 2a). The short human ACSL5-YFP protein was localized in the mitochondria to a greater extent than the long human ACSL5-YFP protein isoform (Figure 2b: Mander’s Coefficient for human ACSL5 short isoform in mitochondria = 0.583 SEM = 0.042 n = 33; Mander’s Coefficient for human ACSL5 long isoform in mitochondria = 0.422 SEM = 0.028 n = 31). Additionally, the human ACSL5-YFP long isoform was localized to a greater extent in the endoplasmic reticulum in comparison to the short human ACSL5-YFP isoform (Figure 2b: Mander’s Coefficient for human ACSL5 short isoform in endoplasmic reticulum = 0.737 SEM = 0.009 n = 31; Mander’s Coefficient for human ACSL5 long isoform in endoplasmic reticulum = 0.837 SEM = 0.007 n = 33). Immunofluorescence validation studies observing subcellular localization of PCAGIG vectors containing human untagged ACSL5 isoforms with organelles of interest, illustrated similar results (data not shown).
ACSL5 protein expression levels and subcellular localization in human myotube cultures from rs2419621 T allele carriers and non-carriers

In human, ACSL5 rs2419621 T allele carriers have been shown to display an increase in ACSL5 mRNA levels in rectus femoris. However, whether the protein expression of ACSL5 isoforms also increases due to the presence of the T allele has not been confirmed. The following set of studies focused on studying ACSL5 in human myotubes from women between the ages of 36 and 61, specifically 5 T allele carriers (1 TT and 4 CT) and 4 non-carriers (Table 1). No significant age differences were observed between T allele carriers vs non-carriers (data not shown). Western blot analyses illustrated a 1.85 fold increase in ACSL5 short isoform levels in T allele carriers in comparison to non-carriers (ACSL5 Volume Intensity T allele carriers = 6.69 ± 0.72 SEM (n = 5) vs non-carriers = 3.62 ± 0.47 (n = 4); Figure 3a). While the short ACSL5 isoform of 683aa was detected in all samples of human muscle cells, the other common human ACSL5 isoform of 739aa was barely present. However, both the long ACSL5 isoform of 739aa, the short 683 aa isoform, and an even shorter uncommon protein isoform of 659aa was present in HepG2 human liver carcinoma cells (Figure 3a and Supplemental Data Figure S3). Therefore, the abundant ACSL5 isoform in skeletal muscle is the ACSL5 short isoform of 683aa. Approximately 60% of ACSL5 colocalized with the mitochondria in human patient muscle cells, a proportion very similar to the proportion of human ACSL5 short isoform colocalization with mitochondria when overexpressed in C2C12 myoblasts (Supplemental Data Figure S4 vs Figure 2b). Both carriers and non-carriers of the rs2419621 polymorphism displayed a similar subcellular localization of ACSL5 (Supplemental Data Figure S4). It is important to note that following measurement of Tom20 staining for area and
intensity to determine mitochondrial number no difference was observed between genotypes (data not shown).

Fatty acid oxidation and oxygen consumption in biopsied muscle tissue and myotubes from rs2419621 T allele carriers and non-carriers

Subsequently, we wanted to determine whether individuals who were rs2419621 T allele carriers also had an increase in fatty acid oxidation levels in vitro and oxygen flux ex vivo (Figure 3b and c). The same patient tissue samples on which western blot analyses were conducted were studied for the following experiment (Table 1). In vitro 14C palmitic acid oxidation experiments on primary myotubes showed a significant 1.46-fold increase in complete fatty acid oxidation in rs2419621 T allele carriers (0.900 ± 0.028 SEM) in comparison to non-carriers (0.615 ± 0.109 SEM) (Figure 3b). High resolution respirometry was conducted in vastus lateralis muscle fibers obtained from four rs2419621 T allele carriers (1 TT and 3 CT females) and four non-carriers (3 CC females and 1 CC male) between the ages of 30 and 66 (Table 1). No age difference was observed between T allele carriers vs non-carriers. A greater increase in complex I and II supported respiration in rs2419621 T allele carriers was observed vs non-carriers (Figure 3c). Specifically, respiration /muscle mass values showed a 1.846-fold increase in rs2419621 T allele carriers (60.59 ± 6.11 SEM) in comparison to non-carriers (32.82 ± 4.12 SEM) (Figure 3c).

Association analysis between ACSL5 rs2419621 genotype and respiratory quotient related measurements post MONET lifestyle intervention in women with overweight or obesity

Based on our in vitro and ex vivo results, we studied whether rs2419621 T allele carriers had greater improvements in their respiratory quotient related measurements in vivo in comparison
to non-carriers following a lifestyle intervention. Specifically, we assessed pre- and post-MONET lifestyle-intervention-study, the respiratory quotient, resting carbon dioxide output (VCO$_2$), resting oxygen uptake (VO$_2$), resting carbohydrate oxidation and resting fatty oxidation in 105 postmenopausal women with overweight or obesity (Table S1). The average age of the population studied was about 58 years with an average post-intervention BMI of 30.32kg/m$^2$ (further descriptive statistics on studied population can be found in Table S1). Women with overweight or obesity carrying the T allele, presented a parameter estimate of $-56.5 \pm SE 24.7$ for change in resting carbohydrate oxidation levels (statistical power of model= 80.2%) and a parameter estimate of $185.6 \pm SE 66.6$ for change in resting fatty acid oxidation levels (statistical power of model= 60.0%) post intervention vs non-carriers. This illustrates their greater reliance on fatty acid oxidation vs carbohydrate oxidation in comparison to non-carriers post-intervention (Table 2).

### 3.6 DISCUSSION

Previous studies have illustrated enhanced fatty acid beta oxidation when ACSL5 was transcriptionally activated in HEPG2 cells [25]. However, to date, functional roles of the two commonly documented human ACSL5 protein isoforms have not been studied. Skeletal muscle, is one of the key tissues that utilizes free fatty acids as a source of energy [4]. This tissue is of interest for studying ACSL5, as the protein contributes towards the uptake of free fatty acids. Thus, the objective of our study was to study the functional role of human ACSL5 isoforms, regarding fatty acid metabolism in skeletal muscle and determine the cellular and molecular basis of responsiveness of rs2419621 T allele carriers to lifestyle interventions.
Functionality studies on human ACSL5 isoforms in C2C12 myoblasts, illustrated that ACSL5 short isoform overexpression led to greater increases in complete and incomplete fatty acid oxidation production levels, in comparison to ACSL5 long protein overexpression. This was paralleled by a greater localization of the short protein isoform in mitochondria in comparison to the long protein isoform. Our current findings support previous in silico studies of human ACSL5 protein, which demonstrated a targeting mitochondrial signal on the short protein, buried by the first encoding exon in the long protein isoform [5]. Thus, increased human ACSL5 short protein localization within the mitochondria allows for the increased conversion of free fatty acids into fatty acyl-CoA molecules, which would then be shuttled into the organelle via carnitine palmitoyl transferases (CPT) for oxidation [26]. Previous work on rodent ACSL5 appear to show its increased role in lipid biosynthesis [8,9]. Our studies in C2C12 cells, illustrate a three-fold increase in rodent ACSL5 localization in the endoplasmic reticulum in comparison to the mitochondria, potentially illustrating its preponderant role in skeletal muscle lipid biosynthesis. Thus to date, while previous work has shown ACSL5 to be present in the endoplasmic reticulum and the mitochondria in rat liver and in human epithelial colorectal adenocarcinoma cells, our current findings shed light on the localization and function of human and rodent ACSL5 within skeletal muscle [8,10,27]. Our studies on human ACSL5 isoforms were conducted on myoblasts instead of myotubes in order to increase transient transfection efficiency with the goal of understanding the function and localization of the human isoforms. Future directions include confirming the functional role of the human ACSL5 long isoform as well as studying whether compensatory mechanisms exist with other fatty acid metabolism related proteins, when overexpressing human ACSL5 isoforms.
Furthermore, confirming the role of the human ACSL5 isoforms in other tissues is important to determine whether the observations from the present study are skeletal muscle-specific.

Previous work in our laboratory has illustrated the potential of the rs2419621 polymorphism to increase recruitment of MyoD transcription factors to the promoter region of ACSL5, resulting in an increased production of the downstream protein [13]. Based on our in vitro findings in C2C12 myoblasts, we hypothesized that rs2419621T allele carriers were more responsive to lifestyle interventions, due to an increase in their fatty acid oxidation levels caused by an increase in ACSL5 short isoform expression. Human myotubes illustrated an increase in the 683aa ACSL5 short isoform production, as well as in their complete fatty acid oxidation levels in rs2419621 T allele carriers vs non-carriers. This increase in cellular fatty acid oxidation was also reflected at the whole-body level, where T allele carriers had a greater dependence on fatty acid oxidation and reduced reliance on carbohydrate oxidation in vivo compared to non-carriers, following the MONET lifestyle intervention. Therefore, the increase in short human ACSL5 isoform could explain the increase in fatty acid oxidation levels observed both in vitro and in vivo. It is interesting to note that while the 683aa protein isoform of ACSL5 was abundant in human myotubes, the expression of the long isoform of 739aa was very low. Furthermore, our results displayed roughly 60% of ACSL5 localized within the mitochondria in human myotubes, which is very similar to the percent mitochondrial localization of transfected human ACSL5 short isoform in C2C12 myoblasts. Thus, regardless of the differentiated state of muscle cells, it appears that the function and localization of human ACSL5 isoforms remains constant. Lastly, no difference in the quantity of mitochondria was noted between genotypes, illustrating that the observed increase in ACSL5 levels wasn’t due to an increase in mitochondrial number in T allele carriers.
In HepG2 cells, the 739aa protein isoform as well as an uncommon 659aa was found (Figure S3). Previous studies on this uncommon ACSL5 isoform of 659aa (ACSL5Δ20), illustrated its role in the regulation of TRAIL-induced apoptosis [27]. Thus, its function might contribute to its abundance in HepG2, as hepatocellular carcinoma cells have been shown to potentially become resistant to TRAIL-induced apoptosis [28,29]. Overall our findings demonstrate an association between rs2419621 genotype, high levels of fatty acid oxidation and the human 683aa short ACSL5 expression in skeletal muscle.

Remarkably, a greater increase in *ex vivo* mitochondrial respiration in myofibers was observed with T allele carriers in comparison to non-carriers. The surprising difference could be attributed to the increase in ACSL5 short isoform expression within the muscle cells of rs2419621 T allele carriers, increasing the production of endogenous fatty acyl-CoA molecules. This consequently increases fatty acid oxidation and acetyl-CoA to support Krebs cycle activity, which provides reducing equivalents to drive mitochondrial respiration. Hence, this could be an indirect mechanism by which carriers of the rs2419621 polymorphism are more responsive to lifestyle interventions in comparison to non-carriers.

The key strength of this study is insight into mechanisms underlying the effects of the rs2419621 genotype on the response to lifestyle interventions in overweight or obese individuals. We investigated the molecular mechanistic effects of the rs2419621 polymorphism on fatty acid oxidation and lifestyle intervention in T allele carriers, by utilising a combination of *in vitro, ex vivo* and *in vivo* methods using biochemical approaches and human population statistical analyses. However, weaknesses that must be acknowledged include an incomplete characterization of the enzymatic activities and potential pH-
dependencies of the short and long isoforms. Thus, future work will need to define their respective pH-dependent activities, their sensitivity to inhibitors such as triacsin, as well as the effects of both human isoforms on lipid biosynthesis. Finally, due to the small number of rs2419621 TT carriers, we were unable to specifically determine whether individuals who are homozygous for the T allele would present a greater response to lifestyle interventions and have a greater increase in fatty acid oxidation in comparison to CT individuals.

3.7 CONCLUSION

Our interdisciplinary approach supports the overall conclusion that ACSL5 rs2419621 [T] carriers lose more fat mass and have increased rate of weight loss during lifestyle interventions. T allele carriers displayed a greater amount of 683aa ACSL5 protein isoform which was found to be localized to a greater extent with the mitochondria, thus contributing to an overall increase in an individual’s fatty acid oxidation and cellular energy expenditure levels. To our knowledge, this is the first time the long and short human ACSL5 isoforms have been considered to have distinct functional roles in cell. Such research provides translational potential, as linking cellular and molecular work to genetic association studies not only provides a means of understanding an individual’s ability to respond to lifestyle interventions, but also aids in defining individualized intervention treatments for weight loss catered towards individual genotype.
3.8 REFERENCES


3.9 FIGURES

Figure 1: a) \(^{14}\)C Palmitic Acid Oxidation into Complete and Incomplete Fatty Acid Oxidation. Fatty acid oxidation experiments were conducted on transfected cells sorted with BeckmanCoulter MoFlo XDP/Beckman Coulter MoFlo Astrios. C2C12 myoblasts were transfected with pCAGIG vectors containing the short (n=4) or long (n=4) ACSL5 isoform cDNA, or pCAGIG empty vectors (n=4) to assess the endogenous \(^{14}\)C palmitate oxidation. Human ACSL5 driven fatty acid oxidation was calculated as: fatty acid oxidation products in presence of exogenous human ACSL5 – fatty acid oxidation products in presence of pCAGIG empty vectors alone. b) Representative western blot image showing protein expression levels of overexpressed human ACSL5 isoforms in C2C12 cells and endogenous ACSL5 mouse.
protein in C2C12 cells. The images are representative of 3 independent experiments. Western blot analysis of 3 independent experiments illustrated no difference in protein expression levels of both overexpressed ACSL5 human proteins and endogenous ACSL5 mouse proteins between samples.
Figure 2: ACSL5 long and short human isoform-YFP tagged colocalization in mitochondria and endoplasmic reticulum in C2C12 myoblasts. a) Immunofluorescence images are shown separate and merged. Images are also shown using a Color Map plugin from Image J where highly correlated pixels from both channels are shown in red while no correlation is shown in blue. Scale bar = 10µM. Images were taken using Zeiss LSM 510/AxioImager.M1 Confocal Microscope with 60 x oil lens. These images are representative...
of 3 independent experiments. b) Comparison of Mander’s Coefficient of colocalization with organelles of interest for ACSL5 human short and long isoforms. Results show significant difference between the localisation of both isoforms (unpaired t-test <0.05) (n=3).
Figure 3: ACSL5 rs2419621 genotype effect on protein levels, fatty acid oxidation and oxygen consumption on human muscle samples. a) Western blot on whole-cell lysates from primary myotubes of *rectus abdominis* illustrating ACSL5 683aa short isoform in patients who are rs2419621 [T] allele carriers (n=5) and non-carriers (n=4). b) $^{14}$C palmitate fatty acid oxidation results showing the effect of genotype on production rate of carbon dioxide and acid.
soluble products (ASP) by primary myotubes from *rectus abdominis* tissue samples. Samples were obtained from the same T allele carriers (n=5) vs non-carriers (n=4) as Fig 3a. c) High resolution respirometry illustrating the effect of genotype on oxygen flux in *vastus lateralis* tissue samples obtained from rs2419621 [T] allele carriers (n=4) and non-carriers (n=4). Mal= malate; Pyr= pyruvate; Cyt C= cytochrome C; FCCP= carbonyl cyanide p-trifluoromethoxyphenyl hydrazine; TMPD= 2mM N,N,N’,N’-Tetramethyl-p-phenylenediamine dihydrochloride.
### Table 1: Ottawa Hospital Weight Management Clinic patient information whose tissue and cells were used for analyses.

#### Patients whose *rectus abdominis* samples were used for *in vitro* analyses

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#### Patients whose *vastus lateralis* samples were used for *ex vivo* analysis

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Table 2: Regression analysis studying the effect of rs2419621 on respiratory quotient related parameters in CT/TT vs CC women with overweight or obesity.

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<td>0.244</td>
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* Multiple linear regression analysis was conducted. Parameter estimate of independent variable=Xt was studied. Biological factors (dependent variables) were studied while adjusting for confounding factors independent for each model (Supplementary Table 2).
CHAPTER FOUR: STUDYING THE ENVIRONMENTAL TOXICANT MEHP AND ITS EFFECTS ON FATTY ACID METABOLISM IN VITRO AND IN VIVO - AN ENVIRONMENTAL ASSOCIATION STUDY AND CELLULAR/MOLECULAR MECHANISTIC APPROACH

IN VITRO AND IN VIVO EVIDENCE OF BIOENERGETIC METABOLISM ALTERATION BY MONO-(2-ETHYLHEXYL) PHthalate

RUNNING TITLE: MEHP INDUCED BIOENERGETICS ALTERATION

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4.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

4.1.1 STATEMENT OF MANUSCRIPT STATUS

The manuscript “In vitro and in vivo evidence of bioenergetic metabolism alteration by mono-(2-ethylhexyl) phthalate” is in preparation for submission to Toxicological Sciences.

4.1.2 CONTRIBUTION STATEMENT

AR participated in the study design and majority of the experiments including the statistical analyses conducted within this paper. AR also wrote the manuscript. SS conducted the work studying cytotoxicity effects of MEHP on C2C12 myoblasts. KM conducted the MEHP exposure studies on C2C12 myoblasts for protein expression analyses using western blots. DP participated in interpreting the data and revised the article critically. MH participated in interpreting the data and revised the article critically. FT participated in the study design, interpreted the data and revised the article critically. All authors read and approved the final manuscript.

4.1.3 COMPETING INTEREST STATEMENT

The authors declare that they have no competing interests.
4.2 ABSTRACT

Introduction: To better understand the potential alteration of muscle bioenergetic metabolism by the obesogenic toxicant mono-(2-ethylhexyl) phthalate (MEHP) the objectives of this research were to determine: 1) the association between urinary MEHP levels and the efficacy of fatty acid utilization in women with obesity who participated in National Health and Nutrition Examination Survey (NHANES) studies. 2) the in vitro effects of MEHP on fatty acid, glucose, and mitochondrial energetics utilization in C2C12 mouse muscle cells.

Methods: The association between urinary MEHP from NHANES participants with plasma fatty acid levels was studied via secondary data statistical analyses. \(^{14}\)C palmitic acid oxidation, Seahorse fatty acid oxidation/glycolysis stress tests and western blot analyses were conducted on C2C12 myotubes exposed to increasing MEHP concentrations.

Results: Increased urinary MEHP in women with obesity was associated with increased plasma arachidonic and gamma-linolenic acid levels. Furthermore, C2C12 myotubes exposed to increasing MEHP concentrations, displayed decreased fatty acid oxidation and mitochondrial bioenergetics, and displayed increased basal glycolytic levels. Hexokinase II protein levels were also upregulated with increasing MEHP exposure in C2C12 myotubes. However, ACSL5 protein expression levels remained constant in MEHP treated myotubes.

Conclusion: MEHP exposure suggests an alteration of fatty acid utilization at the whole-body level in women with obesity and fatty acid and glucose utilization in muscle cells. This suggested that women with obesity may be susceptible to the effects of MEHP by increasing their risks for fatty acid and glucose metabolic alteration.

Keywords: MEHP, toxicity, mitochondrial bioenergetics, metabolism
4.3 INTRODUCTION

Obesogens are defined as chemical compounds that contribute towards impaired lipid metabolism, dysregulation of adipogenesis, and consequently, may contribute to the development of obesity (Grün & Blumberg, 2006). One of these obesogens, di(2-ethylhexyl) phthalate (DEHP), is one of the primary phthalate plasticizers used in North America, and is commonly found in food packaging and medical devices (Environment Canada Health Canada, 1994; US Food and Drug Administration, 2001). DEHP is non-covalently bound into matrices and therefore can leach into the human body and be metabolised via lipases into mono-(2ethyl hexyl) phthalate (MEHP), the major form which is absorbed (Koch et al. 2006). While the average tolerable intake for DEHP is 5.8-19 µg/kg/day, individuals exposed to a medical environment can exceed the average daily intake with an increase of ten-fold by the end of cardiopulmonary bypass in adults (Barry et al. 1989; Environment Canada Health Canada, 1994; Kavlock et al. 2002).

Previous human population studies in women and men, have reported a positive association between urinary phthalate metabolites including MEHP and mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) with obesity, while also demonstrating a positive correlation between DEHP exposure (measured via urinary metabolites) and BMI, waist circumference and adiposity within children (Buser et al. 2014; Deierlein et al., 2016; Smerieri et al., 2015; Stahlhut et al. 2007). Studies have also illustrated higher urinary MEHP to MEHHP ratio within women with greater BMI, suggesting a slower rate of oxidative metabolism of the toxicant (Yaghjyan et al., 2015). A decrease in MEHHP, a product of MEHP oxidation, may be a result of a decrease in activity of selected P450 enzymes, which might affect phthalate
metabolism (Yaghjyan et al., 2015). This may result in the alteration of fatty acid utilization (Yaghjyan et al., 2015) pointing out the importance of studying the effects of MEHP in subjects with obesity.

Studies have shown that elevated intra-myocellular accumulation of lipids in skeletal muscle of subjects with obesity, is a consequence of reduced fatty acid oxidation which contributes towards insulin resistance (Kelley et al. 1999). Rodent and human studies have both illustrated how chronic high fat feeding can lead to elevated incomplete β oxidation, and impairment in mitochondrial oxidative phosphorylation and biogenesis (Koves et al., 2008; Muoio & Newgard, 2008; Schrauwen, 2007; Sparks et al., 2005). Furthermore, rats exposed to increased dietary fat and sucrose, showed a decrease in muscle type-specific mitochondrial production of ATP (Chanseaume et al., 2006; Chanséaume & Morio, 2009). However, more recent evidence has suggested a link between insulin resistance and muscle mitochondrial dysfunction (Aguer & Harper, 2012; DeFronzo & Tripathy, 2009; Mogensen et al., 2007; Petersen et al. 2004; Phielix et al., 2008). Thus, the following studies imply the alteration of muscle mitochondrial bioenergetics during the development of chronic diseases such as obesity, highlighting the importance of studying this tissue.

While little is known about the effects of MEHP on human skeletal muscle, studies have shown a decrease in muscle strength in the elderly with increasing urinary MEHP, which was hypothesized to be a result of MEHP-induced oxidative stress and inflammation (Kim et al. 2016). Furthermore, Corbasson et al. 2016 illustrated an association between increased urinary MEHP and decreased lean mass in adults, where lean mass excluded bone mineral content, thus primarily representing muscle mass (Corbasson et al. 2016). Studies in rats exposed to DEHP have illustrated a reduction in glucose oxidation and glucose/lactate transport within
muscle and impairment in insulin receptor and GLUT4 gene expression levels in L6 muscle cells (Martinelli et al. 2006; P Rajesh & Balasubramanian, 2014; Parsanathan Rajesh & Balasubramanian, 2014). While adipocytes have been studied extensively and results have demonstrated altered adipocyte lipid metabolism and mitochondrial respiration, little is known about the cellular and molecular effects of MEHP on skeletal muscle fatty acid and glucose metabolism as well as mitochondrial energetics.

Thus, we aimed to determine 1) the association between MEHP and plasma fatty acid levels in women with obesity who participated in the National Health and Nutrition Examination Survey (NHANES) 2003-2004 study. 2) the *in vitro* effects of MEHP on fatty acid and glucose utilization and on mitochondrial energetics in C2C12 mouse myotubes.

**4.4 Material and Methods**

*NHANES 2003-2004 Study Data Analysis*

The NHANES study has been previously described (CDC National Center for Health Statistics., 2017). Briefly, NHANES is an American based survey designed to study the health and nutritional status of both adults and children in United States. NHANES provides extensive data on demographic, dietary and biological laboratory/body measurements. Because the continuous NHANES survey for 2003-2004 was the most recent survey that provided data on plasma fatty acid levels, we selected this population for our secondary statistical analyses. Human population statistical analyses focused on studying women with obesity (BMI ≥ 30kg/m²).

Studied variables have been previously described (CDC National Center of Health Statistics., n.d.-a). BMI was measured using standard calculations (weight in kg/ height in m²). Urinary MEHP was measured using high performance liquid chromatograph-electrospray ionization-
tandem mass spectrometry (HPLC-ESI-MS/MS) using isotopically-labeled phthalate metabolites as internal standards. Plasma fatty acid levels were measured as previously described, from 100µl of plasma obtained from individuals who had fasted for ≥ 8 hours.

**NHANES 2003-2004 Study Statistical Analyses**

Backward stepwise regression analyses were conducted to study the association between urinary MEHP and plasma fatty acid levels. Covariates that were included within the regression models, were continuous variables (age and BMI), and categorical variable (ethnicity: Non-Hispanic White, non-Hispanic Black, Mexican American and other Hispanic). Ethnicity was coded by dummy variables while using non-Hispanic White as the reference level. Urinary MEHP was also included to study whether it was a significant factor for plasma fatty acid levels. Prior to conducting the statistical analyses, one extreme outlier, based on standard deviation (SD) = 3, was found for urinary MEHP levels and removed from data analysis. Independent variables considered in the regression analyses were also weighted by using sample weight values provided by NHANES. Cohen’s $f^2$ value was calculated, to measure the effect size for each of the independent variables. Descriptive statistics was also conducted to determine the mean, SD and total participants (n), for all dependent variables studied. All statistical analyses were conducted using Minitab software 17, while a p value < 0.05 was considered statistically significant.

**Cell Culture**

The C2C12 mouse myoblast cell line (ATCC® CRL-1772™) was grown in standard Dulbecco’s modification Eagle’s medium (DMEM; Gibco) containing 10% Bovine Serum (BS; Gibco), 1% L-glutamine (Life Technologies) and 1% penicillin/streptomycin (Life
Technologies). C2C12 cells were differentiated into myotubes for 7 days in DMEM supplemented with 2% BS.

**MEHP Exposure**

MEHP (Accu Standard- please see company’s precautionary and hazard statements for proper use of chemical) acute exposure design consisted of either C2C12 myoblasts or myotubes exposed to 10, 50, 100 and 300 µM of MEHP (in 0.1% DMSO) for 24 hours. Concentrations of MEHP used were physiologically relevant and previously published by Chiang et al. 2016. MEHP solutions were added into culture medium. Cells exposed to 0.1% DMSO and untreated cells served as controls.

**MEHP Cytotoxicity Test**

C2C12 myotubes were exposed to MEHP (10,100 and 300 µM of MEHP) for 24 hours with n=3 independent experiments. 1:500 dilution of Propidium Iodide (Sigma; 1mg/ml) and 1:1000 dilution of Hoechst® 33342 (ThermoFisher; 5mg/ml) were added and mixed into culture medium. Following 10 min incubation at 37°C, cells were imaged using the Zeiss Axio Observer D1 Inverted Dic Fluorescence Microscope (20x, 0.80 NA, Air, Plan-Apo (DIC II) Objective; Blue (Ex:390/22 Em:460/50) and Red (Ex:560/40 Em:630/75)).

**14C-Palmitic acid Oxidation Experiment**

Following 24 hour exposure of C2C12 myotubes in a 12-well plate to 10, 100 and 300 µM of MEHP, total palmitic acid oxidation was assessed using 14C-palmitic acid as described previously (Aguer et al., 2013). Briefly, ASP (acid-soluble products) from both culture medium and cells were extracted (Glatz, Jacobs, & Veerkamp, 1984; Kitzmann et al., 2011; Veerkamp, Van Moerkerk, Glatz, & Van Hinsbergh, 1983). The remainder of the medium in
the glass vials containing benzethonium hydroxide was used for determining CO$_2$ production. Prior to scintillation counting in a PERKIN ELMER Tri-Carb 2910 TR liquid scintillation counter, all experimental vials were incubated overnight in the dark at room temperature. Three experiments were conducted (n=3). Total fatty acid oxidation = CO$_2$ produced + ASP measured from cells and culture medium. All values were adjusted to protein concentration which was determined using the Biorad Protein Assay Dye Reagent.

*Mitochondrial Respiration Measurement from Seahorse Mitochondrial Stress Test, Glycolysis Stress Test and Fatty Acid Oxidation Test*

Following exposure of C2C12 myotubes to 10, 50, 100 and 300 µM concentrations of MEHP for 24 hours in a 96-well Seahorse plate, mitochondrial stress test, glycolysis stress test and fatty acid oxidation tests were conducted following the Agilent company protocol. The 50µM concentration was only used for mitochondrial stress test and glycolysis stress test. As we were only interested in measuring total fatty acid oxidation, etomoxir addition was omitted from the protocol. Prior to initiating the Seahorse XF assay, 30 µl of palmitate-BSA (stock concentration: 1 mM) or BSA (stock concentration: 0.17 mM) as control were added into the appropriate wells. Final concentrations for drugs utilized in the fatty acid oxidation and mitochondrial stress test were: oligomycin (3µM); Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 2µM), antimycin (4µM), rotenone (5.6µM). Final concentrations for drugs utilized in the glycolysis stress test were oligomycin (3 µM) and FCCP (2 µM), glucose (100mM) and 2-deoxy-D-glucose (2-DG; 1M). All experimental results were normalized to results from control untreated cells. Three independent experiments were conducted for mitochondrial stress test, 5 for glycolysis stress test and 3 for fatty acid oxidation test.
Western Blot Analysis

Active Motif nuclear kit- “Preparation of Whole Cell Extract from Cells” protocol was utilised for the extraction of whole cell proteins from C2C12 cells exposed to MEHP (10, 50, 100, 150, 300 µM) for 24 hours, with n=3 independent experiments. Protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific).

A standard western blot protocol was implemented using 12% SDS-PAGE gels. All primary and secondary antibodies were prepared in 5% BSA-PBST. Membrane was incubated with the following primary antibodies: ACSL5 goat polyclonal IgG 1:200 (SantaCruz; sc47999), GAPDH rabbit polyclonal IgG 1:15000 (Abcam; ab22555), B-actin mouse monoclonal IgG 1:1000 (SantaCruz; sc47778), hexokinase I mouse monoclonal IgG 1:1500 (Abcam; ab105213), hexokinase II rabbit monoclonal IgG 1:1000 (Cell Signalling C64G5); Tom20 rabbit polyclonal IgG 1:1000 (SantaCruz; sc-11415). Secondary antibodies used were rabbit anti-goat IgG-HRP 1:5000 (SantaCruz; sc2768), goat anti-rabbit IgG-HRP 1:5000 (SantaCruz; sc2004), and goat anti-mouse IgG-HRP 1:5000 (SantaCruz; sc2031). Amersham ECL prime western blotting detection reagent and VersaDoc MP 4000 system (Biorad) with the Quantity One 4.6.9 Software (Chemi Hi Sensitivity 0.5x Gain Application) were used to detect and analyzed protein bands. As there was an increased variation in protein band intensity between the different western blot membranes for the C2C12 myoblasts exposed to MEHP, the following western blot quantitative analyses were normalized to results from untreated control cells, to minimize error.

C2C12 In Vitro Studies Statistical Analyses

One-Way Anova statistical analyses was conducted using Minitab 16 software.
4.5 RESULTS

*MEHP and plasma omega-6 fatty acid levels in women with obesity participating in the NHANES 2003-2004 Study*

Participant’s characteristics are presented in Table 1. While the majority of the plasma omega-6 fatty acid levels didn’t display a significant association with increasing urinary MEHP, a positive association was observed between urinary MEHP levels and gamma-linolenic acid (0.436 ± SE: 0.182; p=0.019) as well as arachidonic acid (7.62 ± SE: 1.73; p=0.000) in women with obesity (Table 2). Furthermore, r² adjusted model value explained 35% of the individual variation observed in arachidonic acid levels. Interestingly, it is MEHP which has the largest effect size (f²=0.212) in the linear model explaining variance in plasma arachidonic acid levels. However, while r² adjusted model value explained 24% of the individual variation observed in gamma-linolenic acid levels with a major influence of ethnicity, MEHP still influenced the model with an effect size of f²=0.056.

Previous work on the role of arachidonic acid metabolites have illustrated their roles in skeletal muscle tissue physiology including myogenesis (Prisk & Huard, 2003; Sun, Ba, Cui, Xue, & Zeng, 2009). Furthermore, as arachidonic acid is a key component of membrane and cytosolic phospholipids of mammalian cells, we decided to study the effects of MEHP in C2C12 mouse muscle cells (Tallima & El Ridi, 2017).

*Evaluation of MEHP cytotoxicity on C2C12 cells*

Prior to studying the effects of increasing concentrations of MEHP on C2C12 myotubes, its cytotoxicity was studied. There was no significant difference in the number of apoptotic and necrotic cells with increasing concentrations of MEHP exposure on C2C12 myotubes,
measured by condensed nuclei and PI positive cells (Figure 1). Furthermore, cell death always remained below 5% of the cell population.

The effects of MEHP on exogenous fatty acid oxidation and mitochondrial respiration in C2C12 myotubes

To characterize the metabolic effects of MEHP, 14C-palmitic acid oxidation as well as palmitate-induced respiration were studied in C2C12 myotubes. There was an overall decrease in total fatty acid oxidation following the exposure of myotubes to increasing concentrations of MEHP (Figure 2a). Specifically, a decrease was observed between the control 0.1% DMSO (1.183 nmol/hr/mg ± 0.035) and MEHP exposed cells of 10 µM (1.033 nmol/hr/mg ± 0.048), 100 µM MEHP (1.076 nmol/hr/mg ± 0.012) and the highest concentration 300 µM MEHP (1.034 nmol/hr/mg ± 0.022). This reduction in fatty acid oxidation level, was accompanied by a reduction in both maximal respiration as well as spare capacity when studying the muscle cell effects of MEHP on oxygen consumption rate (OCR) (Figure 2b and c). Specifically, a decrease in maximal respiration was observed between 10 µM (1.75 pmol/min/µg ± 0.382) and higher concentrations of 100 µM (0.62 pmol/min/µg ± 0.263) and 300 µM MEHP (0.60 pmol/min/µg ± 0.466). A decrease was also observed in spare capacity, between 0.1% DMSO (1.87 pmol/min/µg ± 0.121), 10 µM (1.85 pmol/min/µg ± 0.177) and the higher concentrations of MEHP, 100 µM (1.10 pmol/min/µg ± 0.279) and 300 µM (0.65 pmol/min/µg ± 0.281). No differences in basal respiration was observed between C2C12 myotube exposure to different MEHP concentrations. Furthermore, metabolic effects were only observed during fatty acid-driven oxidation, as mitochondrial stress test illustrated no differences with exposure to increasing concentrations of MEHP (Supplementary Figure 1).
The effects of MEHP on cellular glycolysis levels

Based on altered fatty acid metabolism in C2C12 myotubes exposed to MEHP, the effects of the toxicant on glucose utilization, specifically, extracellular acidification rate (ECAR) was studied. ECAR measured by mitochondrial stress test, illustrated a trend towards an increase in basal glycolysis levels following increased exposure to MEHP, reaching significance between 50 (0.89 ± 0.12) and 300 µM (1.25 ± 0.0674) MEHP (Supplementary Figure 1). Furthermore, ECAR measured by the glycolysis stress test in C2C12 myotubes, also illustrated a significant increase in basal glycolysis levels between 0.1% DMSO control (1.10 ± 0.097) and 300 µM MEHP (1.40 ± 0.135) (Figure 3).

The effects of MEHP on metabolism related proteins

After examining the effects of MEHP exposure on muscle fatty acid and glucose utilization/metabolism, we studied the toxicant’s effect on metabolism-related protein expression. Hexokinase II, a rate-controlling step in glycolysis and ACSL5, a key protein in fatty acyl-CoA activation from free fatty acids were studied. A significant increase in hexokinase II was observed with increasing concentrations of MEHP exposure on C2C12 myotubes (Figure 4). Specifically, a significant increase in hexokinase II was observed between DMSO control (Volume Intensity: 2.18 ± 0.091) or 10 µM MEHP (Volume Intensity: 2.28 ± 0.116) and 100 µM MEHP (Volume Intensity: 3.17 ± 0.452). This increase however was not significant in myotubes exposed to 300 µM MEHP. Increasing the exposure of C2C12 myotubes to MEHP appeared to have no effect on ACSL5. Furthermore, there was a decrease in ACSL5 levels with increasing MEHP in myoblasts (Supplementary Figure 2), specifically between 10 µM (Volume Intensity: 1.00 ± 0.131) and higher concentrations 100 µM (Volume Intensity: 0.464 ± 0.237) and 300 µM MEHP (Volume Intensity: 0.467 ± 0.201) when adjusted
for 0.1% DMSO control. These results reveal alteration in ACSL5 levels in myoblasts exposed to MEHP toxicant.

4.6 DISCUSSION

MEHP, is the monoester hydrolyzed form of the plasticizer DEHP. While much is known about the metabolic effects of MEHP on the general population, how this toxicant influences the metabolism of an individual with obesity remains to be elucidated. Studies have shown that individuals with obesity have elevated intra-myocellular accumulation of lipids in skeletal muscle, in part due to a reduction in fatty acid oxidation thus contributing towards insulin resistance (Kelley et al. 1999). As previous research by Corbasson et al. 2016, has illustrated an inverse association between increased urinary MEHP and lean mass (which excluded bone mineral content, thus primarily muscle mass), our research was focused on studying the effects of MEHP on muscle cell metabolism. Our results implicate an alteration in glucose and fatty acid utilization by MEHP in muscle cells as well as suggest changes in specific fatty acid utilization at the whole-body level in individuals with obesity.

Our secondary data statistical analyses focused on assessing the association between urinary MEHP and plasma fatty acid levels, given that previous studies illustrated that increased DEHP exposure is reflected in urinary levels of the monoester derivative (Green et al., 2005). As we were interested in analysing the effects of MEHP on fatty acid transport and mitochondrial oxidation alteration, we used plasma fatty acid levels as a proxy for mitochondrial bioenergetic function (Lagerstedt et al., 2001). Specifically, previous prospective studies have shown increased omega-6 fatty acids from erythrocyte and increased omega-6: omega-3 ratio in cord plasma phospholipids to be associated with obesity and weight gain (Donahue et al., 2011; Wang et al., 2016). Our results showed a positive association
between plasma levels of gamma-linolenic acid and arachidonic acid with urinary MEHP levels. Furthermore, we observed that MEHP has a large effect on plasma arachidonic acid levels. Arachidonic acid, is known to produce the metabolites prostaglandins and leukotrienes, involved in myogenesis, as well as muscular inflammation and repair (Korotkova & Lundberg, 2014). Interestingly, increased dietary omega-6 fatty acid levels including arachidonic acid, have also been associated with insulin resistance, leptin resistance and decreased mitochondrial biogenesis (Cheng et al., 2015; Massiera et al., 2010; Phillips et al., 2010; Pisani, Amri, & Ailhaud, 2015; Simopoulos, 2013; Simopoulos, Bourne, & Faergeman, 2013). A recent study by Markworth et al. illustrated how resistant trained men who received increased supplementation of dietary arachidonic acid had increased plasma arachidonic acid and gamma linolenic acid levels (Markworth et al., 2018). Furthermore, an increase in arachidonic acid in muscle was also observed, paralleled by increased mRNA levels of MyoD and myogenin (Markworth et al., 2018). However, as our secondary statistical analyses included esterified fatty acids that are found in the phospholipids, triglycerides and cholesterol esters, increased levels of arachidonic acid and gamma-linolenic acid that we observed with increased urinary MEHP, may also relate to increase in fatty acids channeled towards lipid biosynthesis. Regardless, our findings suggest an alteration of both fatty acid metabolism and utilization of fatty acids at the whole-body level with increased urinary MEHP levels. Due to a potential disruption in their enzymatic activity of MEHP oxidizing cytochrome P450 enzymes, individuals with obesity may be more susceptible to fatty acid metabolic alteration caused by MEHP, as compared to healthy individuals (Yaghjyan et al., 2015). Consequently, this could make them more prone to metabolic dysfunction. However, this will need to be confirmed by future studies.
Based on the known effect of arachidonic acid on skeletal muscle growth and function, as well as our secondary analyses illustrating urinary MEHP’s positive association with plasma levels of arachidonic acid, we studied the effects of MEHP on muscle cells. Previous work conducted by Feige et al. 2007 illustrated a dose-dependent activation of a PPARγ receptor element (PPRE)-firefly luciferase reporter, in the presence of adipogenic transcription factor PPARγ when C2C12 cells were exposed to MEHP, suggesting the toxicant’s potential PPARγ agonist effects (Feige et al., 2007). Interestingly, from our results, C2C12 myotube’s exposure to increased concentrations of MEHP, resulted in a decrease in total fatty acid oxidation levels. Furthermore, this decrease was paralleled with a disruption in mitochondrial respiration, particularly a decrease in maximal respiration and spare capacity measured by oxygen consumption rate. Thus, exposure of C2C12 myotubes to increasing concentrations of MEHP alters fatty acid oxidation and oxidative phosphorylation pathway. Previous research have demonstrated an upregulation of 3T3-L1 adipocyte respiration with increased exposure to MEHP, while exposure of isolated rat liver mitochondria to MEHP showed an inhibition in palmitic acid oxidation as well a reduction in overall mitochondrial respiration (Chiang et al., 2016; Winberg & Badr, 1995). This illustrates MEHP’s tissue-specific effect on mitochondrial respiration and fatty acid metabolism.

In regard to glucose metabolism, a significant increase in basal glycolysis was observed between control cells and cells exposed to 300µM MEHP. This increase in basal glycolysis, primarily representing the production of lactate, illustrates an alteration in glucose utilization which supports previous work in other tissues and species. Particularly, impairment in glucose oxidation has been observed in Chang liver cells and insulin-stimulated L6 rat myotubes with
exposure to increasing concentrations of DEHP and MEHP (Rengarajan et al. 2007; Viswanathan et al. 2017).

Previous findings illustrated a significant increase in leg muscle glucose oxidation within patients with T2D versus non-diabetics (Kelley & Mandarino, 1990). This dysfunction in basal muscle metabolism was believed to contribute to systemic basal metabolic abnormalities in individuals with T2D (Kelley & Mandarino, 1990). Thus, linking MEHP’s in vitro fatty acid metabolic changes to the toxicant’s effects on in vivo fatty acid utilization can be justified.

Hexokinase II, the key enzyme responsible for the conversion of glucose to glucose-6-phosphate and the abundant form in skeletal muscle, was found to be upregulated with exposure of C2C12 myotubes to increasing levels of MEHP. This increase in hexokinase II may contribute to the increased glycolysis observed with exposure of C2C12 myotubes to MEHP. While ACSL5 protein levels remained constant in C2C12 myotubes exposed to MEHP, a significant decrease in ACSL5 protein expression levels was observed within myoblasts. ACSL5 contributes towards the conversion of long-chain free fatty acids to fatty-acyl CoA. The decreased in expression of this key protein within myoblasts, may contribute to altered fatty acid metabolism in proliferative cells. However, this needs to be confirmed by further studies.

The present study focuses on the effects of MEHP at both the cellular and whole-body level, in women with obesity. To our knowledge, this is the first study to observe the effects of MEHP on plasma omega-6 fatty acid (gamma-linolenic acid and arachidonic acid) levels in women with obesity. Future work includes studying the metabolic effects of MEHP in men with obesity.
In conclusion, our current results expands our knowledge on the effects of MEHP on cellular and whole-body metabolism. Increased MEHP exposure contributes towards metabolic dysfunction at both the muscle cell and whole-body level, supporting previous studies on the influence of basal muscle metabolism on systemic metabolic abnormalities in individuals with insulin resistance and glucose metabolism dysregulation. Hence, women with obesity may be more susceptible to the metabolic effects of MEHP.

4.7 REFERENCES


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4.8 FIGURES

Figure 1: MEHP cytotoxicity in C2C12 myotubes - C2C12 seven-day differentiated myotubes were exposed to varying concentrations of MEHP (10, 100 and 300 µM) for 24 hours followed by staining for Hoechst 33342 and Propidium Iodide (PI), both DNA staining dyes. Cells that have undergone apoptosis would result in nuclei condensation which can be detected via Hoechst 33342 while apoptotic and necrotic cells will be detected by PI which is unable to cross the membrane of live cells. No significant difference in cytotoxicity was detected between varying concentrations of MEHP exposure to C2C12 myotubes as seen on bar graph with cell death being less than 5% for all conditions. Images were taken at 20x with Zeiss AxioObserver.D1 Microscope (n=3; 6 images/condition taken with 20x objective).
Figure 2: Effects of MEHP on C2C12 myotube’s fatty acid oxidation and mitochondrial respiration- C2C12 seven-day differentiated myotubes were exposed to varying concentrations of MEHP (10, 100 and 300 µM) for 24 hours. a) assessment of total fatty acid oxidation by measuring palmitate driven exogenous fatty acid oxidation after incubation with radiolabelled $^{14}$C-palmitate. n=3 b and c) Seahorse assessment of fatty acid oxidation by measuring mitochondrial oxygen consumption rate in presence of palmitate (n=3). * represents p < 0.05.
Figure 3: Assessment of the effects of MEHP on cellular glycolysis levels in the presence of exogenous glucose - C2C12 seven-day differentiated myotubes seeded onto an XF96-well plate were exposed to varying concentrations of MEHP (10, 50, 100 and 300 µM) for 24 hours. Extracellular acidification rate (ECAR) indicative of glycolysis when provided with glucose was assessed using the Seahorse XF Cell Glycolysis Stress Test (n=5). * represents p < 0.05.
Figure 4: The effects of MEHP on metabolism related proteins - Western blot analyses on whole-cell lysates from C2C12 seven-day differentiated myotubes exposed to varying concentrations of MEHP (10, 50, 100 and 300 µM) for 24 hours (n=3). * represents p < 0.05.
4.9 Tables

**Table 1:** Characteristics of women with obesity enrolled in NHANES 2003-2004

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**Table 2:** Backward regression analysis studying the modulators of plasma omega-6 fatty acid (Arachidonic Acid (AA) and Gamma-Linolenic Acid (GLA)) levels within adult women with obesity who participated in NHANES 2003-2004 study.

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5 GENERAL DISCUSSION

Once defined as a “high-income country problem”, the advancement of overweight and obesity into a global epidemic has been a critical concern, with approximately 600 million adults and 108 million children documented with obesity worldwide\textsuperscript{1,2}. While treatments established to combat obesity include exercise and dietary interventions, medication and bariatric surgery, inter-individual variation in treatment response exists\textsuperscript{3-5}. Furthermore, overweight and obesity are multi-factorial conditions and thus could develop through the interplay of many complex factors including genetic, epigenetic, behavioral and environmental factors. Hence, there is a growing pressure to further our knowledge of factors that affect adiposity and weight gain, while also identifying weight loss predictors to aid in the treatment of obesity via personalized lifestyle interventions.

Globally, while the frequency of obesity was determined to be about 12\% in adults and 5\% in children, it was shown that across all ages, women had a higher prevalence of obesity in comparison to men\textsuperscript{2}. Thus, the overarching objective of this Ph.D. thesis was to provide further insight into the effects of genetics, behavioral and environmental factors in the development of overweight and obesity, with a key focus on women. Specifically, my research focused on understanding the effects of an individual’s genotype on weight loss during behavioral modifications via a diet and exercise intervention, as well as the effects of the environmental toxicant, mono-(2ethylhexyl) phthalate, on weight gain. Furthermore, the current research offers an interdisciplinary approach, by studying the cellular and molecular mechanisms, as well as utilising human population statistics to determine how these factors influence weight gain and obesity.
Known to be one of the key tissues to utilize free fatty acids as a source of energy, skeletal muscle constitutes around 40% of body mass within a healthy adult\textsuperscript{6,7}. Specifically, in healthy lean individuals while 20-30\% of total resting oxygen uptake is attributed to the skeletal muscle, during maximal physical activity, over 90\% of the whole-body oxygen uptake is attributed to muscle\textsuperscript{7-9}. Furthermore, studies on the effects of obesity in skeletal muscle, have illustrated an elevated intra-myocellular accumulation of lipids within the tissue of individuals with obesity\textsuperscript{10-12}. This was deemed to be a consequence of reduced fatty acid oxidation which contributes towards insulin resistance\textsuperscript{10}. The primary tissue of interest for this Ph.D. thesis was the skeletal muscle. Chapters 2 and 3 focus on studying the metabolic effects of genetic factors on individuals with overweight and obesity during lifestyle interventions, and Chapter 4 focuses on the metabolic effects of environmental factors on individuals with obesity.

Due to the increased demand for successful personalized lifestyle interventions in women with obesity, the necessity in identifying genotypes that are highly sensitive to weight loss, and understanding their impact at a cellular and molecular mechanistic level, is of vital importance\textsuperscript{13}. Thus, Chapters 2 and 3 of the following thesis focus on a key polymorphism of the human ACSL5 gene, rs2419621. Characterised by a cytosine to thymine transition in the promoter region of the gene, rs2419621 has been previously shown by our lab to be associated with increased rate of weight loss in menopausal women with obesity, who had participated in the Ottawa Hospital Weight Management Clinic\textsuperscript{14}. Furthermore, studies conducted to determine the functionality of the polymorphism, illustrated the presence of an additional EBOX site on top of the two commonly found sites, recruiting more myoD transcription factors, thus increasing the regulation of the downstream ACSL5 gene\textsuperscript{15}. However, while the rs2419621 genotype has been shown to play a role in increased mRNA expression and
response to lifestyle interventions of women with overweight and obesity, how this particular polymorphism exerts its effect at a cellular and molecular level is unknown. Furthermore, changes in anthropometric measurements observed in carriers of the rs2419621 polymorphism with obesity, had not been determined prior to our research. Thus, the research described in Chapter 2 took a genetic association study approach, by testing the association between the ACSL5 rs2419621 genotype and response to lifestyle interventions in women with overweight and obesity, by studying pre- and post-intervention changes in anthropometric measurements. The research in Chapter 3 utilized a molecular mechanistic approach to determine the cellular and molecular mechanisms by which the rs2419621 ACSL5 polymorphism exerts its effect.

Findings presented in Chapter 2 demonstrate that the rs2419621 polymorphism plays a key role in response to diet and exercise intervention\(^{16}\). Specifically, carriers of the rare T allele following lifestyle interventions MONET and CAO, had a greater reduction in their visceral fat and fat mass levels, while having a greater increase in their percent lean mass, in comparison to non-carriers\(^{16}\). These observations were noted in postmenopausal women with overweight or obesity. In addition, a greater decrease in BMI following the lifestyle interventions was noted in T allele carriers vs non-carriers when studying postmenopausal women with obesity solely\(^{16}\). The increased weight loss in T allele carriers vs non-carriers can be explained by the greater reduction in adiposity associated with the presence of the rs2419621 polymorphism following a lifestyle intervention. Furthermore, the reduction in fat-related anthropometric measurements post-intervention within women with overweight, illustrates the potent metabolic effect of the ACSL5 polymorphism at the whole-body level.

This observed significant reduction in adiposity in the [T] allele carriers, is further supported by our findings described in Chapter 3 in which we report the association of rs2419621
genotype with respiratory quotient related measurements. Specifically, T allele carriers with obesity who participated in the MONET intervention, presented a greater increase in their fatty acid oxidation levels and a corresponding decrease in their carbohydrate oxidation levels in vivo following the intervention, in comparison to non-carriers. However, while in vivo results explaining the effects of the rs2419621 genotype on overweight and obesity display an increased efficiency of fatty acid oxidation, thus contributing towards improvements in anthropometric measurements, how this occurs at a cellular and molecular level required further elucidation. Additionally, it is important to note that the MONET intervention consisted of 1/3 of the population undergoing an exercise intervention. Hence, more drastic fatty acid oxidation changes may have been noticed if the entire population had participated in resistance training.

Chapter 3 findings provide detailed insight into the molecular mechanisms by which the rs2419621 polymorphism exerts its effect. Specifically, it was observed that carriers of the rare T allele had increased levels of the human 683aa ACSL5 protein isoform in comparison to non-carriers. Our in vitro findings on C2C12 mouse muscle cells overexpressing human ACSL5 have illustrated that the commonly found short isoform of 683aa is localized in the mitochondria to a greater extent in comparison to the long human ACSL5 protein isoform of 739aa, also resulting in a greater fatty acid oxidation levels. This increase in protein expression was paralleled with an increase in both in vitro human muscle cell carbon dioxide production, as well as ex vivo human muscle tissue mitochondrial respiration. Furthermore, confocal imaging of cells from T allele carriers vs non-carriers illustrated no differences in mitochondrial quantity between groups. Thus, Chapters 2 and 3 clearly illustrate that the increased response to lifestyle interventions observed within T allele carriers, their increase
rate of weight loss and improved anthropometric measurements, is in part due to an increase in the human 683aa ACSL5 protein isoform, contributing towards an increased in vitro, ex vivo and in vivo fatty acid oxidation and energy expenditure. Thus, our findings contribute towards an improved understanding of the cellular and molecular functional characteristics of the ACSL5 rs2419621 genotype.

Previous reports have illustrated the importance of acknowledging sex differences in lifestyle treatment of obesity\(^\text{17}\). Specifically, while sex differences in energy metabolism have been observed, the etiology is unclear, with potential contributors ranging from sex steroids to leptin hormone regulation\(^\text{17}\). Although the effects of ACSL5 rs2419621 genotype have been thoroughly studied within post-menopausal women, the polymorphism’s effect on the cellular and molecular mechanisms in men is yet unknown. Moreover, while Adamo et al. illustrated increased weight loss in women, these studies need confirmation within individuals of varying sociodemographic status (i.e., different age groups, race, income). Furthermore, the following projects are focused on individuals who underwent diet interventions and exercise training. Whether similar metabolic effects in vivo will be observed in individuals with overweight and obesity, following a longitudinal observational study, needs to be verified. Statistical analyses conducted on studying the polymorphism’s effect on healthy individuals as well as athletes exposed to continuous rigorous exercise are also of interest for future studies.

The rs2419621 polymorphism increases the recruitment of myoD and was shown to only be affective in the presence of the transcription factor\(^\text{15}\). Specifically, when studying CV-1 African green monkey kidney cells where myoD is not expressed, the polymorphism only affected downstream promoter activity in the presence of the myoD transcription factor\(^\text{15}\). Thus, while the effects of rs2419621 are directed towards skeletal muscle metabolism, indirect
effects of the polymorphism may exist noted by reduced visceral fat in carriers of the T allele. However, further exploration of the polymorphism’s indirect effects is required.

My research focused on establishing the function of the human ACSL5 protein isoforms, specifically regarding fatty acid oxidation and mitochondrial respiration within skeletal muscle. While our findings illustrate that the short protein isoform of 683aa appeared to be the most abundant protein isoform in skeletal muscle samples, this may not be the case in other tissue samples. This was clearly illustrated in HepG2 liver carcinoma cells, which expressed both the two common ACSL5 protein isoforms of 683 and 739aa, as well as the uncommon 659aa protein isoform, which may play a role in sensitization to TRAIL-based apoptosis. Thus, further studies are required to elucidate the functional role of the long protein isoform of 739aa as well as the short protein isoforms 683 and 659aa within different tissues. Regardless, this research illustrates for the first time the possibility that ACSL5 protein isoforms have separate functional roles in metabolism. As diseases such as cancer have been shown to affect the expression of the ACSL5 protein, further studies are required into the roles of the human protein isoforms in such diseases. Generally, ACSL proteins differ in their substrate preference, tissue specificity and subcellular localization. However, studies have shown the presence of compensatory mechanisms following the suppressed expression of one of the ACSL proteins. Therefore, addressing how the rs2419621 polymorphism influences the interplay between other ACSL proteins, namely ACSL 1, 3, 4 and 6, is of interest in the future.

Finally, while behavioural factors can be monitored, and genetic factors such as fatty acid metabolism related polymorphisms are important, it is still important to consider other external factors that aren’t easily regulated such as environmental toxicants. Obesogens are toxicants
that are found in the environment that play a role in increased adiposity\textsuperscript{27}. Interestingly, a feedback loop is generated, in which studies have shown individuals with greater BMI to have decreased clearance of certain toxicants from their system, potentially making them more susceptible to the metabolic dysregulation caused by the obesogen\textsuperscript{28,29}. Specifically, mono-(2ethylhexyl) phthalate (MEHP), the monoester derivative of di(2-ethylhexyl) phthalate (DEHP), has been postulated to affect individuals with obesity to a greater extent vs healthy individuals\textsuperscript{28}. Due to their potential disruption in MEHP oxidizing cytochrome P450 enzymatic activity, individuals with obesity are more susceptible to MEHP’s metabolic effects vs healthy individuals\textsuperscript{28,29}. Consequently, this could make them more prone to diseases such as diabetes and obesity, when exposed to DEHP, one of the primary phthalate plasticizers used in North America\textsuperscript{30}. Consequently, due to the increased prevalence of obesity within women, Chapter 4 focuses on the effects of mono-(2ethylhexyl) phthalate, and its effects on cellular metabolism and fatty acid utilization within women with obesity.

Previous research has documented increased plasma fatty acid levels to have been associated with disruption in fatty acid transport and mitochondrial oxidation disruption\textsuperscript{31}. Our observations from women with obesity who participated in the NHANES study, illustrated an association between increased levels of plasma gamma-linolenic acid and arachidonic acids with increased urinary MEHP. Increased omega-6 fatty acid levels, including arachidonic acid, have also been associated with decreased mitochondrial biogenesis and activity, insulin resistance and leptin resistance\textsuperscript{32–35}. Interestingly, our findings also demonstrate that the treatment of C2C12 mouse muscle cells with MEHP caused a reduction in fatty acid oxidation and mitochondrial bioenergetics. Furthermore, exposing C2C12 cells to MEHP resulted in an increase in basal glycolytic levels, and glycolysis-related protein, hexokinase II.
It is important to note that previous research supports the ideas that the toxicant has tissue-specific effects on mitochondrial respiration. Specifically, research conducted on 3T3-L1 adipocytes has illustrated an increase in mitochondrial respiration following exposure to MEHP, while isolated mitochondria obtained from rat liver illustrated an inhibition in palmitic acid oxidation following exposure to MEHP\(^{36,37}\). However previous work on MEHP/DEHP’s effect on glucose metabolism have illustrated similar observations as our \textit{in vitro} work, where an impairment in glucose utilization was observed, within human Chang liver cells exposed to DEHP, and 3T3-L1 cells following exposure to increasing concentrations of MEHP\(^{36,38}\).

Previous research on the effects of muscle glucose oxidation in type 2 diabetic patients have illustrated a significant increase in basal leg muscle glucose oxidation vs in non-diabetic individuals\(^{39}\). This observation in individuals with diabetes, was believed to contribute towards an overall systemic basal metabolic abnormality\(^{39}\). As a large percentage of the human body is skeletal muscle, known to be the primary site of insulin resistance during type 2 diabetes progression, relating our cellular work to the observations noted at the whole-body level can be justified. Thus, future studies include studying the association between urinary MEHP and fasting glucose/insulin levels in individuals with obesity.

Our \textit{in vitro} and human population studies, illustrate how exposure to MEHP, results in cellular fatty acid and glucose metabolic disruption, as well as altered plasma fatty acid levels. Thus, individuals with obesity may be highly susceptible to the metabolic defects associated with MEHP, increasing their chances of metabolic dysfunction.

A key focus of Chapters 2 and 3 was the ACSL5 protein, known to play a role in esterifying free fatty acids into fatty acyl-CoA molecules, channeling them towards fatty acid oxidation and lipid biosynthesis. Based on our population statistics on women with obesity, we noted a
parallel increase in urinary MEHP and plasma fatty acid levels, specifically arachidonic acid and gamma linolenic acid. Previous work on the role of arachidonic acid metabolites on skeletal muscle have illustrated their function in skeletal muscle tissue physiology including myogenesis\textsuperscript{40,41}. The increase in fatty acid levels, has been shown to be indicative of impaired fatty acid transport and mitochondrial oxidation\textsuperscript{31}. Furthermore, a reduction in fatty acid oxidation and mitochondrial bioenergetics was noted at the muscle cellular level, following increased exposure to MEHP. Thus, we postulated that ACSL5, a key protein known to regulate fatty acid esterification and transport could be dysregulated following exposure of muscle cells to MEHP. Interestingly, no changes in ACSL5 expression were noted in C2C12 myotubes. However, there is the possibility that other ACSL proteins are dysregulated by such toxicant exposure and therefore further research is required to understand MEHP’s regulation of fatty acid metabolism related proteins in skeletal muscle. Interestingly, a significant reduction in ACSL5 protein expression was noted in C2C12 myoblasts exposed to MEHP, implying a certain level of ACSL5 protein dysregulation within proliferative muscle cells. However, further studies in C2C12 myoblasts are needed to confirm the effects of MEHP on muscle cell proliferation. Future studies which are ongoing in our lab, include exposing C2C12 myoblasts to the MEHP toxicant, in the presence or absence of ACSL5 via siRNA techniques, and studying its impact on overall mitochondrial bioenergetics.

Lastly, as environmental toxicants can affect the epigenome of an individual, how MEHP affects the epigenetic regulation of various fatty acid metabolism related genes is of key interest. Specifically, MEHP has been shown to play a role in DNA methylation potentially regulating the expression of FA metabolism-related genes\textsuperscript{42}. Thus, future directions include understanding the epigenetic effects of MEHP within skeletal muscle C2C12 cells, and
whether it regulates the expression of such genes through DNA and histone hyper/hypo methylation, acetylation etc.

The concept of personalized medicine, including personalized genomics, states that the genetic background of an individual can lead to individualised treatments according to a person’s genotype. Treatments would include prescribing specific dietary and behavioural changes to treat diseases such as obesity. While the concept itself appears promising, to date genetic loci initially thought to be of high value for determining susceptibility to obesity, were in fact shown to have small effects on weight gain and only explained a fraction of the total variance. An example is the FTO variation, which was shown to associate with only a modest weight gain. Thus, while identifying key polymorphisms that affect metabolism such as ACSL5’s rs2419621, is of value to the progression of personalized medicine treatments, research has shown the insufficient incorporation of environmental factors in disease etiology. A recent report published by Carlsten et al. emphasized the importance of studying genetic and environmental factors in understanding epigenetics and medical treatment of disease. The report stressed on the underestimation of complex chronic diseases by studying an individual’s genetics solely, while disregarding environmental and social determinants of health. While current research acknowledges the importance of including such factors into personalized medicine, this would result in a complete shift in current public health policies to emphasize the importance of both the physical and social environment of an individual.

It has been noted that studying solely one genotype and toxicant of interest regarding skeletal muscle metabolism, is a limitation of the thesis, and such observations may differ when studying different tissues of interest, as well as within different populations of varying ethnicity, age and gender. However, my Ph.D. research stresses on the importance of
understanding an individual’s personal genome in combatting obesity, while acknowledging that the disease is clearly multi-factorial, and effective personalized medicine treatment must also consider the importance of environmental factors such as toxicants, in the development and progression of this disease. Furthermore, my doctoral research is consistent with the importance of interdisciplinary research approaches, in which biochemical analyses of cellular and molecular metabolism are complemented by human population statistics to study the effects of genetics and environmental factors at the whole-body level. This was done with the overall goal of an improved comprehensive understanding of the effects of ACSL5 rs2419621 polymorphism and the environmental toxicant MEHP on human metabolism.
5.1 REFERENCES


6 APPENDIX

6.1 SUPPLEMENTARY FIGURES/TABLES FOR CHAPTER THREE

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**ELSFKYFRQQDSLYEHIQ**
Figure S1: Protein alignment of Human ACSL5 long (739aa) and short (683aa) protein isoforms with mouse ACSL5 (683aa) and rat ACSL5 (683aa). Both mouse and rat ACSL5 are 81% identical to the Human short protein isoform of ACSL5.
Figure S2: Mouse ACSL5 colocalization in Endoplasmic Reticulum and Mitochondria- a) Immunofluorescence images stained for mouse ACSL5, Calreticulin (for endoplasmic reticulum staining) and Tom20 (mitochondria staining) in C2C12 cells. Images are shown separate and merged, as well as Color Map plugin from Image J where highly correlated pixels from both channels are shown in red while no correlation is shown in blue. Images were taken using the Zeiss LSM 510/Axiolmager.M1 Confocal Microscope 60x oil lens. Images are representative of 3 independent experiments. Scale bar= 20µm. b) Colocalization of mouse ACSL5 with organelles of interest was studied using Mander’s Coefficient. Mander’s Coefficient for ACSL5 in endoplasmic reticulum= 0.809 (SEM=0.003 n=3 analysing a total of 47 cells); Mander’s Coefficient for ACSL5 in mitochondria= 0.297 (SEM=0.020 n=3 analysing a total of 45 cells). Unpaired t-test was used to compare between ACSL5 colocalization with endoplasmic reticulum and mitochondria *p< 0.001.
Figure S3: ACSL5 protein isoform expression within human muscle sample (HMS) and HepG2 cells – Western blots using anti-ACSL5 goat polyclonal antibody (Santacruz) - Lane A: Whole cell lysate of rectus abdominis cells derived-myotubes from HMS 133 rs2419621 CC carrier depicting ACSL5 76 kDa short isoform of 683aa; B: F-Ladder (100, 70, and 55kDa); C: 5ug, D: 10ug, and E: 20ug of whole cell lysate of HepG2 liver carcinoma cells depicting ACSL5 76kDa short isoform of 683aa, ACSL5 82kDa long isoform of 739aa, and 73kDa ACSL5Δ20 isoform of 659aa.
Figure S4: Immunofluorescence images stained for human ACSL5 and Tom20 in human Rectus Abdominis satellite cells derived-myotubes. Images are shown separate and merged as well as Color Map plugin from Image J where highly correlated pixels from both channels are shown in red while no correlation is shown in blue. Images were taken using the Zeiss LSM 510/Axiolmager.M1 Confocal Microscope 20x lens using Z-stack. Scale bar = 20μm. Colocalization of human ACSL5 with mitochondria was analyzed using Mander’s Coefficient and determined to be statistically non-significant between the different genotypes. Image are representative of n = 5 rs2419621 [T] allele carriers and n=4 rs2419621 non-carriers.
Table S1: Descriptive statistics on Montréal Ottawa New Emerging Team participants with obesity or overweight pre/post-intervention.

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Table S2: Best subset regression analysis results to determine covariables for the multiple linear regression analysis. Data sets that were used for dependent variables were data from the Montreal New Emerging Team (MONET) population combining individuals with overweight or obesity.

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6.2 SUPPLEMENTAL FIGURES FOR CHAPTER FOUR

Supplementary Figure 1: The effects of MEHP on cellular endogenous mitochondrial respiration and glycolysis a) C2C12 seven-day differentiated myotubes seeded onto an XF96-well plate were exposed to varying concentrations of MEHP (10, 50, 100 and 300 µM) for 24 hours. The following values were measures by using the Seahorse XF Cell Mitochondrial Stress Test a) oxygen consumption rate (OCR) indicative of endogenous cellular mitochondrial respiration and b) extracellular acidification rate (ECAR) indicative of endogenous glycolysis (n=3).
Supplementary Figure 2: The effects of MEHP on ACSL5 levels in C2C12 myoblasts - Western blot analyses on whole-cell lysates from C2C12 myoblasts exposed to varying concentrations of MEHP (10, 50, 100, 150 and 300 µM) for 24 hours (n=3).
6.3 SUPPLEMENTAL METHODOLOGY FOR CHAPTER THREE

Supplementary Material - Methodology

Cell Cultures

C2C12 growth medium using standard Dulbecco’s modification Eagle’s medium (DMEM) was supplemented with 10% fetal bovine serum (FBS- Gibco), 1% L-glutamine (Life Technologies) and 1% penicillin/streptomycin (Life Technologies).

Human *rectus abdominis* cells were grown in F10 nutrient mixture (Ham’s) medium supplemented with 12% bovine growth serum (BGS), 10 ng/mL epidermal growth factor, 1µM dexamethasone, 25 pmol/L insulin 0.1 µg/mL, 6.3mL (1X) antibiotic-antimycotic and 5µg/mL gentamycin sulfate. Cells were differentiated for 7 days in low glucose DMEM supplemented with 2% horse serum, 5 mL (1X) antibiotic-antimycotic and 5µg/mL gentamycin sulfate.

HepG2 liver carcinoma cell line was cultured in standard Eagle's minimum essential media containing 1g/L D-glucose and 2mM L-glutamine (Gibco), supplemented with 10% FBS (Gibco).

Construction of Vectors

cDNA of the human long and short protein isoform of 739aa and 683aa respectively, were amplified by PCR with the following forward primers and reverse primers:

**ACSL5 long isoform of 739aa:** CCTTCTCTCGAGATGGACGCTCTGAAGCCACCCTGT containing XhoI restriction digest site
ACSL5 short isoform of 683aa: CTGCTGCTGTTTCCTCGAGATGCTTTTTATCTTTAA
CTTTTTGTTTTCCCCA containing XhoI restriction digest site

peYFP-N1 reverse primer:
CAGGTACTTAAGTACCAGATCCTTATCCTGGATGTGCTCA TACAGGCT containing BamHI restriction digest site

pCAGIG reverse primer:
GTACTTAAGTGCGGCCGCCTAATCCTGGATGTGCTCATAAGG containing NotI restriction digest site

**Immunostaining**

*For C2C12 cells overexpressing human ACSL5-YFP vectors and stained for Mitochondria (Tom20) and Endoplasmic Reticulum (Calreticulin):*

Tom20 (FL-145) rabbit polyclonal IgG 1:1000 (Santa Cruz; Sc-11415) in 1% BSA-PBST

Calreticulin rabbit polyclonal IgG 1:1000 (Abcam; ab2907) in 1% BSA-PBST

Alexafluor 647 goat anti-rabbit IgG (H+L) 1:1500 (Life Technologies; A21244) in 1% BSA-PBST

*For double immunostaining to also detect endogenous ACSL5 from C2C12 or untagged human ACSL5:*

ACSL5 (E-12) mouse monoclonal IgG 1:50 dilution (Santa Cruz; sc398310) in 1% BSA-PBST

Alexafluor 546 IgG (H+L) goat anti-mouse 1:1500 (Life Technologies; A11030) in 1% BSA-PBST
For human muscle cells stained for endogenous ACSL5 and mitochondria with Tom20:

Tom20 (F-10) mouse monoclonal IgG at 1:1000 (Santa Cruz Sc17764) in 1% BSA-PBST

Chicken anti-mouse IgG (H+L) Alexafluor 647 at 1:1000 (ThermoFisher A-21463) in 1% BSA-PBST

ACSL5 rabbit polyclonal IgG antibody was used at 2.5ug/ml concentration (ThermoFisher; PA5-52392) in 1% BSA-PBST

Donkey anti-rabbit IgG (H+L) Cy3 at 1:250 (Jackson Immuno; 711-165-152) in 1% BSA-PBST

Microscope settings

Images were acquired with a 63x Plan-Apochromat 1.4 oil objective for C2C12 staining, and 20x objective for human muscle cells, while using the following confocal lasers: Argon (514nm), HeNe (543nm) and HeNe (633nm) and the following filter sets: Chroma Filter Set 49003 (YFP) and Zeiss Filter Set 45 (mCherry, Cy3.5).

Western blot

ACSL5 goat polyclonal IgG 1:200 (SantaCruz; sc47999) in 1% BSA-PBST

Rabbit anti-goat IgG-HRP 1:5000 (SantaCruz; sc2768) in 1% BSA-PBST

GAPDH rabbit polyclonal IgG 1:15000(Abcam; ab22555) in 1% BSA-PBST

Goat anti-rabbit IgG-HRP 1:5000(SantaCruz; sc2004) in 1% BSA-PBST
High Resolution Respirometry of Biopsied Muscle

The following injections were consecutively added into the O2K system chambers containing biopsied muscle: malate (2mM), pyruvate (5mM), glutamate (10mM), ADP (5mM; for complex I-supported respiration), succinate (10mM; for complex I and II supported respiration) and 0.25µM of carbonyl cyanide p-trifluoromethoxyphenyl hydrazine (FCCP) titrated to drive maximal respiration. Complex 4 driven respiration was also studied with the addition of 2mM N,N,N’,N’-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD) and 2mM ascorbate.

Respiratory Quotient Related Measurements

For best subset regression analysis variables were considered based on causative effect on weight loss: age, initial weight, height and changes in transferrin, glucose, CRP, orosomucoid, haptoglobin, insulin, leptin and participation in resistance training. Average energy, protein, carbohydrate, sugar and fat consumed in the three days recorded by the nutrition journal (all recorded as g/day) were also tested as potential covariates. Covariates selected for adjustment were based on the adjusted R-squared and Mallows Cp specific for each model studied.