**Serum Fatty Acid Profiling within Distinct Lipid Fractions Provides
a More Robust Indicator of Insulin Resistance in Humans than
Total Triglyceride and Fatty Acid Profiles**

by

Michael Adam Zulyniak

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ABSTRACT

SERUM FATTY ACID PROFILING WITHIN DISTINCT LIPID FRACTIONS PROVIDES A MORE ROBUST INDICATOR OF INSULIN RESISTANCE IN HUMANS THAN TOTAL TRIGLYCERIDE AND FATTY ACID PROFILES

Michael Adam Zulyniak
University of Guelph, 2014

Advisor:
Dr. David M. Mutch

The need to better predict the development of insulin resistance (IR) and type 2 diabetes (T2D) is necessary as the prevalence of T2D is projected to increase in Canada and cost Canadians ~\$18 billion by 2030. The International Diabetes Federation currently advises the use of total blood triglycerides (TGs) as a predictor for identifying individuals at risk of developing IR and T2D. However, it has been demonstrated that total TG levels do not reliably reflect IR across multiple ethnicities. This suggests that a more accurate marker of IR is required. Interestingly, recent evidence suggests that distinct fatty acids (FAs) in blood TGs can reflect an individual's IR status more accurately than total TGs. It is therefore reasonable to postulate that an analysis of the FAs within TGs and other blood lipids could lead to the discovery of a more accurate marker of IR.

Over the course of three studies, the following thesis aimed to demonstrate that distinct FAs within serum lipids are associated with markers of IR more robustly than total TGs or total FAs. Firstly, a large cross-sectional cohort comprised of young adults was used to demonstrate the associations between individual serum FAs and markers of IR (e.g. fasting glucose). Secondly, a population of Caucasian men varying in IR-risk were examined in order to demonstrate that specific serum FAs within TGs associate with markers of IR

more strongly than total TG levels. Finally, a cohort of individuals was investigated in order to demonstrate the improved ability of phospholipid and TG FA profiles (compared to total FAs) to distinguish individuals varying in metabolic health.

This thesis demonstrated that circulating FAs associate with markers of IR and that these associations vary between individuals of different sex and ethnicity. Furthermore, the findings of this thesis indicate that, compared to total serum TGs, specific FAs within lipid fractions may provide a more accurate means to identify individuals at increased risk of developing IR and T2D. Collectively, this thesis encourages future investigations of FAs within serum lipid fractions in order to uncover an improved method for identifying individuals at risk of IR and T2D.

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List of Abbreviations (Alphabetical):

ALA	α -linolenic acid
ARA	Arachidonic Acid
BMI	Body Mass Index
CCR2	C-C motif Chemokine Receptor 2
CE	Cholesterol Ester
CLA	cis-9, trans-11 Conjugated Linoleic Acid
COX-2	Cyclooxygenase-2
(hs)CRP	(high-sensitivity) C-Reactive Protein
CVD	Cardiovascular Disease
DHA	Docosahexaenoic Acid
DRA	Diabetes Risk Assessment
EPA	Eicosapentaenoic Acid
FA	Fatty Acid
FFQ	Food Frequency Questionnaire
GC	Gas Chromatography
GLUT4	Glucose Transporter 4
HDL-c	High-Density Lipoprotein
HETE	Hydroxyeicosatetraenoic Acid
HGI	Hyperglycaemic/Hyperinsulinaemic
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HOMA%B	Homeostatic Model Assessment of β -cell function
IFN- γ	Interferon- γ
IL-10	Interleukin-10
IL-1Ra	Interleukin 1-Receptor Antagonist
IL-6	Interleukin-6
IP-10	Interferon- γ Inducible Protein 10
IR	Insulin Resistance
JNK	c-Jun Amino-Terminal Kinase
LA	Linoleic Acid

LDL-c	LDL-Cholesterol
LH	Lean Healthy
LPS	Lipopolysaccharide
MCP-1	Macrophage (Monocyte) Chemoattractant Protein-1
MHO	Metabolically Healthy Obese
MUFA	Monounsaturated Fatty Acid
MUO	Metabolically Unhealthy Obese
n-3	Omega-3
n-6	Omega-6
NF κ β	Nuclear Factor κ β
NGI	Normoglycaemic/Normoinsulinaemic
OA	Oleic Acid
OGTT	Oral Glucose Tolerance Test
OPLS-DA	Orthogonal Projections to Latent Structures-Discriminant Analysis
PA	Palmitic Acid
PC	Phosphatidylcholine
PCA	Principal Component Analyses
PDGF- β β	Platelet-Derived Growth Factor β
PE	Phosphatidylethanolamine
PGF _{2α}	Prostaglandin-2 α
PL	Phospholipid
PMA	Palmitoleic Acid
PUFA	Polyunsaturated Fatty Acid
RANTES/CCL5	Regulated upon Activation Normal T-cell Expressed and Secreted
RBC	Red Blood Cells
SA	Stearic Acid
SEM	Standard Error Mean
SFA	Saturated Fatty Acid
SREBP-1c	Sterol Regulatory Element Binding Protein-1c
T2D	Type 2 Diabetes

TG	Triglyceride
TLC	Thin-Layer Chromatography
TLR	Toll-Like Receptor
TNF- α	Tumour Necrosis Factor- α
TNH	Toronto Nutrigenomics and Health
Total-c	Total Cholesterol
TXB2	Thromboxane-B2
VIP	Variables of Importance in Projection
VLDL	Very-Low Density Lipoproteins
WHO	World Health Organization

Chapter 1. Review of Literature

1.1. Type 2 Diabetes: A Growing Concern

Despite being viewed historically as a disease of Western nations, type 2 diabetes (T2D) has become a worldwide health concern [2,3]. While North American and European countries have been managing this burden for decades, developing nations have recently begun to feel the effects as well and the impact has been profound. In China, for example, the prevalence of T2D increased between 1980 and 2010 by more than 500%, and future predictions expect this value to double again by 2025 [3]. As such, projections by the International Diabetes Federation (IDF) anticipate that by 2035, 1 in 10 people in the world will be diabetic [4].

A number of studies have underlined the association between excess adiposity and T2D [5-8], suggesting that the increased incidence of T2D is linked to the growing number of overweight and obese individuals. The World Health Organization (WHO) estimates that the global number of overweight (BMI > 25 kg/m²) and obese (BMI > 30 kg/m²) people will increase from 1.3 billion in 2005 to almost 2 billion by 2015 [9]; suggesting that the number of individuals diagnosed with T2D will continue to rise as well. Furthermore, the increased incidence of T2D will, in turn, impose a greater burden on global health care costs. In Canada alone, where the incidence of T2D is expected to more than double between 2000 and 2020, annual healthcare costs associated with T2D are anticipated to rise from \$5.7 to \$12.1 billion [2]. In short, the economic burden of T2D treatment in Canada and the rest of the world is growing and will continue to do so unless improved

[11,12]. This link was demonstrated by Hotamisligil and colleagues, who reported that obese women were more IR than lean healthy controls, and subsequently demonstrated improved insulin sensitivity following weight loss [13]. Accordingly, adiposity (or fat mass) has also been associated with increased IR and T2D risk. Boyko *et al.* demonstrated this in a cohort of 520 men and women, where individuals with the greatest level of adiposity were found to have the highest likelihood of developing T2D [14]. Interestingly, despite the potential of physical activity to reduce an individual's risk for T2D [15], Rana *et al.*, reported that obesity is a stronger contributor to T2D risk than physical inactivity [16]. In light of these investigations and others, obesity and T2D are often investigated in tandem and numerous pathways linking the two metabolic conditions have been uncovered [6].

Obesity is commonly associated with a state of positive energy-balance, whereby the quantity of energy consumed by an individual is greater than their energy expenditure [17,18]. Therefore, as the most energy-rich nutrient, an increased consumption of dietary fat (~38 kJ/g), compared to carbohydrate or protein sources (both providing ~17 kJ/g) [19], has been associated with an elevated likelihood of developing obesity and/or T2D [20-22]. For example, Black and colleagues recently showed that individuals who regularly consumed a "high-fat" diet (35% fat, 44% carbohydrate, 21% protein) were found to have elevated BMI, % body fat, and IR compared to individuals who consumed "low-fat" diets (20% fat, 65% carbohydrate, 15% protein) [23]. This excess energy is typically stored as TG-rich lipid droplets in adipose tissue [24,25] and, if the state of positive energy balance is sustained, fat cells (i.e., adipocytes) hypertrophy in order to permit the continued uptake and storage of energy [26,27]. However, if the diameter of adipocytes increases beyond a specific diameter (~120 μm), something that is observed in obese individuals, then

adipocytes enter a state of hypoxia as oxygen and carbon dioxide can no longer be efficiently recycled between adipocytes and blood vessels [28]. With the onset of hypoxia, adipocytes up-regulate the expression of hypoxia-inducible factor-1 (HIF-1), which in turn up-regulates the expression and secretion of inflammatory proteins (including, toll-like receptor (TLR) 4, tumour necrosis factor (TNF)- α , c-Jun amino-terminal kinase (JNK), interleukin (IL)-6, C-C motif chemokine receptor (CCR)2, and macrophage chemoattractant protein (MCP)-1) [6,29-31]. Inflammatory cytokines then suppress the activity of key proteins in the insulin signalling pathways of adipocytes and induce a state of IR [32]. This prevents circulating insulin from inhibiting cellular lipolysis and results in an increased secretion of free (or non-esterified fatty acids (FFAs)) into circulation [27]. Increased adipocyte lipolysis is reflected in obese individuals by an elevation of total serum FFA levels [33,34]. Furthermore, hypoxic adipocytes and elevated FFA levels signal the down-regulation of anti-inflammatory cytokines, such as interleukin-(IL) 10 and adiponectin; two cytokines that reduce inflammation and improve insulin sensitivity [29,35]. Inflammatory cytokines and circulating FFAs also trigger the recruitment of macrophages to adipose tissue [30]. Macrophages secrete additional pro-inflammatory cytokines, such as TNF- α and JNK [36,37], which exacerbate the pro-inflammatory state and further impair insulin signalling in adipocytes. Concomitant with the increase in pro-inflammatory cytokines, elevated concentrations of FFA in blood alter the composition and fluidity of cell membranes in adipose, skeletal muscle, liver and other tissues [38]. This change in cell membrane fluidity impairs the ability of glucose transporters (i.e. GLUT4) to translocate to the cell membrane in response to insulin and, therefore, reduces the cell's ability to uptake glucose [39]. Collectively, these events lead to a systemic elevation of FFAs and pro-

inflammatory cytokines that encourage IR in several tissues and impede the ability to uptake blood glucose [6]. If untreated, circulating glucose levels will continue to rise and pancreatic β -cells will become unable to secrete sufficient levels of insulin to maintain glucose homeostasis in the fasted (<7.0 mmol/L and/or HbA1c $< 6.5\%$) and/or post-prandial state (e.g., ≤ 11.1 mmol/L, 2-hour post 75-g oral glucose tolerance test) [1]. When this happens, an individual is diagnosed with T2D [1,40].

Therefore, given the strong connection between FFAs, obesity, and T2D, it is reasonable to assume that an individual's progression towards IR may be influenced by their intake of dietary fats [41-43]. Moreover, it also appears that the influence of diet towards an individual's risk of IR and T2D may vary according to the type of fat consumed [41-43]. This has been appreciated by observing and comparing the unique dietary patterns of individuals around the world (e.g. Western, Mediterranean, and marine-based), and has allowed researchers to tease out the contributions of the different types of fat towards T2D. By doing so, researchers have hypothesized that the type of fat consumed may have a greater impact on the health of an individual than the total quantity of fat consumed [44]. Evidence now supports this notion and has demonstrated that while some dietary fats promote the onset of IR and T2D, others have a favourable impact on insulin sensitivity [41-43].

1.1.2. Diet and Insulin Resistance

One of the first studies to investigate the link between dietary patterns and metabolic health in humans was conducted by Kinsell and colleagues, who compared the effects of plant and animal-based fats on lipid metabolism [45]. These authors

demonstrated that the substitution of animal fats with plant fats reduced circulating cholesterol levels [45]. This suggested that it was not simply the consumption of fats *per se* that influenced serum cholesterol levels, but rather the type of fat consumed [45]. Subsequent studies have further demonstrated that different types of fats influence IR in distinct manners as well. For example, Xiao and colleagues demonstrated that three isocaloric mixtures of emulsions differing in their source of fat (e.g., processed/dairy, plant, or animal) influenced the level of insulin sensitivity in overweight and obese non-diabetic individuals in a unique manner [46]. More specifically, although all fat emulsions elevated fasting insulin levels, the dairy fat emulsion significantly reduced insulin sensitivity while the animal-based fat emulsion reduced the secretion of insulin in response to glucose infusion [46]. Lovejoy *et al.* demonstrated a similar relationship between dietary fats and fasting insulin levels in a cohort of men and women who consumed one of three isocaloric diets that differed in fat content [47]. The authors found that overweight participants who consumed meals rich in fats common to dairy- and animal-products were at the greatest risk of developing IR [47]. Despite these associations between dietary fats and IR, the mechanisms by which fats influence T2D remain uncertain. However, investigations of diets differing in fat content have provided valuable information and allowed us to better understand how different types of fat influence glucose homeostasis and, ultimately, IR and T2D risk.

1.1.3. The Contribution of Dietary Fats to Insulin Resistance

Despite the variety of dietary habits across continents, nutritional researchers investigating T2D have primarily focused on three dietary patterns: Western,

Mediterranean, and marine-based (commonly associated with South Asian, Japanese, and Inuit populations) [48]. Although seemingly narrow in focus, these three diets have provided valuable insight into how dietary habits and specific types of fat contribute towards IR and T2D. Indeed, each of these diets possesses a predominant type of fat or a unique combination of fats. As such, investigations of these three dietary patterns have provided evidence showing the strong associations between dietary fats and T2D.

Before discussing the three dietary patterns in greater detail, it is important to briefly introduce the three major classes of dietary fats. In the human diet there are three primary classes of dietary fats: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) (**Figure 1.1.**). Each of these classes represents a group of fatty acids (FAs) with specific structural characteristics. Briefly, SFAs do not have double bonds within their carbon backbone, while MUFAs and PUFAs possess one or multiple double bonds, respectively. MUFAs can be further divided into *cis*- and *trans*-subclasses, with the prefix denoting the orientation of the double-bond [49]. Similarly, PUFAs can be further categorized into omega-3 (n-3) and omega-6 (n-6) FAs depending on the position of their first double bond from the methylated end. Within these classes there exist a number of FAs that vary by the length of their carbon backbone and, in the PUFA class, the number of double-bonds that they possess. An understanding of these molecular structures has allowed nutritional investigators to hypothesize how and why certain dietary patterns and classes of FAs (e.g., SFA, MUFA, and PUFA) associate with IR and T2D.

Figure 1.1. – Types of Fatty Acid. There are three classes of fatty acids (FAs). Saturated fatty acids (SFAs) do not contain any carbon-carbon double bonds. Conversely, monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) have one or more double bonds, respectively. MUFAs can be further divided according to the orientation of their double bond, *cis* or *trans*. Similarly, two groups of PUFAs exist, and are distinguished according to location of the first double bond (relative to the methyl end (CH₃)). For example, a PUFA with the first double bond on the 3rd or 6th carbon would distinguish it as a n-3 or n-6 PUFA, respectively.

Western Diet. The Western diet is common to industrialized nations and has been repeatedly associated with an increased risk of obesity, IR, and T2D [50-52]. It is typified as having a high percentage of refined carbohydrates and fats from dairy-, processed-, and animal-based sources, which are rich in SFAs, *trans*-MUFAs, and n-6 PUFAs [50-53]. As such, investigations of the Western diet have suggested that the aforementioned FA classes are associated with an increased risk of IR and T2D [50-53]. For example, van Dam *et al.* compared the Western diet to that of a prudent diet (characterized by a reduced intake of SFAs, *trans*-MUFAs, and n-6 PUFAs) in a population of over 42,000 men to determine their respective influence on T2D risk [54]. At the 12-year follow-up, the prudent diet was found to modestly reduce T2D risk while the Western diet was associated with a significantly increased risk of T2D [44]. Similar results were demonstrated by Fung *et al.*, who

investigated the dietary patterns of 69,554 women [52]. The Western diet was positively associated with T2D risk compared to other dietary patterns that provided a smaller proportion of red and processed meats that are common to Western dietary patterns [52]. Interestingly, more recent investigations have suggested that the consumption of a Western diet in early-life can influence an individual's risk of T2D in adulthood. For example, Malik and colleagues reported this when women (n = 37,038) who consumed a Western style diet during adolescence (compared to a more prudent diet) were found to present an increased number of risk markers for T2D in adulthood [55]. Despite these, and other studies demonstrating an association between Western dietary patterns and T2D risk, the precise mechanisms by which the SFAs, *trans*-MUFAs, and n-6 PUFAs classes influence IR and T2D in humans remain uncertain. However, animal-model investigations have uncovered some of the underlying mechanisms of Western dietary fats. For example, rats fed SFA-rich diets, compared to those on a low fat diet, were impaired in their ability to up-regulate fat-responsive genes (namely, pyruvate dehydrogenase kinase 4 (PDHK4) and (cytosolic thioesterase 1 (CTE1)), which are necessary for FA metabolism. Furthermore, rats consuming the SFA-rich diets presented trends toward a reduced level of skeletal muscle insulin sensitivity [56]. These findings coincided with those of Prada *et al.*, where rats consuming a Western diet for 10 days had larger adipose tissue stores and developed a significant degree of IR in skeletal muscle (via inhibition of the IRS-1/phosphatidylinositol 3-kinase/protein kinase B (IRS-1/P3K/PKB) pathway) [57]. Collectively, these studies suggest that SFAs reduce the ability of cells to oxidize fat and respond to insulin, as well as encourage the accumulation of fat in adipose tissue stores. Although comparatively few studies have investigated *trans*-MUFA and IR, research by Ibrahim and colleagues

suggested that the consumption of *trans*-MUFAs reduce cell membrane fluidity and in doing so reduce insulin sensitivity [58]. Interestingly, investigations of n-6 PUFAs suggest that they may in fact be beneficial towards insulin sensitivity and reduce T2D risk. Indeed, Lee *et al.*, demonstrated that while a high-fat diet rich in SFAs encouraged IR in rats, a diet rich in n-6 PUFAs did not [41]. Cellular investigations from the same study further suggested that SFAs and n-6 PUFAs are incorporated into different lipid fractions and that these lipid fractions have opposing influences on IR [41]. Therefore, it appears that diets rich in SFAs and *trans*-MUFAs do indeed promote the development IR. This suggests that a closer examination of these FA classes in specific lipid fractions may provide a greater degree of understanding concerning the relationship between Western dietary fats and T2D.

Mediterranean Diet. Investigations of the Mediterranean diet have been largely favourable towards IR and T2D risk [43,59-61]. The Mediterranean diet, unlike the Western diet, contains a higher proportion of nutrients and fats from fruits, vegetables, legumes, and whole-grain products [43,59-61]. These food products make the Mediterranean diet a rich source of *cis*-MUFAs, particularly those found in olive oils and nuts [43,59-61]. To assess the benefit of the Mediterranean diet towards T2D risk, Martinez-Gonzalez and colleagues assessed the dietary patterns of 13,180 Spanish university students using a 136-item food frequency questionnaire (FFQ) [61]. The results demonstrated that an increased adherence to a Mediterranean diet was associated with a reduced risk of T2D [61]. A more recent study conducted by Salas-Salvado and colleagues, where individuals were randomly assigned to groups consuming either a low-fat diet or one that encouraged the consumption of Mediterranean foods, confirmed the findings of

Martinez-Gonzalez *et al.* [62]. At the 4-year follow up, individuals consuming the Mediterranean diet had a 52% reduced risk of developing T2D compared to the control group [62]. To better understand the influence of the Mediterranean diet on markers of IR, Pérez-Jiménez *et al.* performed a crossover study in a cohort of 59 young men and women [63]. Their findings demonstrated that the *cis*-MUFAs common to the Mediterranean diet had a significant impact on improving measures of fasting insulin and insulin-stimulated glucose uptake, relative to Western dietary fats [63]. This suggests that the consumption of *cis*-MUFAs reduces an individual's likelihood of developing T2D by favourably influencing glucose homeostasis. Although the mechanisms directly responsible for these favourable effects are not yet fully appreciated, it may be a combination of reduced fat storage (via increased fat oxidation) [64,65] and decreased inflammation [66], both of which are associated with a lower risk of IR and T2D. Concerning fat oxidation, which is associated with T2D risk [67,68], research by both Kien *et al.* and Soares *et al.* demonstrated that healthy young adults consuming a drink rich in Mediterranean dietary fat (i.e., *cis*-MUFA) had an increased level of fat oxidation compared to individuals consuming a formula rich in Western dietary fats [65,69]. Soares *et al.*, further demonstrated that the positive influence of Mediterranean fats (namely, olive oil) on fat oxidation may be partly explained by the concomitant increase in diet-induced thermogenesis following its consumption, particularly since this increase in thermogenesis was not observed in subjects who consumed Western dietary fats [65]. This difference in thermogenesis would require a greater amount of energy and may, therefore, partly explain the increased level of fat oxidation observed in individuals consuming a Mediterranean diet [65]. A second mechanism may be the positive association between Mediterranean dietary patterns and

adiponectin concentrations [66]. Adiponectin is an anti-inflammatory cytokine (or adipokine), which is secreted by adipose tissue and has been positively associated with insulin sensitivity [70]. Therefore, the upregulation of adiponectin in *cis*-MUFA rich diets could certainly have a favourable influence on improving glucose homeostasis and reducing T2D risk [66]. In conclusion, a Mediterranean diet rich in *cis*-MUFAs appears to have a favourable influence on IR and reduces an individual's risk for T2D; however, the mechanisms associated with the improvements in T2D risk are not yet fully understood. As such, future studies should aim to investigate individual *cis*-MUFAs in order to better understand the mechanisms behind these favourable effects and determine if all *cis*-MUFAs contribute similarly and equally towards reducing T2D risk.

Marine-based Diet. Diets that are largely supplemented by marine animals, such as those consumed by Arctic and Japanese communities, have also been investigated for their influence on IR and T2D [71,72]. These diets contain a large proportion of n-3 PUFAs (relative to the Western and Mediterranean diet) and have been largely associated with more favourable anti-inflammatory and blood lipid profiles (e.g., elevated HDL-C and reduced TG levels), and improved measures of insulin sensitivity [42,72-75]. In 1998, the diet of Japanese school children was investigated in order to determine if a shift from a marine-based diet to a Western-style diet was associated with a corresponding change in metabolic health [76]. It was shown that this dietary shift was indeed associated with, and strongly contributed to, an increased incidence of childhood obesity and T2D [76]. More recently, Nanri and colleagues observed that the total quantity of fish and seafood consumed in a population of over 20,000 Japanese men was significantly and inversely associated with T2D risk [77]. Interestingly, this association was not found in their

population of Japanese women (n= 29,759). This suggested to the authors that the consumption of marine animals (or n-3 PUFA) may be ineffective at reducing T2D risk in overweight women [77]. Although other studies have also failed to demonstrate a favourable association between fish oils and glucose homeostasis risk [78,79], a number of these had shorter supplementation periods or smaller doses of n-3 PUFAs than studies that demonstrated a favourable effect. Indeed, the majority of studies that used longer supplementation periods demonstrated that the consumption of marine animals had a significant impact on improving glucose tolerance and reducing T2D risk in men and women, regardless of their level of adiposity [80-83]. A study by Feskens and colleagues, for example, demonstrated the favourable effects of prolonged fish consumption (as determined by an FFQ) in a population of 175 men and women that on glucose tolerance and T2D risk could not be accounted for by other confounders such as age, sex, BMI, or total energy or carbohydrate intake [80]. Interestingly, as with the Western and Mediterranean diet, the mechanisms by which n-3 PUFAs elicit their beneficial effects are not yet fully understood; however, a number of pathways have been presented, such as their influence on adipocyte size and adiponectin concentrations. The ability of fish oils to reduce adipocyte size [84,85] is intriguing since adipocyte volumes are positively associated with the development of T2D in overweight and obese populations [86]. Furthermore, adipocyte size is inversely associated with plasma concentrations of adiponectin [87], which may, therefore, partly account for the favourable influence of marine animals on adiponectin secretion [88]. Lastly, like other dietary fats, n-3 PUFAs are incorporated into the cell membrane; however, n-3 PUFAs increase the fluidity of cell membranes due to their conformational structure [85,89]. This change in membrane

structure has been found to be positively associated with the quantity of glucose transporter 4 (GLUT4) protein within the cell, as well as the translocation of GLUT4 to the cell surface in response to insulin [90,91]. This increased ability of tissue cells to uptake glucose likely contributes to the reduced risk of T2D that is associated with the consumption of marine-animals. In short, the majority of research suggests that fish oils, and n-3 PUFAs, reduce the risk of T2D and that this is accomplished, in part, by their favourable influence on adipocyte morphology and glucose homeostasis. Interestingly, although fish are the primary sources of n-3 PUFAs in marine based diets, the benefits and mechanisms by which other sources of n-3 PUFAs such as plants (e.g., flax), elicit their effects are comparatively less well supported. As such, future investigations should also examine these dietary sources of n-3 PUFAs in order to gain a better understanding of how their increased consumption may also influence an individual's risk of developing T2D.

In summary, the Western, Mediterranean, and marine-based diets differ in their association with T2D risk. Furthermore, it appears that the specific FA classes consumed within each diet (i.e., SFA, *cis/trans*-MUFA, or n-3/n-6 PUFA) each influence an individual's risk of IR and T2D differently. However, despite this knowledge, we have yet to fully appreciate why these FA classes associate differently with the development of T2D and if all FAs within these classes associate equally with T2D risk. Indeed, it may be that certain FAs within these classes are more strongly correlated with T2D risk than others, this is an interesting avenue of research and may allow us to better understand how these dietary fats influence IR and T2D. However, in order to answer these questions, a more thorough investigation of dietary FAs and lipid fractions is required.

1.2. Lipidomics for Type 2 Diabetes Research

While genomics, transcriptomics, and proteomics have been widely used to improve our understanding of obesity and T2D, lipidomics is a tool that has been used comparatively less [92]. Lipidomics, which is a subset of metabolomics, can provide an understanding of the role of FAs and lipids towards the development of obesity and health-related complications, such as IR and T2D. This is because circulating lipids and FAs reflect an individual's lifestyle (e.g. diet and exercise) as well as their gene and protein activity, all of which can influence IR and T2D [93,94]. Lipidomic techniques have provided valuable information concerning obesity- and T2D-related changes in adipocyte [95], macrophage [96], skeletal muscle [97], lipoprotein lipid composition [98] and liver FA metabolism [99], to name a few, and have allowed researchers to better understand the contribution of obesity towards T2D. Such methods have also allowed researchers to unravel the underlying mechanisms by which treatment options, such as exercise, metformin, and rosiglitazone improve the status of T2D patients [100,101]. For example, rosiglitazone was found to reduce the abnormally high levels of circulating FAs and lipids in T2D patients, while metformin does not [101]. As well as providing a better understanding of the aetiology and treatment options of T2D, lipidomic techniques have revealed that quantifying specific FAs within lipid fractions (e.g. TG, PL, etc.) can provide a more accurate indication of IR and T2D [10,102]. For example, Wong *et al.* demonstrated that a combination of lipidomic-based data collected from blood (namely, patterns of FAs in distinct lipid fractions) and clinical risk factors (e.g., sex, age, waist circumference, TGs, HbA1c%) could more accurately predict an individual's likelihood of having T2D with 10.5% greater accuracy than the bioclinical risk model alone [102]. Similarly, Rhee *et al.*

performed a lipidomic-based analysis on fasted serum samples from 378 individuals to determine if specific FA patterns in lipids might provide an alternative method for predicting T2D risk [103]. Rhee and colleagues identified nine distinct FA patterns within specific lipid fractions that were associated with an increased risk for T2D and four that were inversely associated [103]. Furthermore, it was found that specific combinations of FAs within phospholipids (PL) and TGs provided the strongest associations with T2D risk, particularly shorter SFAs [103]. Such studies not only encourage the investigation of individual FAs but also suggest that by investigating distinct lipid fractions that a more robust method to predict an individual's IR and T2D status might be found.

This suggests that in the field of T2D research, lipidomics can provide a powerful approach to better understand how different lipids influence and associate with an individual's risk of T2D. Furthermore, work by Kotronen *et al.* suggests that analyzing FAs within specific blood lipid fractions can provide an even greater understanding of how FAs associated with T2D and uncover stronger associations between them [10]. For example, by focussing on the TG lipid fraction, Kotronen *et al.* demonstrated that TGs containing specific SFAs reflected an individual's IR status more accurately than total TGs [10]. Such studies, whereby researchers focus their investigation on specific lipid fractions, can be referred to as FA or lipid profiling [104,105]. These techniques allow researchers to better understand the contributions of distinct FAs within a given lipid fraction towards T2D and have the potential to uncover more robust associations between specific FAs and T2D. However, in order to appreciate the roles of individual lipids in FA profiling studies, an understanding of the contributions of circulating lipid fractions towards IR and T2D is required.

1.2.1. Contribution of Specific Lipid Fractions towards Insulin Resistance

In FA profiling investigations, researchers have the option of studying a number of different lipid classes. However, most common to obesity and T2D investigations are the PL, cholesterol ester (CE), TG, and free (or non-esterified) fatty acid (FFA) lipid fractions due to their abundance in fasted plasma samples [106-109]. In comparison to the total concentrations of ceramides and diacylglycerides (DAGs) (0.0053 and 0.036 mmol/L, respectively) [109], total PLs (2.79 ± 0.17 and 4.29 ± 0.31 mmol/L), CEs (1.97 ± 0.20 and 4.60 ± 0.83 mmol/L), TGs (0.86 ± 0.20 and 1.95 ± 0.55 mmol/L), and FFAs (0.67 ± 0.07 and 1.13 ± 0.21 mmol/L) in healthy and T2D individuals, respectively, are more abundant [106-109]. This suggests that the PL, CE, TG, and FFA lipid fractions are easier to measure in both a research and clinical environment. Furthermore, aside from their abundance, the PL, CE, TG, and FFA lipid fractions can provide valuable insight into the dietary habits of an individual over the previous hours and weeks (**Figure 1.2.**). Such information can be used to gain a better understanding of how an individual's diet, reflected in their blood lipid profile, may influence their risk of T2D.

The study of these four lipid fractions (PL, CE, TG, and FFA) is of great importance as recent evidence suggests that dietary FAs do not sequester equally between lipid fractions [41,110,111]. Indeed, although the majority of FAs ingested are in TGs, once broken down by lipases, FAs are transported to tissues in the body and can be incorporated into any lipid fraction [112-114]. For example, n-3 PUFAs consumed as TGs were found to primarily incorporate into the PL and CE lipid fractions of serum, rather than the TG and FFA lipid fractions [110]. This notion is important because the lipid fraction in which a FA is incorporated may subsequently influence whether the FA will have a favourable or

unfavourable influence on IR and T2D [115]. Indeed, a number of investigations have demonstrated that a FA can be positively, negatively, or not associated with T2D risk depending on the lipid fraction that it is associated with [115-117]. For example, of the 21 FAs uncovered by Yang and colleagues within the PL, CE, TG, and FFA lipid fractions that distinguished healthy and T2D individuals, 8 FAs could only discriminate the two groups when measured within a specific lipid fraction [116]. Such evidence suggests that an improved method for predicting individuals at increased risk of developing T2D can be achieved if lipid fractions are examined individually. However, a greater understanding of these lipid fractions is important in order to better understand how they uniquely influence the development of IR and T2D.

Figure 1.2. - Lipids in Circulation. Four commonly investigated lipid fractions in human blood are free fatty acids (FFAs), triglycerides (TGs), cholesterol esters (CEs) and phospholipids (PLs). The FFA lipid fractions demonstrates the greatest degree of variability and can change in hours, while TGs, and PLs and CEs can reflect an individual's dietary habits over the preceding days and weeks.

Phospholipids (PL). PLs are the most abundant lipid fraction in circulation and are involved in a number of biological processes throughout the body, including cell membrane structure and fluidity, FA storage and transport, and cholesterol homeostasis [106,118-120]. Due to their involvement in the aforementioned biological processes, PLs are of interest for IR and T2D researchers. However, little evidence exists concerning the association between total serum PL levels and T2D. Indeed, the study by Hallgren *et al.* in 1960, which investigated differences in total plasma PL concentrations between healthy (197 ± 12 mg/100mL) and T2D (302 ± 22 mg/100mL) men [109], is one of the few studies to have done so. Nonetheless, the findings of Hallgren *et al.* indicated that there was a significant association between total serum PL concentrations and T2D risk. This suggests that a more detailed examination of PLs is warranted in order to better understand its association with T2D.

Additionally, dividing total PLs into individual PL species may provide additional insight. Each PL species is similarly constructed by binding two FAs (by esterification) and a unique head group, such as choline or ethanolamine (through a phosphodiester bond), to a glycerol molecule [106,118]. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the two most abundant PLs in circulation (60-80% and ~25% of total PLs, respectively) and are the major structural components of cell membranes [120-122]. Interestingly, within cell membranes, PCs and PEs are positioned in such a way whereby PCs are primarily localized to the outer plasma membrane while PEs are found to be more richly distributed within the inner plasma membrane [123]. The reason for this localization is not yet understood but evidence has suggested that the ratio of total PC/PE

abundance in hepatocytes is associated with impaired liver function and non-alcoholic fatty liver disease, which often coexists with IR [6,123-125]. This suggests that the ratio of total blood PC/PE requires further investigation in the field of T2D research, as it may provide additional insight regarding the impairment of insulin signalling pathways and provides an alternative means by which to predict IR [125]. PC is also the only PL that is known to be required for hepatic production and secretion of lipoproteins (e.g., very-low density lipoproteins (VLDL-c) and HDL-c), which are required to transport TG and cholesterol around the body [121]. This is relevant because T2D patients commonly have elevated circulating levels of VLDL-c and reduced HDL-c compared to non-T2D individuals [126,127]; suggesting that hepatic PC synthesis and secretion levels may be associated with T2D risk. Aside from the recent evidence presented by Graessler *et al.*, which suggested that small reductions in fasting glucose levels are associated with reductions in total PC levels [128], few investigations have reported on differences in total blood PC concentrations between healthy and T2D individuals. As such, future investigations may find it interesting to investigate the PC fraction more closely in order to better understand the associations between hepatic lipoprotein synthesis, serum PC, and IR. Comparatively less is known about PEs but one aspect of PEs does suggest an area of interest for IR and T2D researchers. PEs possess a cone-shaped structure, unique from PCs, which make them important for membrane fluidity and shape [122,129]. In line with this, reductions in erythrocyte membrane fluidity and content of PEs has been reported in T2D patients [130]. However, significant elevations of circulating PEs (+19.6 % and +31.3 %) have also been observed in pre-diabetic and T2D individuals, respectively, relative to non-T2D individuals [131]. This suggests that further investigations of PEs are required in order to better

understand its relationship with T2D. In short, despite the lack of empirical evidence demonstrating a direct link between total PL, PC, and PE concentrations with T2D risk, aspects of the most abundant PL species, PC and PE, suggest that they may influence the development of IR. Therefore, it is likely that a closer investigation of PLs will provide valuable insight concerning their association with IR and T2D and provide an alternative means by which to predict their development.

Cholesterol Esters (CE). Un-esterified or “free” cholesterol is most abundant in cell membranes, where it provides rigidity to the structure [132-134]. However, the fluidity and rigidity of the membrane is dynamic and has the ability to fluctuate. One method for doing so involves cleaving cholesterol from the cell membrane, which in turn reduces cell membrane fluidity [134]. A single FA can be esterified to the “free” cholesterol molecule by acyl-CoA:cholesterol acyltransferase (ACAT), forming a cholesterol ester (CE) [134,135]. CEs can then be assembled in the liver and secreted within lipoproteins, primarily LDL-c (42% of LDL-c mass) and VLDL-c (12.4% of VLDL-c mass), into circulation for transport of cholesterol and FAs to other tissues [126,136,137]. This is relevant because blood levels of LDL-c and VLDL-c are positively associated with T2D [1,138]. These associations may be partly explained by the increased levels of total circulating FFA levels observed in IR and T2D individuals [6], because FFAs stimulate the liver to increase LDL- and VLDL-lipoprotein production and secretion [139,140]. The rise in LDL-c and VLDL-c production can also be further stimulated by an increase in ACAT activity in cells in response to elevated glucose levels (as observed in IR and T2D patients) [6]. This, in turn, can further stimulate the production of LDL-c and VLDL-c by synthesizing greater amounts of CE

[138,141]. Collectively, this increase in LDL-c and VLDL-c secretion would cause a greater abundance of total circulating CE levels in the blood of T2D patients.

As with total PL investigations, few studies have investigated the association between total serum CE levels and T2D risk; however, a study by Meikle *et al.* suggested that a significant association does indeed exist between total CE concentrations and the development of T2D [131]. More specifically, CE concentrations were found to be elevated by +21.2% and +32.7% in pre-diabetic and T2D individuals, respectively, relative to non-diabetic individuals [131]. Due to this positive association between CE and T2D, the CE fraction could provide valuable insight regarding the development of IR and T2D. As such, investigations of the CE lipid fraction may uncover a valuable marker for predicting the development of T2D.

Triglycerides (TG). TGs are the common storage form of FAs throughout the body [142,143]. They are composed of one glycerol molecule bound to 3 FAs [143]. TGs in circulation are synthesized in the liver, in response to elevations of blood glucose, insulin, and total FFA concentrations, each of which is elevated in IR and T2D individuals [142,144]. Elevated blood glucose and insulin concentrations stimulate the activity of sterol regulatory element binding proteins (SREBPs) [139,142,145]. SREBPs are transcription factors responsible for up-regulating hepatic lipogenic enzymes responsible for TG synthesis from FFA [142]. Once constructed, TGs are packaged into VLDLs and secreted into circulation [144,146]. Therefore, TG synthesis is correspondingly activated in at-risk and T2D individuals with elevated glucose and insulin levels, leading to elevated levels of blood TGs in IR (+47.6%) and T2D (+53.0%) individuals relative to non-T2D individuals [131,144,146-148]. Indeed,

correlations between fasting TG levels and markers of T2D, such as fasting insulin concentrations and estimates of IR, have been well documented [1,70,149]. For this reason, total blood TGs are included in numerous IR and T2D predictive models [1,150-154]. D'Agostino and colleagues, for example, demonstrated in a cohort of 872 participants that individuals who remained healthy after a 5-year period had significantly lower TG levels at baseline compared to those that developed T2D (16% of cohort) [153]. In short, it appears that total blood TG concentrations are strongly associated with T2D and appear to be a valuable marker for predicting an individual's likelihood of developing T2D. Furthermore, the associations between TG levels and circulating insulin concentrations (in response to glucose levels) and hepatic lipogenesis give them a unique ability to reflect two pathways that are associated with the development of IR and T2D (i.e., glucose and lipid homeostasis) and, therefore, provide TGs with a robust ability to associate with and predict IR and T2D. Despite the strong association between total blood TG and T2D, it appears that distinct FAs within the TG fraction provide an even greater degree of accuracy for predicting T2D risk [10]. This positions TGs as a lipid fraction of great interest for FA profiling investigations aiming to improve T2D prediction methods.

Free Fatty Acids (FFA). FFAs are FAs that are not esterified to another molecule, such as glycerol or cholesterol, and are also referred to as non-esterified FAs (NEFAs) [155]. FFAs are liberated from a lipid head group and are subsequently bound to albumin, which facilitates their transport in blood [155]. Although the mechanisms by which FFAs contribute to early symptoms of IR are not fully understood, it is generally agreed that the development of obesity ($\text{BMI} > 30 \text{ kg/m}^2$) imposes a state of chronic low-grade systemic

inflammation [156,157], which can, in turn, stimulate adipocytes to increase TG lipolysis [6,158] and, thereby, elevate circulating FFA concentrations [159], [6,158]. The association between elevated FFA levels and the onset of IR was demonstrated by Roden and colleagues in a small population of healthy men who were fitted with both euglycaemic and hyperinsulinaemic clamps and provided with a FFA lipid emulsion to elevate blood FFA levels [160]. Three hours after ingesting the emulsion, skeletal muscle demonstrated a 50% reduction of oxidative glucose metabolism and glycogen synthesis, and a significant decrease in glucose uptake [160]. Interestingly, Santomauro *et al.* demonstrated that an overnight reduction in total circulating FFA levels, via pharmaceutical intervention (*Acipimox*), could reduce the degree of IR in diagnosed T2D obese individuals [161]. A 60-70% reduction of circulating FFAs was observed and accompanied by a 50% reduction in fasting insulin levels and a 30% improvement in glucose uptake following a 2-hour oral glucose tolerance test (OGTT) [161]. In short, total circulating FFA levels appear to have a profound association with IR and glucose homeostasis. This association is reflected in the ability of total FFA levels to predict IR and T2D risk [104,162-165]. In a cohort of 3,671 healthy normoglycaemic individuals, Charles *et al.* demonstrated that individuals with the highest total blood FFA levels at baseline were the most likely to develop impaired glucose tolerance compared to individuals with lower levels of FFAs [165]. Paolisso *et al.* went one-step further and demonstrated that total FFA levels can be used to predict an individual's risk of developing T2D [163]. At baseline, fasting FFA levels and OGTT responses were measured in non-T2D Pima Indians [163]. Four-years later, Paolisso and colleagues determined that individuals with elevated baseline levels of fasting blood FFAs (485 $\mu\text{mol/L}$) were 2.3 times more likely to develop T2D than individuals with lower baseline

levels of fasting FFAs (248 $\mu\text{mol/L}$) [163]. Collectively, this suggests total blood FFA levels can be an informative marker for monitoring the development of IR and T2D.

In conclusion, the PL, CE, TG, and FFA lipid fractions each present a strong association with, and an ability to monitor, the development of IR and T2D. Furthermore, each of these lipid fractions appears to uniquely contribute, either through one or multiple tissues, towards the regulation of glucose homeostasis. This suggests that an investigation of multiple lipid fractions could provide a more complete understanding of how dietary fats contribute towards IR. Moreover, researchers seeking to identify a panel of clinically relevant predictive markers for T2D should consider examining multiple lipid fractions simultaneously as this is likely to provide a more comprehensive assessment of the body's current state of health. However, to examine these lipid fractions more closely requires an investigation of the FAs residing within them. Such an investigation is likely to provide a better understanding of the role of individual dietary FA towards IR and reveal those FAs that most strongly associate with T2D.

1.2.2. Influence of Distinct Fatty Acids on Insulin Resistance

It now appears that not all lipid fractions contribute in a similar manner towards the development of IR and T2D. Therefore, it is reasonable to hypothesize that the lipid fraction that a FA is associated with could influence the effect of the FA on glucose homeostasis. Furthermore, we now know that the influence of a FA towards IR and T2D is dependent on numerous structural characteristics, including carbon chain length, degree of

saturation, and the location and configuration of double bonds (**Figure 1.1**) [103,166]. However, it should also be noted that not all FAs within the same FA class (e.g. all SFAs or PUFAs) should be assumed to contribute equally to a condition, such as T2D [167,168]. As such, the following section aims to demonstrate that FAs within the same FA class can differ in their association with IR and that distinct FAs can provide a stronger method of prediction for individuals at increased risk of IR and T2D.

Saturated Fatty Acids (SFA). An increased consumption of SFAs and an observed elevation of SFAs in circulation have been historically associated with poor health [50-52]. In cases of obesity and T2D, SFAs were believed to unequivocally promote IR [169,170]. This was recently proposed again by Ebbesson and colleagues who showed that all SFAs, regardless of chain length, had a negative impact on fasting glucose and insulin, and 2-hour OGTT glucose measurements in women and men [171]. However, a number of recent studies have presented contradictory evidence and suggested that it is the shorter chain SFAs (i.e., 14:0 and 16:0), rather than the long chain SFAs (such as 18:0, 22:0, and 24:0), that are responsible for the negative health consequences associated with high SFA consumption [167,172,173] (**BOX 2**). For example, Shaw *et al.* reported that preadipocytes treated with 18:0 upregulated the expression of anti-inflammatory and insulin sensitizing genes [167], while *in vivo* studies performed by Louheranta *et al.* and Kelly *et al.* concluded that 18:0 rich diets did not impair insulin

BOX 2: Common Saturated Fatty Acids (SFAs)

Common Name	Formula
Myristic Acid	14:0
Palmitic Acid	16:0
Stearic Acid	18:0
Arachidic Acid	20:0
Behenic Acid	22:0
Lignoceric Acid	24:0

sensitivity [172]. A more comprehensive investigation of all circulating FAs demonstrated that SFAs associated with HOMA-IR less significantly as chain length increased [173]. More specifically, 14:0 and 16:0 were found to significantly associate with HOMA-IR, while longer SFAs (such as 22:0 and 24:0) did not [173]. Collectively, these studies encourage a closer examination of SFAs in order to better understand how SFA chain length influences its association with markers of IR and T2D, and whether specific SFAs are more strongly associated with IR and T2D compared to others. The SFAs 14:0, 16:0, and 18:0 will be discussed in greatest detail due to the amount of evidence regarding their associations with, and influence towards, IR and T2D.

An abundance of shorter chain SFAs, such as 14:0 and 16:0, appears to be inversely associated with IR [47,173]. This was demonstrated by Lovejoy *et al.*, who reported that circulating levels of 14:0 and 16:0 were found to negatively influence insulin sensitivity in a small cohort of men and women [47]. Furthermore, Vessby and colleagues demonstrated that elevated levels of 14:0 and 16:0 predicted the development of T2D in a cohort of 1,828 middle-aged men [174]. In both of these studies, longer chain SFAs such as 18:0 were not found to predict the development of T2D; suggesting that 14:0 and 16:0 have unique effects on glucose homeostasis compared to longer SFAs. Komatsu and colleagues sought to better understand the independent effects of distinct FAs, including 14:0 and 16:0, on insulin secretion [175]. Upon treating rat pancreatic β -cells with either 14:0 or 16:0, it was noted that both of these SFAs significantly augmented the quantity of insulin released but that other SFAs ranging in length from 6:0 to 22:0 did not [175]. This suggests that elevations of 14:0 and 16:0 have a more detrimental influence on β -cells [176,177]. Furthermore, this study suggested that SFA chain length is not the sole determinant of their influence on IR,

as SFAs that were either shorter or longer than 14:0 and 16:0 failed to demonstrate the same effect. While studies aimed at specifically examining the association between 14:0 and T2D have not been reported, investigations of 16:0 are more prominent and point towards potential mechanisms of influence. Two studies in particular, by Kien *et al.* and Reynoso *et al.*, suggested a role for 16:0 in the development of obesity and impaired insulin signalling, respectively. The findings of Kien *et al.*, which suggested that dietary 16:0 negatively associates with total FA oxidation [69], is interesting since reduced FA oxidation has been associated with an increased risk of IR [67,68,178]. Furthermore, Reynoso *et al.*, demonstrated that the treatment of rats with 16:0 impaired insulin signalling [179]. Upon closer investigation, it was determined that 16:0 inhibited the activation of insulin receptor substrate-1 (IRS-1) in rat skeletal muscle, which is necessary for GLUT4 translocation and insulin-stimulated glucose uptake [179]. In short, although the mechanisms by which 14:0 influences IR are not yet known, 16:0 appears to impair insulin sensitivity and FA oxidation in skeletal muscle cells, both of which are commonly observed in T2D patients [180]. This suggests that measuring 16:0, rather than total SFA concentrations, may provide a more precise marker of an individual's risk for IR and T2D.

Unlike 14:0 and 16:0, which are positively associated with IR, investigations of longer chain SFA, such as 18:0, are less conclusive. Indeed, similar to the studies presented by Lovejoy *et al.* and Vessby *et al.* earlier, Louheranta and colleagues demonstrated that a diet with elevated levels of 18:0 had no effect on insulin sensitivity or glucose tolerance in healthy women after 4 weeks [172]. Unfortunately, the mechanisms explaining the difference in association between 14:0, 16:0 and 18:0 with IR and T2D are not understood. However, one reason may stem from the more favourable influence of 18:0 on resolving

inflammation [168]. Indeed, as a known contributor towards IR and T2D [156,181,182], an inverse association between 18:0 and inflammation could account for the inability of 18:0 to positively influence IR and T2D in healthy populations. However, other studies have shown a negative influence of 18:0 on IR, suggesting that further research is necessary in order to better understand how 18:0 is associated with T2D. It certainly appears that of the aforementioned SFAs, 16:0 may serve as the SFA to best assess an individual's risk for IR and T2D. Interestingly, previous work by Salomaa *et al.* supports this theory, as 16:0 was found to be the only SFA capable of distinguishing non-T2D individuals from those that were diagnosed as either IR, newly diagnosed T2D, or previously diagnosed T2D [183].

While Salomaa and colleagues quantified total FA levels, FA profiling studies have suggested that examining these SFAs in specific lipid fractions may provide a more robust predictor of IR and T2D. For example, Louheranta and colleagues examined if the lipid fraction in which a SFA is incorporated can contribute to its influence on IR [184]. Their investigation demonstrated that this was correct, and that circulating SFAs in TG (specifically, 16:0) associated with the development of IR more strongly than those sequestered in PL and CE fractions [184]. A later study, by Kotronen *et al.* confirmed this and further demonstrated that TG in serum with a higher proportion of 16:0 can be used to predict IR more accurately than total serum TG concentrations (i.e., a routine marker to assess IR risk in clinical practice) [10].

Collectively, studies of 14:0, 16:0, and 18:0, have demonstrated the value of considering distinct SFA rather than total SFA. In doing so, the individual FAs that most strongly associate with IR and T2D can be measured independently in order to provide a stronger indicator of IR and T2D. Furthermore, the lipid fraction in which a SFA is

incorporated also appears to play an important role in determining how a specific SFAs associates with IR and T2D.

Monounsaturated Fatty Acids (MUFA). Unlike SFAs, investigations of naturally occurring *cis*-MUFAs have often revealed that they are associated with reductions in inflammation and IR (**BOX 3**) [43,59-61]. The Mediterranean diet, provides a high proportion of 18:1c9 (i.e., *cis*-18:1n9) and demonstrates a protective benefit against T2D [43,59-61]. For example, elevated levels of 18:1c9 were inversely associated with estimates of IR (2-hour OGTT) in a cohort of 538 adults [185]. Additionally, Coll *et al.*, provided evidence suggesting that 18:1c9 has the ability to inhibit the negative influence of 16:0 on skeletal muscle insulin sensitivity [186]. This was demonstrated when C2C12 skeletal muscle cells treated with 16:0 and 18:1c9 prevented the inhibition of insulin signalling (via IRS-1) and down-regulation of FA oxidation genes (e.g., peroxisome proliferator-activated receptor- γ -coactivator-1 α (PGC-1 α)) [186] that was observed in cells treated with 16:0 alone [187]. Maedler *et al.* demonstrated similar protective *cis*-MUFA qualities when the impairment of β -cell proliferation and insulin secretion in C2C12 cell by 16:0 was restored by a follow-up treatment with either MUFA, 18:1c9 or 16:1c9 (i.e., *cis*-16:1n-7) [188]. This suggests that elevated levels of circulating 18:1c9 may protect cells from IR and be a valuable marker to

assess insulin sensitivity alongside other FAs, such as 16:0, which are more closely associated with IR. However, as demonstrated by

BOX 3: Common Monounsaturated Fatty Acids (MUFAs)

Common Name	Formula
Palmitoleic Acid	16:1c9
Oleic Acid	18:1c9
Elaidic Acid	18:1t9
Vaccenic Acid (<i>cis</i> or <i>trans</i>)	18:1c\t11

Maedler *et al.*, other *cis*-MUFAs, such as 16:1c9, could also prove a valuable marker of IR.

A closer investigation of 16:1c9, however, reveals some uncertainty regarding its contribution to IR. Indeed, although a number of studies have suggested that 16:1c9 is inversely associated with IR, an equal number of large population-based studies have suggested a positive association between 16:1c9 and T2D risk. For example, in a population of 3,630 men and women, Mozaffarian *et al.* demonstrated that men (n = 1,510) showed a strong association between circulating levels of PL 16:1c9 and fasting glucose and insulin levels, as well as HOMA-IR [189]. Interestingly, a similar association between 16:1c9 and markers of IR was not found in female (n = 2,120) [189]. An earlier study by Hodge *et al.*, however, found that levels of PL 16:1c9 were significantly associated with T2D risk in both sexes (n = 3,737) [190]. These contradictory findings suggest that the role of 16:1c9 may be more complex than originally believed. Indeed, evidence suggests that 16:1c9 may act as an adipose-derived hormone, or lipokine, that improves insulin sensitivity in skeletal muscle, whole-body glucose metabolism, and reduces liver lipogenesis and TG storage [191]. Such findings were supported in a study by Yang and colleagues [192], where 16:1c9 was orally administered on a daily basis for 4-weeks to mice genetically pre-disposed to develop T2D [192]. Upon study completion, it was found that chronic 16:1c9 administration improved insulin sensitivity (as measured by an OGTT), down-regulated liver lipogenic gene expression (e.g., SREBP-1), and reduced liver TG storage compared to control mice and those supplemented with 16:0 [192]. Furthermore, Yang and colleagues noted a reduction in adipose tissue concentrations of the pro-inflammatory marker TNF α [192], a cytokine associated with IR [6]. Therefore, although epidemiological investigations remain inconclusive regarding the influence of 16:1c9 towards IR and T2D, animal-model

investigations suggest that 16:1c9 reduces hepatic lipogenesis and possesses insulin sensitizing qualities that discourages the development of IR and T2D. Certainly future research is required in order to better understand these contradictions but such discrepancies lend further support to the use of targeted FA approaches in order to better understand the distinct contributions of individual *cis*-MUFAs towards the development of IR and T2D.

Unlike 18:1c9 and 16:1c9, which are both *cis*-oriented MUFA, *trans*-MUFA demonstrate less favourable associations with IR and T2D (**BOX 3**). Granados *et al.*, for example, compared the influence of 18:1c9 and 18:1t9 (i.e., *trans*-18:1n9) on insulin sensitivity in C2C12 skeletal muscle cells [193]. Following a 24-hour incubation with either FA, C2C12 cells treated with 18:1t9 demonstrated a 35% reduced ability to uptake glucose compared to cells treated with 18:1c9 [193]. Work by Cromer and colleagues, which investigated the influence of 18:1c9, 18:1t9, and 18:1t11 (*trans*-18:1n7) on glucose oxidation in rat adipose cells, reported similar findings [194]. Primary cell cultures grown from rat adipose tissue and treated with 18:1c9 presented significantly higher levels of glucose oxidation when compared to adipose tissue cells treated with either 18:1 t9 or 18:1 t11 [194]. These studies suggest that *trans*-MUFA have a negative influence on IR and T2D. However, as discussed by Odegaard and Pereira, current research concerning the relationship between *trans*-MUFA and T2D risk is far from unequivocal, as there have been only a limited number of investigations aimed specifically at the relationship between *trans*-MUFA and T2D [195]. As such, the potential for FA profiling to provide a better understanding of the associations between *trans*-MUFAs and T2D risk cannot be overstated.

In conclusion, FA profiling investigations of distinct MUFAs, both *cis*- and *trans*-MUFAs, have provided a great deal of understanding of how individual MUFAs (such as 18:1c9 and 16:1c9) influence the development of T2D. Furthermore, it can also be appreciated that investigations of distinct MUFAs can reveal unique associations with IR and T2D that may not be evident in investigations of total MUFAs. Lastly, although the influence of MUFAs in specific lipid fractions towards IR have not been largely investigated, a predictive model compiled by Yang and colleagues suggested that lipid fractions may indeed affect associations between MUFAs and T2D. Indeed, in an attempt to distinguish T2D individuals from healthy controls, Yang *et al.* revealed that fasting levels of 18:1c9 in both the PL and FFA lipid fractions, but not CE or TG, were important for distinguishing the two groups [116]. Therefore, despite the valuable information already obtained, future studies may reveal even stronger associations between MUFAs and IR, and gain a better understanding of their influence on T2D, if MUFAs within individual lipid fractions are investigated more closely.

Polyunsaturated Fatty Acids (PUFA). Epidemiological studies have largely suggested that total circulating PUFA levels are not associated with risk of T2D [190,196]. This was demonstrated by both Wang *et al.* and Hodge *et al.* in large population cohorts composed of 2,909 and 3,737 middle aged adults, respectively [190,196]. Furthermore, upon dividing PUFA into n-3 and n-6 species (discussed in section 1.1.3.), the inability to predict the development of T2D remained [190]. The inability of total n-3 and n-6 PUFA levels to predict the development of T2D was later demonstrated again by Patel and colleagues in a

cohort of 383 individuals between baseline measures (between 1993-1997) and 2005 [197]. Nonetheless, a number of other studies have demonstrated associations between total n-3 and n-6 PUFA levels and T2D, as well as the individual PUFAs that compose these species (**BOX 4**). This suggests that although total PUFAs and total n-3 and n-6 PUFA levels may not be able to predict the development of T2D that they are associated with markers of IR, and that a closer examination of distinct PUFAs is warranted.

n-3 PUFA. The n-3 subclass of PUFAs is largely associated with improved insulin sensitivity. Ebesson and colleagues, for example, found that plasma concentrations of total n-3 PUFAs were inversely associated with fasting insulin, total TGs, and HOMA-IR, as well as 2-h insulin and glucose levels (OGTT) in healthy Alaskan Inuit (n = 447) [198]. In an independent study, Huang *et al.* demonstrated that elevated levels of total n-3 PUFA in plasma were associated with improved measures of HOMA-IR in Japanese T2D individuals [199]. Furthermore, it was reported that plasma levels of the three most abundant n-3 PUFAs in healthy individuals (18:3n-3, 20:5n-3, and 22:6n-3) were significantly reduced in T2D individuals and that circulating levels of 20:5n-3 and 22:6n-3 were inversely associated with HOMA-IR [199].

Studies suggest that 18:3n-3 is inversely associated with IR [183,200,201]. Salomaa and colleagues demonstrated this in a cohort of individuals varying in glucose tolerance (healthy = 325, impaired = 97, and T2D = 98), where 18:3n-3 abundance

BOX 4: Common Polyunsaturated Fatty Acids (PUFAs)

Common Name	Formula
α-Linolenic Acid (ALA)	18:3n-3
Eicosapentaenoic Acid (EPA)	20:5n-3
Docosahexaenoic Acid (DHA)	22:6n-3
Linoleic Acid (LA)	18:2n-6
Arachidonic Acid (AA)	20:4n-6

significantly increased as glucose tolerance improved [183]. However, few studies have actively investigated associations between circulating levels of 18:3n-3, rather than the dietary intake of 18:3n-3, with IR and T2D. As such, the mechanisms by which 18:3n-3 positively associate with glucose tolerance are not unequivocal, as they are largely based on dietary associations with IR. Nonetheless, dietary supplementation studies have suggested that 18:3n-3 may reduce liver lipogenesis and TG accumulation by down-regulating fatty acid synthase (FAS), SREBP-1 and G-6-PDH activities [202,203], as well as improving insulin sensitivity and GLUT4 content in T2D mice [204]. Interestingly, although evidence presented by Chicco *et al.* suggested that elevated levels of plasma 18:3n-3 can prevent the development of IR in rats fed a high-sucrose diet [205], Anderson *et al.* suggested that, longer n-3 PUFAs (i.e., 20:5n-3 and 22:6n-3) are more strongly associated with improvements in IR [206]. Specifically, elevated levels of 20:5n-3 and 22:6n-3 were both found to favourably alter fasting glucose (-35 %) and insulin (-38 %) levels, and HOMA-IR (-60 %) more strongly than 18:3n-3 [206]. This suggests that although circulating levels of 18:3n-3 appear to be inversely associated with IR, that plasma levels of 20:5n-3 and 22:6n-3 are more strongly associated with IR and T2D risk, and may provide a more robust means to predict their development.

In the epidemiological study by Hodge *et al.* discussed previously, both 20:5n-3 and 22:6n-3 were found to more strongly predict the development of T2D compared to circulating levels of 18:3n-3, total n-3 PUFAs, and total PUFAs [190]. As such, it might be expected that an investigation of these two n-3 PUFAs would present even stronger associations with IR and T2D; however, this does not appear to be the case. Indeed, it appears that elevated levels of circulating 20:5n-3 and 22:6n-3 are more strongly

associated with the resolution of inflammation (which can improve insulin sensitivity) rather than the development IR and T2D *per se*. Kalupahana *et al.*, demonstrated this when increasing circulating levels of 20:5n-3 in mice was found to improve fasting plasma glucose levels (induced by high levels of 16:0) but not fasting insulin and HOMA-IR [207]. Upon closer investigation, Kalupahana *et al.*, found that the adipose tissue of “rescued” mice had reduced levels of inflammation and lipogenesis, and elevated markers of FA oxidation and circulating adiponectin (relative to un-treated mice); suggesting a reduced inflammatory state [207]. Further support for the role of 20:5n-3 was presented by Figueras *et al.*, when T2D rat models treated with 20:5n-3 for 4-weeks demonstrated reduced levels of fasting plasma insulin and decreased levels of gene expression in skeletal muscle and adipose tissue for the inflammatory markers TNF- α and IL-6 [208], both of which are associated with T2D [209]. These results suggest that increased levels of 20:5n-3 may not increase insulin signalling directly, but rather improve the inflammatory status of tissues that are associated with the development of IR and T2D. Therefore, although further evidence is required, it appears that 20:5n-3 may be more strongly associated with adipose and skeletal muscle inflammation than IR. Concerning 22:6n-3, evidence suggests that its favourable influence on insulin sensitivity is induced in a similar, and possibly a more robust, manner to that of 20:5n-3, i.e., through the resolution of inflammation. This was demonstrated by Oliver and colleagues when macrophage, pre-treated with either 20:5n-3 or 22:6n-3 for 5-days, were inflamed (via lipopolysaccharide, LPS) and co-cultured with adipocytes (3T3-L1) [210]. In comparison to 20:5n-3, incubation with 22:6n-3 elicited a larger reduction in secreted pro-inflammatory markers from macrophage (NF κ B and TNF α) and adipocytes (IL-6 and TNF α) and maintained healthy levels of glucose uptake

and GLUT4 translocation [210]. This suggests that, much like 20:5n-3, 22:6n-3 attenuates inflammation; however, 22:6n-3 appears to elicit a stronger improvement of IR compared to 20:5n-3. Therefore, of the three most abundant n-3 PUFAs in circulation, 22:6n-3 presents the strongest association with IR and T2D; however, a more targeted examination of 22:6n-3 should be undertaken. This would allow researchers to determine if the improvements in IR associated with 22:6n-3 are solely the result of its anti-inflammatory effects or interactions with insulin signalling pathways can be uncovered as well. In doing so, its association with and predictive ability for IR and T2D risk could better understood.

n-6 PUFA. As mentioned earlier, total n-6 PUFAs have not demonstrated an ability to predict T2D risk [82]. However, investigations of distinct n-6 PUFAs, namely the two most abundant in circulation, 18:2n-6 and 20:4n-6, have been associated with IR and T2D.

Plasma levels of 18:2n-6 are of particular interest as they have demonstrated a significant inverse association and strong ability to predict T2D in two independent studies [190,197]. Studies actively investigating the distinct role of 18:2n-6 suggest that it imparts its influence on insulin sensitivity by stimulating energy consuming pathways in skeletal muscle [211]. Lam *et al.* demonstrated this by incubating L6 skeletal muscle cells with FAs (specifically, 16:0 or 16:0 with 18:2n-6) for 24-hours, and measuring changes in the expression of key fatty acid oxidation genes (e.g., AMP-activated protein kinase (AMPK)) [211,212]. Briefly, the degree of IR imposed on skeletal muscle cells incubated with 16:0 was reversed by co-incubation with 18:2n-6, which also resulted in increased expression of AMPK [211]. This suggests that 18:2n-6 can stimulate the expression of primary energy metabolism genes in skeletal muscle cells. Furthermore, AMPK activation is a primary

target for exercise and some T2D medications (e.g., metformin and rosiglitazone) aimed at improving glucose metabolism in individuals with T2D [213,214]. This suggests that, as well as providing a significant ability to predict T2D risk in individuals, 18:2n-6 may also have the potential to provide insight into the metabolic health of skeletal muscle.

Unlike 18:2n-6, circulating levels of 20:4n-6 appear to be unable to predict the development of T2D [190,197]. However, in the study by Hodge and colleagues, 20:4n-6 was found to be significantly elevated at baseline in individuals (n=346) that eventually developed T2D [190]. This suggests that 20:4n-6 may have a negative influence on insulin sensitivity and, therefore, encourage the development of IR and T2D. Caspar-Bauguil *et al.* further demonstrated this hypothesis when circulating 20:4n-6 was found to be inversely associated with fasting adiponectin concentrations and positively associated with levels of fasting glucose and HOMA-IR in a small cohort of severely-obese women [215]. As a possible mechanism, Tebbey and colleagues proposed that 20:4n-6 negatively influences glucose homeostasis by inhibiting the expression and half-life of GLUT4 [216]. A follow-up study by the same lab presented an interesting alternative 2 years later. Long *et al.* clarified that 20:4n-6 was not directly responsible for the reduction of GLUT4, rather it was an effect of elevated prostaglandin-E₂ (PGE₂) levels – a product of 20:4n-6 metabolism via the cyclooxygenase (COX) pathway – because cells treated with a 20:4n-6 and a COX2 inhibitor did not show a reduction in GLUT4 expression [217]. This is particularly interesting as it suggests that 20:4n-6 may not, in fact, have a direct influence on IR. Evidence suggests that 20:4n-6 itself may have anti-diabetic qualities. Song *et al.*, demonstrated such a relationship with mice genetically predisposed to T2D [218]. Upon direct administration of 20:4n-6 into the plasma of T2D mice, a 30% improvement in insulin-stimulated glucose

uptake was reported [218]. Therefore, collectively, it remains unclear whether 20:4n-6 associates with T2D in a positive or inverse manner as previous investigations have proposed both. Future investigations that aim to better understand this association might consider investigating 20:4n-6 more closely and independently from its metabolic products (e.g. PGE2). Through the use of a 20:4n-6 analogue such as eicosatetraynoic acid (ETYA), which cannot be metabolized, the association between 20:4n-6 and T2D risk may become more apparent and its validity as a marker of T2D risk determined.

The inability of total PUFA, total n-3 PUFA, and total n-6 PUFA levels initially suggested that PUFAs may not be associated with and incapable of predicting IR and T2D risk. However, a closer investigation of these PUFA subclasses revealed that numerous associations exist between distinct PUFAs - namely 18:3n-3, 20:5n-3, 22:6n-3, 18:2n-6, and 20:4n-6 - and markers of IR. Furthermore, a number of these PUFAs were found to influence the development of IR through distinct mechanisms (i.e., reduce hepatic lipogenesis or resolution of adipose tissue inflammation). This is interesting, as it suggests that individual PUFAs may associate with the IR-status of specific tissues in the body and, therefore, provide information that may be clinically relevant when determining the optimal treatment option. A number of discrepancies were also revealed concerning associations between distinct PUFAs and IR. This may be partly explained by the limited number of FA profiling studies that have actively investigated PUFAs to determine if their associations with IR vary depending on the blood lipid fraction that they are incorporated into. Indeed, FA profiling studies that have been conducted have demonstrated that while longer PUFAs associate with T2D risk in PL, CE, and FFA fractions [103,116] the same PUFAs appear to be inversely associated with T2D risk in the TG fraction [103]. Therefore,

as demonstrated by the SFA and MUFA classes, newer and stronger associations between distinct PUFAs and IR may be revealed if PUFAs within individual lipid fractions were investigated.

In conclusion, although the total abundance of some FA classes in circulation suggest a lack of association with IR and T2D, a closer inspection of individual FAs within these classes revealed robust associations with IR and an ability to predict the development of T2D. Furthermore, an investigation of distinct FAs within these FA classes may provide a better understanding of how FAs influence IR and T2D by offering insight into the tissues and insulin signalling pathways affected. It was also suggested that a number of FAs within each FA class associated more strongly with IR and T2D when investigated in one lipid fraction over another, which provides further support to the proposed investigation of FAs in a lipid-specific manner.

1.3. Implications and Value of Fractionated Lipid Research

Our understanding of dietary fats and lipids has grown since the pioneering work of Kinsell and colleagues. Nonetheless, gaps in our knowledge regarding the contribution of individual FAs towards the development of IR and T2D remain. This suggests that alternative research techniques, such as lipidomics and FA profiling, are necessary in order to better understand these gaps.

Although total plasma TG levels associate with T2D risk, a more accurate method of prediction is possible through the use of lipidomic-based and FA profiling techniques. Individual FAs within specific lipid fractions can be uncovered that are more robustly associated with IR and predict T2D with greater accuracy. It has been suggested that

current clinical prediction criteria for T2D can be significantly improved by incorporating a panel of distinct FAs from a number of different lipid fractions [219]. Furthermore, it is interesting to consider the additional information that can be obtained from a panel of FAs varying in lipid incorporation, rather than the total concentration of a single lipid, such as TG. Circulating lipids and a number of FAs are associated with distinct pathways and tissues involved in the progression towards IR and T2D. As such, a panel of FAs may provide a greater understanding of the IR status of tissues and insulin signalling pathway in the body, which might then be used to direct and tailor a more targeted treatment option for patients.

In conclusion, research suggests that individual lipid fractions can provide a greater degree of understanding concerning the development of IR and T2D than dietary patterns. Furthermore, it appears that the use of lipidomics and FA profiling techniques can reveal underlying associations between distinct FAs and T2D risk that may not be uncovered by examining the abundance of total FA classes. Collectively, this suggests that the investigation of distinct FAs within individual lipid fractions is a valuable avenue of research that has to date been largely overlooked. Although future research is necessary, evidence suggests that an improved method for predicting T2D risk in comparison to current clinical practices is possible and can provide valuable information for improving patient outcomes.

Chapter 2. Overall Rationale and Hypothesis of Thesis

Bioclinical parameters such as BMI and fasting lipid levels (e.g. TG) have long been used to assess an individual's state of well being and risk of future health complications. However, recent evidence suggests that there is a large degree of inter-individual variability in bioclinical measurements and an individual's risk of developing metabolic diseases. For example, evidence now indicates that associations between total circulating TG concentrations and markers of IR can vary significantly between individuals of different ethnicity and sex, which can, in turn, make it difficult to establish standardized cut-off values to reliably assess T2D risk. This suggests that an alternative and more accurate means of predicting IR and T2D would be of great benefit to clinicians and the public.

Interestingly, some evidence exists demonstrating that examining distinct FAs within serum TG may be a more accurate method of predicting IR and T2D risk [10]. This concept was recently reinforced when distinct lipid molecules (namely, PLs with specific FA compositions) were found to increase the accuracy of T2D risk assessment by ~10%, relative to current clinical practices [219]. These studies suggest that the investigation of FA profiles in circulating lipid fractions can offer new insight into how distinct FAs from specific lipids fractions associate with IR and may lead to the identification of an improved method for predicting T2D risk.

Therefore, the overall hypothesis of this thesis was that **distinct FAs within specific blood lipid fractions are more strongly associated with IR than measures of total FAs and lipid levels**. To address this, three specific hypotheses were proposed and presented in the forthcoming chapters:

1. The strength of associations between individual FAs and markers of IR varies according to ethnicity and sex (*Chapter 3*);
2. FAs within a specific lipid fraction associate more strongly with markers of IR than the total abundance of the lipid (*Chapter 4*); and
3. Distinct FAs within lipid fractions can more robustly distinguish metabolically healthy and unhealthy individuals than the total abundance of FAs (*Chapter 5*).

The specific objectives associated with these hypotheses are detailed within the forthcoming chapters.

Chapter 3. Rationale and Objective

Previous studies have demonstrated that FAs are associated with IR. Briefly, SFAs and n-6 PUFAs have been associated with IR [50-52] while *cis*-MUFAs and n-3 PUFAs are largely associated with improved insulin sensitivity [43,60,198,199]. However, relationships between distinct FAs and markers of IR have not been investigated and compared across a number of ethnicities. This is important in order to demonstrate that associations between FAs and markers of IR in one population may not be applicable to another population, and that the FA criteria used to predict T2D risk might be improved if sex- and ethnic- specific criteria are considered. Therefore, the study "*Ethnic- and Sex-Specific Associations Between Plasma Fatty Acids and Markers of Insulin Resistance in Healthy Young Adults*" set out to confirm my 1st specific hypothesis by: (1) confirming that associations between total FAs and markers of IR can be seen across multiple ethnicities; and (2) that these associations vary according to sex and ethnicity.

Chapter 3. Ethnic- and Sex-Specific Associations Between Plasma Fatty Acids and Markers of Insulin Resistance in Healthy Young Adults

Ralston JC*, **Zulyniak MA***[§], Nielsen DE, Clarke S, Badawi A, El-Sohemy A, Ma DWL and Mutch DM. Ethnic- and sex-specific associations between plasma fatty acids and markers of insulin resistance in healthy young adults. *Nutrition & Metabolism*. 2013, 10(42). doi:10.1186/1743-7075-10-42.

*Contributed equally to the manuscript

§Conceptualization, data analysis and interpretation, and writing of the manuscript.

3. Abstract

Although evidence indicates that fatty acids (FA) can affect insulin resistance (IR), not all FA contribute equally to the process. Indeed, monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) are reported to reduce IR, whereas saturated FA (SFA) and trans FA appear to increase IR. However, it is not yet clear how individual FA are associated with markers of IR, and whether these relationships are influenced by ethnicity and/or sex. Therefore, the goal of this study was to examine the ethnic- and sex-specific relationships between plasma FA and markers of IR in a cohort of healthy young Caucasian, East Asian, and South Asian adults. Gas chromatography was used to quantify fasting plasma FA from young Canadian adults (22.6 ± 0.1 yrs) classified as Caucasian ($n = 461$), East Asian ($n = 362$), or South Asian ($n = 104$). Linear regression models were used to investigate associations between plasma FA and markers of IR (i.e. fasting insulin, glucose, and HOMA-IR), according to ethnicity and sex. Numerous significant associations ($P < 0.05$, adjusted for multiple testing) were identified between individual FA and markers of IR, with the majority identified in Caucasians. For SFA, positive associations were found between 14:0 and fasting insulin and HOMA-IR in Caucasian and East Asian populations, and 18:0 and fasting glucose in Caucasians only. Several positive associations were also found for specific MUFA (18:1t11 and 18:1t6-8 with HOMA-IR, and 18:1c9 with fasting glucose) and PUFA (18:2n-6 with fasting glucose and 18:2c9t11 with HOMA-IR) in Caucasian adults only. Most of the aforementioned associations were stronger in males compared to females. Interestingly, no significant associations were found between FA and markers of IR in South Asian adults. We report numerous associations between plasma FA and markers of IR in Caucasian and East Asian populations, but not in South Asian individuals.

Furthermore, these associations appeared to be more robust in men. This demonstrates the importance of investigating associations between FA and markers of IR in an ethnic- and sex-specific manner in order to better understand the contribution of plasma FA to the development of IR and type 2 diabetes.

3.1. Background

Type 2 diabetes (T2D) is a health concern affecting 285 million individuals worldwide, and is expected to affect 439 million individuals by 2030 [220]. T2D is a chronic metabolic disorder characterized by insufficient insulin production, which is preceded by a state of insulin resistance (IR) [221,222]. It is well established that high fat diets contribute to the development of IR [117,196]; however, not all fatty acids (FAs) influence IR equally. For example, high circulating levels of saturated fatty acids (SFAs) and n-6 polyunsaturated fatty acids (PUFAs) are associated with elevated fasting levels of insulin and glucose [171,223,224]. Interestingly, these same FA were reported to be more abundant in diabetic patients compared to healthy individuals [224]. Conversely, certain n-3 PUFAs such as α -linolenic acid (18:3n-3, ALA), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) have been shown to improve glucose homeostasis and provide protective effects against the development of IR by increasing production of insulin-sensitizing adipokines (e.g., adiponectin) and reducing the pro-inflammatory state of adipocytes and macrophages [210,225,226]. This reinforces that the associations between individual FA and the development of IR are distinct.

While several excellent cross-sectional studies have examined the relationships between FA and markers of cardiometabolic risk [173,227-229], it is notable that most of

these previous studies have not investigated to what extent these associations vary with ethnicity. This is significant as it is now acknowledged that specific ethnicities may be more susceptible to develop metabolic diseases than others [230]. For example, associations between common inflammatory markers (e.g. C-reactive protein and interleukin-6) and IR appear to vary according to ethnicity [230], which could contribute to ethnic-specific health disparities. Additionally, individuals of African, Latino, Japanese, and Hawaiian descent residing in America are more susceptible to developing diabetes than Americans of European decent [231]. Recent work by Goff et al. also demonstrated that individuals of South Asian, African-Caribbean and European origins differ significantly in their habitual dietary intake (e.g. total daily energy intake, % fat intake, and % sugar intake) and that these characteristics are associated with differences in basal insulin sensitivity and secretion [232]. Such ethnic-specific differences are likely not explained by a single factor, but rather a myriad of complex gene-environment interactions. As such, there is a need to conduct further analyses in order to elucidate whether the relationships between FAs and markers of IR differ between ethnicities.

Furthermore, evidence also demonstrates that plasma FA profiles vary between men and women [189,233]. These dissimilarities in plasma FAs are often attributed to fundamental differences between men and women, such as total adiposity and lean muscle mass, location of fat deposition (i.e. visceral versus subcutaneous), and systemic hormone concentrations [233,234]. This suggests that greater insight regarding the relationships between FAs and markers of IR may be obtained by further stratifying each ethnicity by sex [235]. Therefore, the goal of the present study was to conduct a cross-sectional analysis in

young Canadian adults of different ethnicity in order to uncover ethnic- and sex-specific relationships between fasting plasma FA concentrations and markers of IR.

3.2. Methods

Study Participants. Participants recruited from the University of Toronto campus (2004-2009) for the Toronto Nutrigenomics and Health (TNH) study were used for the current investigation [236,237]. Participants were between the ages of 20-29 years and classified as either Caucasian (n = 461; 132 men / 329 women), East Asian (n = 362; 95 men / 267 women), or South Asian (n = 104; 41 men / 63 women) by self-reported ethnicity. Participants were excluded from the investigation if they had a body mass index (BMI) greater than 30 kg/m², did not complete a food frequency questionnaire (FFQ), or were diagnosed with diabetes or cancer. The TNH study was approved by the Research Ethics Boards at the University of Toronto and the University of Guelph. All subjects provided informed written consent.

Clinical Measurements. Plasma samples were collected following a 12-hour overnight fast, and were used for analysis of glucose and insulin at LifeLabs Laboratories (Toronto, Canada). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the HOMA Calculator v2.2.2 (www.dtu.ox.ac.uk/homacalculator/index.php).

Food Frequency and Health Questionnaires. Subjects were asked to complete a one-month 196-item FFQ, adapted from the Willet questionnaire [236,238]. This FFQ data was used to

estimate total dietary intake of fat (in grams) per day. Subjects also completed lifestyle and general health questionnaires, which were used to calculate a physical activity score.

Plasma Fatty Acid Analysis. Fasting plasma samples were assessed using gas chromatography (GC) as previously described [239]. Briefly, 5 μ g of a C17:0 internal standard was added to plasma samples and mixed with a 2:1 chloroform:methanol solution containing KCl. Following an overnight incubation at 4°C, samples were centrifuged and the organic layer was dried under a gentle stream of nitrogen gas. Extracted lipids were saponified with 0.5 M KOH in methanol for 1 hour at 100°C, and cooled for 10 minutes at room temperature. 14% BF₃-MeOH and hexane were added to samples, prior to methylation, for 1 hour at 100°C. After samples had cooled, double-distilled water was added, and samples were centrifuged at 1000 rpm for 10 minutes. The upper-layer was extracted and dried under nitrogen gas, prior to reconstitution in hexane. Samples were analyzed using an Agilent Technologies 7890A GC system (Agilent Technologies, Mississauga, CA). All FA peaks were identified by comparison to retention times of FA methyl ester standards. Only those FA that were consistently detected across all plasma samples were considered for the current study (i.e., 24 of the 62 FAs that can be measured by GC). Absolute FA values were calculated by comparing individual FA peaks to the internal standard and are reported as μ g FA / mL plasma $\pm$ SE.

Statistical Analysis. All statistical analyses were conducted with JMP Genomics software Version 5.1 (SAS Institute, Cary, NC). The Shapiro-Wilk test was used to assess the distribution of all variables. Fasting insulin was subsequently log transformed for all

analyses. Fourteen outliers were identified by performing a Jackknife test and removed prior to the analyses. Individual associations between FAs and markers of IR (i.e. fasting insulin and glucose, and HOMA-IR) were examined using mixed-effects linear regression models, which accounted for both fixed effects (age, sex, BMI, physical activity, and average daily fat intake (in grams) per day) and one random effect (date of GC processing). For analyses of sex-specific associations, sex was removed from the aforementioned list of variables. We used a two-step process to determine statistical significance: (1) ethnic-specific associations between FAs and markers of IR were considered significant only if they satisfied a Bonferroni correction for multiple testing; followed by (2) an investigation of sex-specific differences for those FAs that were statistically significant in the previous step. A P-value < 0.05 was considered statistically significant.

3.3. Results

Study Participant Characteristics. Anthropometric and clinical characteristics of the 998 study participants are shown in **Table 3.1**. All subjects were considered to be within the normal healthy range for all parameters. No subjects were diabetic or affected by cancer, and only 2 subjects were smokers.

Associations Between Fatty Acids and Markers of Insulin Resistance. Our GC platform is able to detect 62 distinct FAs; however, for the current study we only considered FAs that were consistently detected in all plasma samples. Using this judicious approach, we investigated associations between 24 individual FAs and various markers of IR in three ethnic groups of young healthy adults (**Tables 3.2-3.5**). Interestingly, all of the statistically significant

associations identified indicated positive relationships between FAs and markers of IR. These significant associations were further investigated in a sex-specific manner.

Saturated Fatty Acids (SFAs). Several SFAs were examined (14:0, 15:0, 16:0, 18:0) for associations with markers of IR (**Tables 3.3-3.5**). In the Caucasian population, plasma 14:0 levels were positively associated with both fasting insulin ($r^2 = 0.19$; $P = 0.0001$) and HOMA-IR ($r^2 = 0.17$; $P < 0.0001$). Similar associations were also seen in the East Asian population, where 14:0 was positively correlated with both fasting insulin ($r^2 = 0.13$; $P = 0.0088$) and HOMA-IR ($r^2 = 0.11$; $P = 0.0024$); however, these associations were borderline significant when adjusting for multiple testing. After separating the Caucasian and East Asian populations by sex, 14:0 remained positively associated with insulin in Caucasian men and women ($r^2 = 0.31$; $P = 0.0049$, and $r^2 = 0.13$; $P = 0.0049$, respectively) and East Asian men ($r^2 = 0.15$; $P = 0.0233$). The relationship between 14:0 and insulin was not significant in East Asian females ($r^2 = 0.14$; $P = 0.0515$). For HOMA-IR, 14:0 remained positively associated in both Caucasian men and women ($r^2 = 0.33$; $P = 0.0006$, and $r^2 = 0.12$; $P = 0.0031$, respectively) and East Asian men and women ($r^2 = 0.17$; $P = 0.0259$, and $r^2 = 0.10$; $P = 0.0142$, respectively). No associations were seen with 14:0 in the South Asian group. We also found that 18:0 was positively associated with fasting glucose levels ($r^2 = 0.10$; $P = 0.0007$) in the Caucasian population. Furthermore, this association remained significant after separating Caucasians into men ($r^2 = 0.15$; $P = 0.0112$) and women ($r^2 = 0.05$; $P = 0.0225$). No significant associations were found between 18:0 and markers of IR in the East Asian or South Asian populations. No associations were identified between plasma 15:0 or 16:0 with markers of IR in the three ethnicities examined.

Monounsaturated Fatty Acids (MUFAs). Numerous MUFAs were examined for associations with markers of IR, including 16:1c9, 18:1t6-8, 18:1t9, 18:1t10, 18:1t11, 18:1c9, 18:1c11 and 18:1c12 (**Tables 3.3-3.5**). Of the aforementioned MUFAs, 18:1t11 was positively associated with HOMA-IR in the Caucasian population ($r^2 = 0.16$; $P = 0.0006$). Separating Caucasians by sex revealed that the positive association between 18:1t11 and HOMA-IR remained significant in both men ($r^2 = 0.31$; $P = 0.0049$) and women ($r^2 = 0.12$; $P = 0.0125$). No significant associations were detected between 18:1t11 and markers of IR in East Asians or South Asians. Additionally in the Caucasian population, 18:1t6-8 was positively associated with HOMA-IR ($r^2 = 0.16$; $P = 0.0015$), and this relationship also remained significant in both Caucasian men ($r^2 = 0.30$; $P = 0.0167$) and women ($r^2 = 0.12$; $P = 0.0317$). Similarly, this relationship was not observed in East Asians or South Asians.

Next, we found that 18:1c9 was positively associated with fasting glucose in the Caucasian population ($r^2 = 0.09$; $P = 0.0017$); however, this relationship remained significant only in men ($r^2 = 0.20$; $P = 0.0004$), and not women ($r^2 = 0.04$; $P = 0.1294$), when separated by sex. No associations were found between 18:1c9 and markers of IR in East Asian or South Asians. No significant associations were detected between markers of IR and 16:1c9, 18:1t9, 18:1t10, 18:1c11 or 18:1c12.

Polyunsaturated Fatty Acids (PUFAs). Twelve PUFAs were examined for their associations with markers of IR (18:2c9t12, 18:2t9c12, 18:2n-6, 18:3n-6, 18:3n-3, 18:2c9t11, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3) (**Tables 3.3-3.5**). Significant associations with PUFA were only detected in the Caucasian population. 18:2n-6 was positively associated

with fasting glucose ($r^2 = 0.10$; $P = 0.0005$), while 18:2c9t11 was positively associated with HOMA-IR ($r^2 = 0.16$; $P = 0.0020$). Further sex-specific investigations demonstrated that the positive association between fasting glucose and 18:2n-6 remained significant in both men ($r^2 = 0.15$; $P = 0.0163$) and women ($r^2 = 0.05$; $P = 0.0086$). The positive associations between 18:2c9t11 and HOMA-IR also remained significant in both men ($r^2 = 0.30$; $P = 0.0097$) and women ($r^2 = 0.11$; $P = 0.0281$). No associations with these PUFAs were found in South Asians or East Asians. No significant associations with markers of IR were found for 18:2c9t12, 18:2t9c12, 18:3n-6, 18:3n-3, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3, 22:5n-3, or 22:6n-3.

Table 3.1 - Study population characteristics (Mean $\pm$ SE).

	Caucasian	East Asian	South Asian
Number (M/F)	461 (132/329)	362 (95/267)	104 (41/63)
Age (years)	23.1 $\pm$ 0.1 ^a	22.1 $\pm$ 0.1 ^b	22.3 $\pm$ 0.2 ^b
BMI (kg/m ²)	22.7 $\pm$ 0.1 ^a	21.6 $\pm$ 0.1 ^b	22.8 $\pm$ 0.3 ^a
Total Fat (g/d)	68.4 $\pm$ 1.2 ^a	61.6 $\pm$ 1.3 ^b	60.7 $\pm$ 2.7 ^b
Log Insulin (pmol/L)	1.57 $\pm$ 0.01 ^a	1.59 $\pm$ 0.01 ^a	1.71 $\pm$ 0.02 ^b
Glucose (mmol/L)	4.72 $\pm$ 0.02 ^a	4.78 $\pm$ 0.02 ^a	4.90 $\pm$ 0.03 ^b
HOMA-IR	0.78 $\pm$ 0.02 ^a	0.82 $\pm$ 0.02 ^a	1.08 $\pm$ 0.05 ^b

A nonparametric analysis of variation (ANOVA) test was performed for each parameter. Significant results were subsequently assessed by a nonparametric Wilcoxon test. Ethnic groups with dissimilar superscript text (e.g. a vs. b) are statistically different ($P < 0.05$) for the given parameter (mean $\pm$ standard error). M/F = male/female. BMI = body mass index. HOMA-IR = Homeostasis Model of Assessment of Insulin Resistance.

Table 3.2 - Baseline fatty acid levels in young healthy adults of Caucasian (n = 461), East Asian (n = 362) and South Asian (n = 104) descent.

	Mean Concentration		
	Caucasian	East Asian	South Asian
<i>Saturated Fatty Acids</i>			
14:0	16.31 ± 0.48 ^a	13.67 ± 0.54 ^b	15.26 ± 1.00 ^{a,b}
15:0	4.59 ± 0.09 ^a	3.85 ± 0.11 ^b	4.21 ± 0.20 ^a
16:0	422.74 ± 6.38 ^{a,b}	420.03 ± 7.20 ^a	400.01 ± 13.43 ^b
18:0	127.38 ± 1.61 ^a	135.49 ± 1.82 ^b	130.87 ± 3.39 ^{a,b}
<i>Monounsaturated Fatty Acids</i>			
16:1c9	37.86 ± 0.96 ^a	34.71 ± 1.08 ^a	29.91 ± 2.02 ^b
18:1t6-8	2.08 ± 0.08 ^a	1.96 ± 0.08 ^a	2.03 ± 0.16 ^a
18:1t9	4.34 ± 0.13 ^a	4.06 ± 0.15 ^a	4.24 ± 0.28 ^a
18:1t10	4.37 ± 0.14 ^a	4.12 ± 0.16 ^a	4.08 ± 0.30 ^a
18:1t11	4.52 ± 0.12 ^a	4.06 ± 0.13 ^b	4.40 ± 0.24 ^a
18:1c9	365.19 ± 7.55 ^a	376.02 ± 8.52 ^a	341.75 ± 15.89 ^b
18:1c11	32.68 ± 1.03 ^a	35.55 ± 1.16 ^b	29.22 ± 2.17 ^c
18:1c12	4.50 ± 0.13 ^a	4.28 ± 0.15 ^a	3.76 ± 0.28 ^a
<i>Polyunsaturated Fatty Acids</i>			
18:2c9t12	4.62 ± 0.18 ^a	4.04 ± 0.20 ^b	4.10 ± 0.37 ^{a,b}
18:2t9c12	2.58 ± 0.05 ^a	2.62 ± 0.06 ^a	2.57 ± 0.11 ^a
18:2n-6	592.14 ± 7.58 ^a	645.99 ± 8.56 ^b	606.51 ± 15.97 ^a
18:3n-6	6.55 ± 0.18 ^a	5.38 ± 0.20 ^b	7.94 ± 0.38 ^c
18:3n-3	13.52 ± 0.30 ^a	14.99 ± 0.34 ^b	15.54 ± 0.64 ^b
18:2c9t11	4.70 ± 0.10 ^a	3.86 ± 0.11 ^b	4.28 ± 0.20 ^b
20:2n-6	4.09 ± 0.08 ^a	4.26 ± 0.09 ^b	3.68 ± 0.17 ^c
20:3n-6	26.01 ± 0.49 ^a	20.19 ± 0.56 ^b	24.06 ± 1.04 ^a
20:4n-6	117.11 ± 1.72 ^a	111.56 ± 1.94 ^b	125.62 ± 3.61 ^a
20:5n-3	10.77 ± 0.39 ^a	12.63 ± 0.44 ^b	10.89 ± 0.82 ^{a,b}
22:5n-3	6.72 ± 0.14 ^a	7.28 ± 0.16 ^b	7.23 ± 0.30 ^{a,b}
22:6n-3	27.54 ± 0.57 ^a	33.86 ± 0.64 ^b	25.51 ± 1.20 ^a

A nonparametric analysis of variation (ANOVA) test was performed for all parameters, and significant results were subsequently assessed by a nonparametric Wilcoxon test. Ethnic groups with dissimilar superscript text (e.g., a vs. b) are statistically different ($P < 0.05$) for the given measure ($\mu\text{g/mL} \pm \text{standard error}$).

Table 3.3 - Associations between fatty acids and markers of insulin resistance in young healthy Caucasian adults (n = 461).

	HOMA-IR		Insulin (log)		Glucose	
	r ²	P-value	r ²	P-value	r ²	P-value
14:0	0.17	< 0.0001	0.19	0.0001	0.08	0.0390
15:0	0.16	0.0022	0.17	0.0073	0.08	0.0376
16:0	0.15	0.0089	0.17	0.0780	0.08	0.0214
16:1c9	0.15	0.1443	0.16	0.5274	0.07	0.3644
18:0	0.15	0.0216	0.16	0.1018	0.10	0.0007
18:1t6-8	0.16	0.0015	0.17	0.0437	0.07	0.4216
18:1t9	0.15	0.0334	0.16	0.1003	0.07	0.3634
18:1t10	0.16	0.0041	0.17	0.0089	0.08	0.1178
18:1t11	0.16	0.0006	0.18	0.0029	0.09	0.0136
18:1c9	0.15	0.0122	0.16	0.0848	0.09	0.0017
18:1c11	0.14	0.2349	0.16	0.6690	0.08	0.0314
18:1c12	0.16	0.0038	0.17	0.0118	0.07	0.4610
18:2c9t12	0.15	0.0405	0.17	0.0690	0.07	0.3508
18:2t9c12	0.15	0.0394	0.16	0.1262	0.15	0.0754
18:2n-6	0.15	0.1586	0.16	0.4673	0.10	0.0005
18:3n-6	0.14	0.3024	0.16	0.5425	0.08	0.0865
18:3n-3	0.15	0.0239	0.16	0.0804	0.09	0.0051
18:2c9t11	0.16	0.0020	0.17	0.0073	0.08	0.0480
20:2n-6	0.15	0.0731	0.16	0.2296	0.08	0.0915
20:3n-6	0.15	0.0135	0.17	0.0609	0.07	0.3305
20:4n-6	0.14	0.7651	0.16	0.7115	0.08	0.0125
20:5n-3	0.15	0.1953	0.16	0.4116	0.07	0.2991
22:5n-3	0.14	0.5481	0.16	0.722	0.07	0.2311
22:6n-3	0.14	0.6422	0.16	0.3711	0.07	0.992

Bold font indicates fatty acids which demonstrated statistically significant associations that satisfied a Bonferroni correction for multiple testing.

r² = Pearson's correlation coefficient

HOMA-IR = Homeostasis Model of Assessment of Insulin Resistance

Table 3.4 - Associations between fatty acids and markers of insulin resistance in young healthy East Asian adults (n = 362).

	HOMA-IR		Insulin (log)		Glucose	
	r ²	P-value	r ²	P-value	r ²	P-value
14:0	0.11	0.0024	0.13	0.0088	0.10	0.0614
15:0	0.09	0.1060	0.12	0.1775	0.10	0.1762
16:0	0.09	0.1127	0.12	0.2928	0.10	0.2815
16:1c9	0.21	0.0873	0.12	0.2311	0.10	0.0785
18:0	0.09	0.1854	0.12	0.5403	0.10	0.2818
18:1t6-8	0.08	0.4737	0.11	0.6167	0.11	0.0565
18:1t9	0.09	0.2372	0.12	0.4811	0.11	0.0867
18:1t10	0.09	0.2883	0.12	0.4064	0.10	0.1989
18:1t11	0.08	0.8989	0.11	0.9035	0.10	0.2031
18:1c9	0.09	0.2495	0.12	0.2969	0.11	0.0355
18:1c11	0.09	0.3086	0.12	0.1099	0.10	0.4933
18:1c12	0.08	0.3911	0.12	0.3966	0.11	0.0535
18:2c9t12	0.09	0.0666	0.12	0.1023	0.10	0.2928
18:2t9c12	0.09	0.1503	0.12	0.2362	0.10	0.6072
18:2n-6	0.08	0.5441	0.12	0.3801	0.10	0.8027
18:3n-6	0.09	0.2841	0.12	0.2322	0.11	0.0171
18:3n-3	0.09	0.1225	0.12	0.1447	0.10	0.5359
18:2c9t11	0.09	0.2516	0.12	0.3460	0.12	0.0085
20:2n-6	0.08	0.4447	0.12	0.3905	0.10	0.8557
20:3n-6	0.09	0.0508	0.13	0.0354	0.10	0.0894
20:4n-6	0.08	0.7148	0.11	0.9917	0.10	0.3656
20:5n-3	0.08	0.4941	0.12	0.4479	0.10	0.8266
22:5n-3	0.08	0.9893	0.11	0.9204	0.10	0.6846
22:6n-3	0.08	0.6374	0.12	0.5313	0.10	0.4428

r² = Pearson's correlation coefficient.

HOMA-IR = Homeostasis Model of Assessment of Insulin Resistance

Table 3.5 - Associations between fatty acids and markers of insulin resistance in young healthy South Asian adults (n = 104).

	HOMA-IR		Insulin (log)		Glucose	
	r ²	P-value	r ²	P-value	r ²	P-value
14:0	0.15	0.0518	0.10	0.0897	0.13	0.0164
15:0	0.12	0.4915	0.08	0.6001	0.08	0.5987
16:0	0.12	0.4176	0.08	0.6002	0.09	0.2739
16:1c9	0.12	0.6737	0.08	0.7724	0.09	0.2273
18:0	0.12	0.8328	0.08	0.9534	0.09	0.3685
18:1t6-8	0.12	0.4670	0.08	0.4406	0.14	0.0109
18:1t9	0.12	0.3505	0.08	0.2504	0.13	0.0145
18:1t10	0.11	0.5304	0.07	0.5077	0.12	0.2557
18:1t11	0.13	0.4832	0.08	0.5813	0.09	0.3856
18:1c9	0.13	0.2700	0.09	0.3395	0.08	0.3988
18:1c11	0.12	0.3661	0.08	0.4598	0.08	0.7893
18:1c12	0.12	0.8614	0.08	0.9561	0.08	0.7816
18:2c9t12	0.12	0.4865	0.08	0.7288	0.09	0.3157
18:2t9c12	0.12	0.6536	0.08	0.5053	0.09	0.2049
18:2n-6	0.12	0.5885	0.08	0.7771	0.08	0.6857
18:3n-6	0.12	0.5773	0.08	0.8202	0.09	0.2618
18:3n-3	0.19	0.0057	0.14	0.0093	0.09	0.2911
18:2c9t11	0.12	0.2849	0.08	0.3788	0.04	0.3001
20:2n-6	0.13	0.2273	0.09	0.2951	0.03	0.9353
20:3n-6	0.13	0.1943	0.09	0.2922	0.03	0.9261
20:4n-6	0.13	0.2706	0.10	0.1549	0.03	0.7612
20:5n-3	0.12	0.8226	0.08	0.8758	0.04	0.5325
22:5n-3	0.12	0.6271	0.08	0.7156	0.04	0.3235
22:6n-3	0.12	0.3889	0.08	0.3763	0.04	0.5587

r² = Pearson's correlation coefficient

HOMA-IR = Homeostasis Model of Assessment of Insulin Resistance

3.4. Discussion

In this study, we conducted a cross-sectional analysis to determine whether the relationship between individual FAs and markers of IR varied in an ethnic- and/or sex-specific manner in a population of healthy young Canadian adults. Previous investigations have examined the relationships between FAs and markers of IR in older adults, as well as in unhealthy men and women from geographically distinct populations [232,240]; however, we believe that this is the first study to investigate these relationships in a population of healthy young men and women differing in ethnicity but living in the same geographical region. This enabled us to observe ethnic-specific associations between FAs and markers of IR while avoiding the recognized confounder of geographical location. Indeed, we observed that numerous associations between FAs and markers of IR were only seen in certain ethnicities. We hypothesize that this could be due to underlying genetic differences that could influence FA composition in an ethnic-specific manner [241,242]. Moreover, we also examined whether sex could influence the relationships between FAs and markers of IR within each ethnicity. We noted that although men and women showed similar relationships between FAs and markers of IR, the correlations in men were typically stronger compared to women. This aligns with previous work by Frias et al., who demonstrated that elevations of plasma free FA concentrations impaired insulin sensitivity in the tissues of men, but not women [233]. This suggests that underlying sex-specific characteristics (e.g. hormone levels, adiposity, lifestyle habits, and quantity of muscle mass) are influencing this relationship [243,244]. In short, our study has identified numerous ethnic-specific associations between FAs and markers of IR, and the majority of these associations appeared to be more robust in men.

Of additional interest, we noted that South Asian subjects had significantly higher levels of fasting insulin and glucose concentrations compared to Caucasian subjects despite having a similar BMI ($22.8 \pm 0.3 \text{ kg/m}^2$ versus $22.7 \pm 0.1 \text{ kg/m}^2$). This finding seems to agree with past work in which differences in glucose metabolism between South Asian and European individuals residing near San Francisco (CA, USA) or southeast England were studied [232,240].

Saturated Fatty Acids (SFAs). Of the SFAs examined, 14:0 (myristic acid) was positively associated with fasting insulin and HOMA-IR in the Caucasian population, and with fasting insulin in the East Asian population. Furthermore, these associations remained significant when examining men and women separately within each ethnicity. This agrees with past work by Lovejoy et al., which examined the associations between relative FA levels in the cholesterol ester (CE) and phospholipid (PL) fractions of red blood cells (RBC) and markers of IR (i.e. fasting insulin and glucose, and HOMA-IR) in 38 young men and women [117]. Lovejoy and colleagues reported that of the FAs examined, relative abundance of CE 14:0 demonstrated the greatest number of positive associations with markers of IR and that these associations were similarly reflected in the PL fraction [117]. Together, our findings and those of Lovejoy et al. suggest that, across multiple ethnicities and within both genders, 14:0 appears to be strongly correlated with markers of IR.

We also found that 18:0 (stearic acid) was positively associated with fasting glucose and HOMA-IR in men and women in our Caucasian population. Similar results were reported by Ebbesson et al. who demonstrated that the relative abundance of 18:0 in RBC was positively associated with fasting glucose [171]. Additionally, Wang et al. found that

18:0 in CE and PL fractions were positively associated with the development of diabetes in middle-aged adults [196]. Although we observed associations with 18:0 in the Caucasian population, we did not detect associations in East Asian or South Asian subjects. Again, this emphasizes the importance of examining associations between FAs and markers of IR in an ethnic-specific fashion.

Monounsaturated Fatty Acids (MUFA). Previous work by Mozaffarian et al. demonstrated that the relative abundance (expressed as % FA) of 16:1c9 (palmitoleic acid) in the PL fraction of plasma was positively associated with markers of IR in men (i.e. fasting insulin and glucose, and HOMA-IR), but not women, in a European-majority multi-ethnic population [189]. We could not confirm this trend between 16:1c9 and markers of IR in our population; however, this may be explained by differences in study design. Specifically, we examined total plasma FAs whereas this previous study examined FAs in plasma phospholipids. Despite this, analyzing total plasma FAs has uncovered additional associations with longer-chain MUFAs that will ultimately contribute to our understanding of how MUFAs may influence markers of IR. We observed a positive association between 18:1c9 (oleic acid) and fasting glucose in both Caucasian and East Asian populations; however, this association appeared to be stronger in Caucasians. Interestingly, previous studies examining the relationship between 18:1c9 and IR have generated inconsistent results. For example, Ryan et al. demonstrated that diets rich in 18:1c9 can reduce IR in T2D adult males [43], while Mayer-Davis et al. revealed a positive association between dietary 18:1c9 and IR [245]. Even further uncertainty was provided by Lovejoy et al. and Hekmatdoost et al., who both reported no evidence of a relationship between 18:1c9 and

markers of IR [47,246]. A novel aspect of our study was that we analyzed the relationships between 18:1c9 and markers of IR in a sex-specific manner, which may provide a partial explanation of the discrepancy existing in the literature. We found that the relationship between 18:1c9 and fasting glucose was driven by Caucasian men and not women. We do not believe that this association has been recognized before and underlines potential sex-specific differences regarding the relationship between 18:1c9 and markers of IR.

Interestingly, we observed associations between 18:1t6-8 (sum of 18:1t6, t7, t8) and HOMA-IR in the Caucasian population, which to our knowledge has not been previously reported. Furthermore, this association appeared to be stronger in men compared to women. Although we adjusted our regression models to account for total fat intake, we acknowledge that FFQ can introduce inaccuracies in self-reporting. Therefore, we cannot exclude that this sex difference in 18:1t6-8 may be related to differences in dietary habits (e.g. ruminant milk and meat consumption [247]) that were inaccurately reported by participants.

We also found that 18:1t11 (*trans*-vaccenic acid) was positively associated with HOMA-IR in Caucasians, but not in East or South Asian populations. This finding is in agreement with a previous study reporting that vaccenic acid levels isolated from serum triglycerides were positively associated with fasting insulin and HOMA-IR in Caucasian males classified as either normoinsulinaemic/normoglycaemic or hyperinsulinaemic/hyperglycaemic [223]. Conversely, Takeuchi et al. recently reported that healthy young Japanese men and women (22.8 ± 3.0 yrs) supplemented with 0.6% *trans*-FAs for 4-weeks demonstrated no effect on fasting measures of insulin and glucose

[248]. While the findings of these two previous studies may initially appear to conflict, the results of our current research may shed some light on this issue. Indeed, our data may suggest that *trans*-FAs are more strongly associated with adverse effects on glucose metabolism in Caucasians, and less so in East and South Asian populations.

Polyunsaturated Fatty Acids (PUFAs). In the PUFA class, we found that 18:2n-6 (linoleic acid) was positively associated with fasting glucose in our Caucasian population, but not in the East Asians or South Asians groups. Previous studies investigating the relationships between 18:2n-6 and markers of IR have often generated inconsistent results. For example, several previous studies have found no evidence of an association between 18:2n-6 and markers of IR in diabetic Japanese adults [173] or overweight French adolescents [115]. In contrast, Salomaa et al. observed that 18:2n-6 in serum CE was lower in diabetic patients compared to healthy controls [183]. Our data therefore adds to the current literature as we have uncovered a novel association with 18:2n-6 in a young healthy population; however, it is clear that further investigation of the relationship between 18:2n-6 and markers of IR is required.

We also found that 18:2c9t11 (cis-9, trans-11 conjugated linoleic acid, CLA) was positively associated with HOMA-IR in Caucasians. These results appear to conflict with previous findings demonstrating a beneficial effect of 18:2c9t11 on insulin sensitivity in middle-aged healthy adults [249]; however, the different ages between these two populations makes it difficult to directly compare the studies. The majority of research examining CLA has been conducted in rodents and has either not focused on specific CLA isoforms or has generated inconsistent results [250,251]. Since findings from rodent

models cannot always be extrapolated to humans, this reinforces the need for further examination of the effects of 18:2c9t11 on IR in humans. To our knowledge, our's is the first study to demonstrate that the 18:2c9t11 isoform in plasma is positively associated with markers of IR in Caucasians, but that this association does not exist in East Asian and South Asian populations. Furthermore, a closer investigation revealed that 18:2c9t11 was positively associated with HOMA-IR in Caucasian men and women. Interestingly, this relationship was more significant in Caucasian men compared to women, which further points towards a role of sex-specific characteristics on associations between FAs and markers of IR.

Challenges and Limitations. We acknowledge that there are several limitations to consider with the present study. Firstly, we did not separate total FAs into individual lipid classes, which would provide further insight into dietary habits (i.e. namely FA consumption) in relation to ethnicity and sex. Thus, we were unable to determine whether a FAs in a specific lipid fraction was driving the associations with markers of IR. Future investigations examining FAs and markers of IR should consider separating total FAs into distinct lipid classes, as well as considering different biological materials (e.g. serum or erythrocytes) in order to better understand the relationships between FAs and IR. Secondly, the smaller sample size for the South Asian population may have limited our ability to detect potential associations in this ethnic group, and further studies in larger populations are still required. Thirdly, it is important to note that the current study was performed in young healthy adults. As such, it remains to be seen whether these relationships also exist in at-risk (e.g. obese and T2D) populations. Finally, our cross-sectional analysis did not allow us to draw

conclusions on cause and effect. To address this, prospective studies are required to examine whether the plasma FA profile in young adults can predict risk for developing T2D later in life.

3.5. Conclusion

In summary, we have demonstrated that circulating levels of specific FAs appear to be positively associated with markers of IR. Moreover, we have revealed potential ethnic-differences regarding these relationships (e.g. significant associations were detected in Caucasian and East Asian young adults, but not South Asians). Our data also suggests that the associations identified may be stronger in men compared to women. This suggests that underlying sex-specific differences (e.g. hormone levels, adiposity, lifestyle habits, and quantity of muscle mass) may be influencing these associations. Taken together, our results illustrate the significance of investigating associations between FAs and IR in an ethnic-specific and sex-specific manner in order to fully understand whether specific plasma FAs could contribute to the development of IR and T2D.

Chapter 4. Rationale and Objective

Study 1 confirmed my 1st hypothesis by demonstrating that associations between FAs and markers of IR vary according to ethnicity and sex. In light of this, the following study examined a population composed of a single ethnicity and sex. Since men demonstrated the strongest associations in Study 1, a population comprised of Caucasian males was used in the following study in order to eliminate sex- and ethnicity-related variability.

Study 2 was carried out in order to support my 2nd specific hypothesis: that FAs within a lipid fraction associate more strongly with markers of IR compared to total lipids. This is important in order to demonstrate that distinct FAs within a given lipid fraction can be a more robust biomarker of IR-status than the total abundance of that lipid. Due to the use of total blood TGs as a marker of T2D risk in clinical practice, TGs were selected as the lipid fraction of interest. Therefore, the following study "*Vaccenic Acid in Serum Triglycerides is Associated with Markers of Insulin Resistance in Men*" aimed to demonstrate that distinct FAs in the TG lipid fraction can provide a more accurate representation of an individual's IR-status than total TG levels.

Chapter 4. Vaccenic Acid in Serum Triglycerides is Associated with Markers of Insulin Resistance in Men

Zulyniak MA[§], Ralston JC, Tucker AJ, MacKay KA, Hillyer LM, McNicholas PD, Graham TE, Robinson LE, Duncan AM, Ma DW, Mutch DM. Vaccenic Acid in Serum Triglycerides is Associated with Markers of Insulin Resistance in Men. *Appl Physiol Nutr Metab.* 2012, 37(5):1003-1007.

[§]*Conceptualization, FA analysis, data analysis and interpretation, and writing of the manuscript.*

4. Abstract

Serum triglyceride levels are associated with metabolic disorders; however, it remains unclear whether the fatty acid (FA) composition of triglycerides is also changed. Although there were no differences in circulating triglyceride levels between normoglycaemic/normoinsulinaemic and hyperglycaemic/hyperinsulinaemic men, inspection of individual FAs revealed that vaccenic acid (18:1n-7) was enriched with hyperglycaemia/hyperinsulinaemia. Moreover, vaccenic acid levels were positively correlated with insulin and HOMA-IR. This reinforces that examination of individual FA in the context of insulin resistance is warranted.

4.1. Introduction

Triglycerides (TG) are integral for fatty acid (FA) trafficking and energy storage throughout the body. Importantly, elevated serum TG levels are widely associated with metabolic status, which has led to their use as an indicator of compromised health associated with metabolic disorders, such as obesity and type 2 diabetes (T2D) [252].

While elevated serum TG is widely used as a clinical marker, it remains unclear whether the FA composition of TG is also changed with metabolic disorders. Therefore, studying FA composition of TG will generate novel insight regarding the metabolic adaptations arising with conditions like obesity and T2D. Furthermore, this compositional analysis may uncover new predictors of insulin resistance (IR). This was recently exemplified by Kotronen *et al.*, who reported that specific FA patterns in serum TG molecules (e.g. TG containing 16:0/16:0/18:1) were more precise indicators of IR than total TG [10]. Therefore, the aims of the present exploratory study were to (i) determine

whether men characterized as normoglycaemic/normoinsulinaemic (NGI) or hyperglycaemic/hyperinsulinaemic (HGI) could be distinguished by serum TG levels and/or global TG FA profiles; and (ii) examine whether individual FAs in the TG fraction are associated with markers of insulin sensitivity.

4.2. Materials and Methods

Subjects. Subject recruitment, screening, classification as NGI (n = 10) or HGI (n = 10), and bioclinical data collection were previously described [253]. Criteria for NGI and HGI classifications were established using the Canadian Diabetes Association Guidelines (2008) [254] and previous work by Pereira *et al.* examining insulin sensitivity [255]. Of the 28 subjects originally recruited by Tucker *et al.*, the present analyses focused solely on the 20 men in order to eliminate gender diversity. The study received clearance by the University of Guelph Human Research Ethics Board and all participants provided written consent.

Pre-Study Diet. All subjects maintained a 3-day food diary prior to blood collection. This was analyzed to assess dietary variability between the NGI and HGI groups according to energy, carbohydrate, protein, total fat, saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), and cholesterol intake.

Analysis of Fasted Serum Triglyceride, Glucose, and Insulin. Total serum TG levels were measured from fasting venous blood samples at Guelph General Hospital (Guelph, Canada) using an auto analyzer (Synchron CX systems, Beckman Coulter, Mississauga, Canada). All blood samples were obtained from subjects at baseline (i.e. prior to the study intervention

outlined in Tucker et al. [253]). Glucose and insulin levels were measured from fasting venous blood samples as previously described [253]. The HOMA Calculator v2.2.2 was used to estimate IR (HOMA-IR) and β -cell function (HOMA%B).

Triglyceride Fatty Acid Compositional Analysis. All solvents and reagents were obtained from Fisher Scientific (Toronto, Canada). Isolation, extraction, and quantification of FA from TG was performed as previously described [256]. Briefly, lipids were extracted from serum samples and spotted onto Silica-G TLC plates (Analtech, Newark, USA). TG lipid bands were collected and methylated at 100 °C for 1.5 h. Samples were analyzed using an Agilent Technologies 7890A GC system (Agilent Technologies, Mississauga, CA). Peaks were identified by comparison to FA methyl ester standards suspended in hexane. Individual FA values were calculated as a percentage of total peak area. All data is reported as % FA $\pm$ SE.

Statistical Analyses. Statistical analyses were conducted using R software (Version 2.13.2). Mann-Whitney tests were used to examine differences between NGI and HGI for bioclinical parameters, total TG levels, and individual FAs. Principal component analyses (PCAs) were used as a means to illustrate the ability of bioclinical parameters and global TG FA profiles to distinguish NGI and HGI men. Regression models were used to identify associations between individual FAs and bioclinical parameters. Data was assessed for normality using quantile-quantile plots. Outliers were identified by examining residual plots and Cook's distances (values > 1 were inspected) for each regression model [257]. A P-value < 0.05 was considered significant.

4.3. Results

Subject Characteristics and Pre-Study Dietary Intake. Statistically significant differences between NGI and HGI groups were observed for BMI (NGI = 26.9 ± 3.2 kg/m², HGI 35.7 ± 6.5 kg/m², P-value = 0.0004), waist circumference (NGI = 96.2 ± 2.5 cm, HGI = 118.5 ± 3.8 cm, P-value = 0.0003), fasting glucose (NGI = 4.5 ± 0.2 mmol/L, HGI = 4.9 ± 0.1 mmol/L, P-value = 0.0172), fasting insulin (NGI = 33.7 ± 3.0 pmol/L, HGI = 101.1 ± 7.7 pmol/L, P-value = 0.0002), and estimates of IR (HOMA-IR; NGI = 0.61 ± 0.05 , HGI = 1.86 ± 0.14 , P-value = 0.0002) and β -cell function (HOMA%B; NGI = 90.5 ± 9.4 , HGI = 152.1 ± 11.9 , P-value = 0.0013). A PCA plot visually confirmed that these parameters were sufficient to distinguish NGI from HGI subjects (**Figure 4.1A**). Notably, there was no difference in total TG levels between the groups (NGI = 1.16 ± 0.12 , HGI = 1.80 ± 0.34 , P-value = 0.143). Furthermore, 3-day food diaries indicated no dietary differences in energy or macronutrient intakes between NGI and HGI subjects [253].

Triglyceride Fatty Acid Compositional Analysis. PCA depicted that global TG FA profiles (i.e. all FA indicated in **Table 1**) were insufficient to distinguish NGI and HGI subjects (**Figure 4.1B**). Closer inspection of individual TG FAs revealed that statistically significant differences between groups were only observed for vaccenic acid (18:1c11) (**Table 4.1**). Specifically, vaccenic acid levels were higher in HGI compared to NGI men (P-value = 0.043). Palmitic acid (16:0) demonstrated a trend to be elevated in HGI males (P-value = 0.075).

Relationship between Triglyceride Fatty Acid Composition and Bioclinical Parameters. We next examined whether the levels of vaccenic and palmitic acid in TG were associated with

bioclinical parameters associated with T2D. Palmitic acid was not associated with any parameters; however, vaccenic acid was positively correlated with both fasting insulin (P-value = 0.014, $r = 0.597$) and HOMA-IR (P-value = 0.010, $r = 0.616$) (**Figure 4.2.**).

Table 4.1. - Fatty acid compositional analysis of serum triglycerides

Fatty Acid			% Composition of Triglyceride		
	Molecular Name	Common Name	NGI	HGI	P-value
SFA	14:0	Myristic Acid	2.24 ± 0.26	2.26 ± 0.28	0.912
	16:0	Palmitic Acid	26.95 ± 0.15	31.21 ± 1.76	0.075
	18:0	Stearic Acid	8.26 ± 0.73	7.86 ± 0.79	0.971
	Total		37.54 ± 1.85	41.32 ± 2.18	0.075
MUFA	16:1n7	Palmitoleic Acid	3.38 ± 0.12	3.95 ± 0.38	0.218
	18:1c9	Oleic Acid	34.64 ± 1.10	33.11 ± 1.30§	0.447
	18:1c11	cis-Vaccenic Acid	2.75 ± 0.08§	3.14 ± 0.15	0.043*
	Total		40.50 ± 1.30	36.89 ± 3.27	0.393
PUFA	18:3n-3	<i>α</i> -Linolenic Acid	1.74 ± 0.33	1.81 ± 0.28	0.579
	20:5n-3	Eicosapentaenoic acid	ND	ND	ND
	22:6n-3	DHA	ND	ND	ND
	18:2n-6	Linoleic Acid	16.58 ± 1.83	16.97 ± 1.38	0.853
	20:2n-6	Eicosadienoic Acid	ND	ND	ND
	20:3n-6	DGLA	ND	ND	ND
	20:4n-6	Arachidonic Acid	1.55 ± 0.12	1.65 ± 0.33§	0.604
	22:4n-6	Adrenic Acid	2.17 ± 0.71	1.70 ± 0.61§	0.278
	Total		22.04 ± 2.02	21.80 ± 1.55	0.912
	Total n-3		1.74 ± 0.33	1.81 ± 0.28	0.579
	Total n-6		20.30 ± 1.85	19.99 ± 1.41	1.000
	Total n-3/n-6		8.50 ± 1.31	9.04 ± 1.22	0.481

Total saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated (PUFAs) fatty acids refer to the sum of the individual fatty acids (FAs) detected in the class. Total n-3 and n-6 are the sum compositions of each group in the PUFA class. Significant differences in FA composition between normoglycaemic / normoinsulinaemic (NGI) and hyperglycaemic / hyperinsulinaemic (HGI) groups were determined using Mann-Whitney, $P < 0.05$ was significant. FA comparisons between NGI and HGI groups contain $n = 20$ subjects (NGI = 10, HGI = 10) unless indicated by § superscript (in which case $n = 9$). Outliers were determined by FAs acids detected at $< 1.0\%$ of total FAs are indicated by *ND*.

Figure 4.1. - Principal component analysis (PCA) of subjects according to bioclinical parameters and triglyceride (TG) fatty acid profile.

(A) Subjects were separated into normoglycaemic / normoinsulinaemic (NGI) and hyperglycaemic / hyperinsulinaemic (HGI) groups using bioclinical parameters (BMI, waist circumference, fasting insulin and glucose, HOMA-IR, and HOMA%B), as expected. (B) Subjects were then analyzed according to their TG fatty acid (FA) profile (i.e. which corresponds to all FAs listed in Table 1). The analysis demonstrated that TG FA composition was insufficient to distinguish NGI and HGI subjects. NGI and HGI subjects are represented by open (○) and closed (●) circles, respectively.

Figure 4.2. - Associations between vaccenic acid and bioclinical parameters.

Triglyceride (TG) vaccenic acid (18:1c11) was positively associated with fasting insulin (**A**) and HOMA-IR (**B**). Associations were identified with regression analyses adjusted for BMI and age. Data points with Cook's distances > 1 and discordant within the quantile-quantile plot were considered outliers and removed from analysis. Following outlier analysis, a single data point was removed from each model, resulting in n = 19.

4.4. Discussion

Prior to undertaking a detailed prospective investigation regarding the relationship between serum TG FA composition and IR, we elected to first retrospectively examine a study examining men classified as low-risk (NGI) or high-risk (HGI) for developing IR. Therefore, the present study represents an extension of a study previously conducted by Tucker *et al.* [253]. The present exploratory study postulated that total TG levels and/or TG FA profiles would differ between NGI and HGI men.

We initially compared the two NGI and HGI groups from a global perspective using bioclinical and TG FA profiles. Our first PCA plot confirmed that, as expected, the two groups of men could be distinguished using the bioclinical parameters originally used to classify them. However, in contrast to our expectations, the second PCA plot visually demonstrated that global TG FA profiles failed to distinguish the two groups of men, which suggested that global TG profiles did not substantially differ between the two groups. This finding agrees with a previous study by Yang *et al.* in which a lipidomics approach was used to classify T2D and healthy subjects (which are more distinct phenotypes than the NGI and HGI groups included in the current study). Yang and colleagues demonstrated that although serum TG FA composition was a poor discriminator of T2D and healthy subjects, the FA composition of phospholipid and cholesterol ester fractions appeared to discriminate the two groups more effectively [116]. Thus, our study and that of Yang *et al.* suggest that serum TG FA composition does not substantially change with IR.

Having determined that our NGI and HGI groups could not be distinguished based on the global FA profiles in serum TG, we subsequently examined whether individual FAs within TG differed between NGI and HGI subjects. We found no evidence for differences in

n-3 and n-6 FAs between the two groups. Alpha-linolenic and linoleic acids were the major n-3 and n-6 FAs, respectively, while only trace levels (< 1%) of docosahexaenoic acid were observed. Our compositional analysis of n-3 and n-6 FAs agree with those previously reported by Vidgren *et al.* [258]. Upon inspection of individual FAs we found that vaccenic acid (18:1n-7) levels were significantly elevated in HGI subjects, while palmitic acid (16:0) showed a trend to be elevated in the HGI group. These associations with IR were previously proposed in a review by Lovejoy [259]. As such, vaccenic and palmitic acid were more closely examined to determine their relationship with risk factors associated with IR.

Regression analyses revealed that palmitic acid was not associated with BMI, waist circumference, fasting insulin, glucose, HOMA-IR, or HOMA%B. While this may appear to contrast with past research demonstrating positive associations between palmitic acid levels and IR, it is important to recognize that this previous research identified these associations using palmitic acid levels measured in total lipids or the phospholipid fraction [46,174]. In contrast, vaccenic acid was positively associated with both fasting insulin and HOMA-IR. This is of particular relevance in light of recent contributions that have demonstrated associations between vaccenic acid and altered glucose and lipid metabolism.

While the precise basis of the relationship between vaccenic acid and health status remains elusive [260], Ståhlman *et al.* recently reported that a greater proportion of vaccenic acid at the sn2 position of plasma TG was associated with T2D in women [98]. While further research is required in order to determine the purpose of vaccenic acid's incorporation into TG, the authors speculated that the amount of vaccenic acid in TG may reflect altered hepatic lipid metabolism [98]. Although the current study and the previous study by Ståhlman *et al.* did not distinguish the isoform of vaccenic acid (i.e. *cis* or *trans*) in

TG, work by Alstrup and colleagues demonstrated that both isoforms negatively influenced glucose metabolism and the secretion of insulin in isolated mouse β -cells [261]. Furthermore, Alstrup *et al.* speculated that vaccenic acid isoforms may interact with Ca^{2+} channels in islet cells, which could elevate insulin secretion despite reductions in glucose oxidation [261]. In light of our findings and those from previous work, we propose that the relative amount of vaccenic acid in TG may be indicative of the transition from healthy to hyperinsulinaemia to T2D.

We recognize that the primary limitation of the current exploratory study lies with the small sample size. However, we are confident of our study conclusions because of independent reports linking changes in vaccenic acid levels in blood TG with IR. Nevertheless, we propose that subsequent prospective studies using a larger sample size consisting of subjects with different levels of insulin sensitivity are required to definitively demonstrate that vaccenic acid levels in the TG fraction reflect IR severity. Furthermore, we cannot completely exclude the possibility that our results may be influenced by subtle differences in food consumption between the two groups of subjects. Although macronutrient intake did not significantly differ between the two groups according to 3-day food diaries, future studies should consider recording participant dietary habits over a longer period of time to account for differences in the intake of specific food groups.

4.5. Conclusion

We report that total TG levels and global TG FA profiles do not distinguish NGI and HGI men, despite statistically significant differences in bioclinical parameters. However,

vaccenic acid levels in TG were elevated in HGI subjects and may, therefore, play a role in the development of IR and reflect changes in insulin secretion and hepatic lipid metabolism. This exploratory study reinforces the value of incorporating a lipidomics-based approach in future clinical studies in order to investigate the contribution of specific FAs to the development of IR.

Chapter 5. Rationale and Objective

Study 2 confirmed my 2nd hypothesis by demonstrating that distinct FAs within the TG fraction associated more strongly with markers of IR compared to total TGs. This also further reinforced my overall thesis hypothesis, by demonstrating that specific FAs, such as *cis*-vaccenic acid (18:1c11) within TGs, associate more strongly with markers of IR compared to total lipid concentrations. Therefore, having confirmed my first two hypotheses in studies 1 and 2, I set out to support my final specific hypothesis.

To accomplish this, a new study cohort was established that allowed me to compare the profiles of lean and obese individuals differing in metabolic status (i.e., lean healthy (LH), metabolically healthy obese (MHO), and metabolically unhealthy obese (MUO)) according to published cardiometabolic criteria. The formation of this cohort allowed me to minimize variability between study participants, both biological (e.g., sex, ethnicity, age, BMI, and fat %) and technical (as discussed in **Appendix A**). By designing and building this study cohort I aimed to support my 3rd specific hypothesis by demonstrating that a stronger characterization of these three groups could be obtained by investigating FAs in specific lipid fractions rather than total FA levels. By doing so, my goal was to better understand the underlying influences of specific FAs toward metabolic health and present the most robust FAs for distinguishing metabolically healthy and unhealthy individuals.

Chapter 5: A Distinct Fatty Acid Profile Underlies the Reduced Inflammatory State of Metabolically Healthy Obese Individuals

Perreault M*, **Zulyniak MA***[§], Badoud F, Stephenson S, Buchholz AC, Badawi A, and Mutch DM. A distinct fatty acid profile underlies the reduced inflammatory state of metabolically healthy obese individuals. *PLOS ONE*. 2013. *In press*.

*Contributed equally to the manuscript.

§Conceptualization, patient recruitment, clinical study management, FA analysis, FA data analysis and interpretation, and writing of the manuscript.

5. Abstract

Background. Obesity is associated with numerous health complications; however, a subgroup of obese individuals (termed the metabolically healthy obese or MHO) appear to have lower risk for complications such as type 2 diabetes (T2D) and cardiovascular disease. Emerging evidence suggests that MHO individuals have reduced inflammation compared to their metabolically unhealthy obese (MUO) counterparts. As it is recognized that fatty acids (FAs) have a strong relationship with inflammation, the current study aimed to uncover if the reduced inflammation observed in MHO individuals is mirrored by a more favourable FA profile.

Methods. Fasted serum samples were collected from lean healthy (LH), MHO, and MUO participants (n = 10/group) recruited from the Diabetes Risk Assessment study. A panel of pro- and anti-inflammatory markers were measured by immunoassay. Total serum FA profiling, as well as the FA composition of circulating phospholipids (PL) and triglycerides (TG), was measured by gas chromatography. ANOVA and Mann-Whitney-Wilcoxon tests were used to assess statistical significance between the groups ($P < 0.05$).

Results. MHO and MUO individuals had similar BMI and body fat %; however, lipid parameters in MHO individuals more closely resembled that of LH individuals. MHO individuals had circulating levels of high sensitivity C-reactive protein (hsCRP) and interleukin-6 (IL-6) similar to LH individuals, while levels of platelet derived growth factor- $\beta\beta$ (PDGF- $\beta\beta$) were intermediate to that of LH and MUO individuals. FA profiling analysis combined with discriminant analysis modelling highlighted a panel of nine FAs (consisting

of three saturated, three monounsaturated, and three polyunsaturated FAs) in PL and TG fractions that distinguished the three groups. Specifically, saturated FAs (myristic (14:0) and stearic acid (18:0)) levels in MHO individuals resembled that of LH individuals.

Conclusion. Our results suggest that the reduced inflammatory state of MHO individuals compared to MUO individuals may stem, in part, from a more favourable underlying FA profile.

5.1. Introduction

The low-grade chronic inflammation characteristic of obesity plays a significant role in the development of downstream complications, such as type 2 diabetes and cardiovascular disease [7,8,262]. However, evidence suggests that not all obese individuals are at a similar risk for these complications [263,264]. Obese individuals who are seemingly protected from downstream complications are classified as metabolically healthy obese (MHO). While our molecular understanding of the MHO phenotype remains limited, clinical research has shown these individuals are more insulin sensitive and present a favourable lipid status compared to their metabolically unhealthy obese (MUO) counterparts (also referred to as “metabolically abnormal obese”) [265]. Recent observations also suggest that MHO individuals may have a reduced inflammatory status compared to MUO individuals [264,266,267].

Few studies have investigated the inflammatory profile associated with MHO. In 2005, Karelis et al. first noted that post-menopausal MHO women had lower levels of circulating high sensitivity C-reactive protein (hsCRP) and inflammation-sensitive protein alpha-1 antitrypsin (A1AT) compared to MUO women [266]. Subsequently, Klöting et al. reported that MHO individuals had lower circulating levels of various inflammatory markers (e.g., CRP, progranulin, chemerin, and retinol-binding protein-4) compared to MUO subjects [264]. More recently, Phillips and Perry demonstrated that MHO individuals had lower concentrations of a number of pro-inflammatory markers (e.g., complement component 3, CRP, tumour necrosis factor- α , interleukin-6, and plasminogen activator inhibitor-1) and higher adiponectin compared to MUO individuals of similar adiposity [267]. Together, these studies provide evidence that MHO have reduced inflammation

compared to their MUO counterparts; however, the mechanisms responsible for this disparity remain to be elucidated.

It is now widely appreciated that FAs can influence whole-body inflammation by regulating the production and secretion of cytokines, chemokines, and eicosanoids [268,269]; however, not all FAs act similarly. Saturated and trans fats tend to be positively associated with inflammation, while monounsaturated and polyunsaturated fats typically have beneficial effects [239,270,271]. As such, elucidating the FA profile in MHO individuals will provide important insight to help us better understand the basis for their reduced inflammatory state.

Total circulating levels of free FAs (i.e., FFAs) were reported to be lower in MHO compared to MUO individuals [272,273]; however, it remains unknown if individual FA levels differ between MHO and MUO individuals. This is relevant given that past research has shown that measuring FAs in specific lipid fractions (e.g., phospholipid, PL; triglyceride, TG) can provide novel insight to help understand the changes in FA metabolism that are associated with inflammation [96,115]. For example, Pietiläinen et al. employed a global FA profiling approach to show that expanding adipose tissue is characterized by a FA profile that may favour inflammation [96]. While such an approach has not been used to study MHO, this is warranted given that the expression of lipogenic genes was recently shown to differ between MHO and MUO individuals [273]. As such, we expect that using a FA profiling approach will generate novel insight to help understand if FAs contribute to the reduced inflammatory state seen in MHO individuals.

In the current study we first set out to confirm that MHO individuals from our cohort were characterized by a reduced inflammatory state and then subsequently

examined whether this was associated with a distinct circulating FA profile. Together, the knowledge generated by this research will help unravel the underlying basis for the reduced level of inflammation seen in MHO individuals, and may ultimately be used to develop tailored dietary strategies to more appropriately manage obesity-related complications.

5.2. Material and Methods

Study population. Individuals were recruited into the Diabetes Risk Assessment (DRA) study (Clinical Trial No. NCT01884714) from Guelph, Ontario and the surrounding communities using study posters and newspaper advertisements (**Appendix B**). Persons expressing interest in the study were screened over the phone and excluded if they met any one of the following criteria: 1) below 35 or above 70 years of age; 2) diagnosed with an acute or chronic autoimmune inflammatory disease, infectious disease, viral infection, and/or cancer; or 3) regular alcohol consumption exceeding 2 drinks/day (1 drink = 10g alcohol). The research protocol was approved by the University of Guelph Research Ethics Board (REB#10AP033). All participants signed a written consent form (**Appendix C**).

Anthropometric Measurements. All measures related to adiposity (i.e., height (m), body weight (kg), waist and hip circumferences (cm), fat mass (% and kg) and fat-free mass (% and kg)) were obtained in the Body Composition and Metabolism Laboratory at the University of Guelph (www.uoguelph.ca/bodycomp). Body mass (to the nearest 0.1 kg) was measured with subjects wearing only a bathing suit and swimming cap, using the digital BOD POD scale (BOD POD Air Displacement Body Composition system; Life Measurement

Inc., CA, USA). The scale was calibrated weekly against standardized 20-lb weights. Height was measured to the nearest 0.5 cm, using a wall-mounted stadiometer (Seca Corp., Ontario, Canada). Body mass index (BMI) was calculated from height and mass (kg/m^2).

Fat mass and fat-free mass were measured using the BOD POD. The instrument was calibrated twice in the morning of each data collection day: once with the test chamber empty and once by placing a cylinder of known volume (49.980 L) in the chamber. Raw body volume was measured with subjects wearing only a bathing suit and swimming cap, with no jewelry. Subjects were instructed to sit quietly, limit movement, and breathe normally while in the test chamber. Body volume was measured twice and the average was used to determine body density. If the 2 measurements differed by more than 150 mL, a third measurement was taken and the average of the 2 closest was then used. The final step involved in determining body density was the measurement of thoracic gas volume; subjects were instructed to sit quietly and plug their noses while breathing through a disposable tube connected to the rear of the instrument. The subjects were instructed to make 3 quick, light pants, after 4 or 5 normal breaths. Percentage body fat was calculated from density using the Siri equation. All measurements were performed by the same trained person. The coefficient of variation for percentage body fat measurements was $2.2 \pm 2.3\%$.

Bioclinical Measurements. Blood samples were collected from all participants following an overnight fast (~ 12 hrs). Serum samples were sent to LifeLabs Medical Laboratory Services (Guelph, ON, Canada) for the analysis of glucose (mmol/L), insulin (pmol/L), total-triglycerides (TG; mmol/L), total-cholesterol (Total-c; mmol/L), high-density lipoprotein

cholesterol (HDL-c; mmol/L), low-density lipoprotein cholesterol (LDL-c; mmol/L), glycosylated haemoglobin (HbA1c), and hsCRP (mg/L). Estimates of the insulin sensitivity (HOMA-IR) and β -cell function (HOMA%B) were calculated using the HOMA Calculator v2.2.2 [274].

Systolic and diastolic blood pressure was measured (in duplicate) at rest using an automated blood pressure monitor (Intellisense, OMRON Healthcare, Bannockburn, IL, USA).

Classification of Groups. Thirty participants were classified into LH, MHO, and MUO groups based on their adiposity and metabolic status ($n = 10/\text{group}$). Adiposity status was determined using the revised BMI cut-offs proposed by Shah and Braverman, where lean was considered $< 28\text{kg/m}^2$ for males and $< 24\text{kg/m}^2$ for females, and obese was considered $\geq 28\text{kg/m}^2$ for males and $\geq 24\text{kg/m}^2$ for females [275]. Metabolic status was determined using criteria adapted from that originally proposed by Karelis *et al.* [276] in order to account for sex-specific differences and medication. An individual was considered “metabolically healthy” if 3 or more of the following criteria were met: HDL-c $> 1.0\text{mmol/L}$ for males and $> 1.3\text{mmol/L}$ for females; TG $< 1.7\text{mmol/L}$ without use of lipid-lowering drugs; Total-c $< 5.2\text{mmol/L}$; LDL-c $< 2.6\text{mmol/L}$; and HOMA-IR < 1.95 without use of anti-diabetic drugs. Each group was comprised of 7 women and 3 men. LH, MHO, and MUO groups were matched for age, while the MHO and MUO groups were matched for BMI and percentage body fat.

Inflammatory Marker Analysis. A panel of pro- and anti-inflammatory markers were measured in fasted serum samples. Interleukin-10 (IL-10), monocyte chemotactic protein-1 (MCP-1/CCL2), tumour necrosis factor- α (TNF- α), and high-molecular weight adiponectin (HMW adiponectin) were measured, in duplicate, using immunoassay kits according to the manufacturer's instructions (BioLegend, San Diego, CA, USA or R&D Systems, Minneapolis, MN, USA) and read using a SynergyMX plate reader (Biotek, Winooski, VT, USA). Interleukin-6 (IL-6), interleukin-1 receptor antagonist (IL-1Ra), interferon- γ (IFN- γ), regulated upon activation normal T-cell expressed and secreted (RANTES/CCL5), platelet derived growth factor- $\beta\beta$ (PDGF- $\beta\beta$), and interferon- γ inducible protein 10 (IP-10) were measured, in duplicate, by multiplex bead immunoassay and read using the Bio-Plex suspension array system according to the manufacturer's recommendations (Bio-Rad, Mississauga, ON, Canada).

Fatty Acid Analysis. All solvents and reagents were obtained from Fisher Scientific (Toronto, ON, Canada). The isolation, extraction, and quantification of total FAs, as well as fractionated FAs (i.e., in PL and TG fractions), from fasted serum samples were performed as previously described [223]. Briefly, samples were spiked with 10 μ L of a 1 μ g/ μ L C17:0 internal standard. Both total and fractionated FAs were extracted with chloroform:methanol (2:1, v/v). Samples were then flushed with nitrogen and placed at $\sim 4^{\circ}\text{C}$ over night. The next day, samples used for total FA analysis were methylated at 100°C for 1.5 hrs. For the analysis of FAs in isolated lipid fractions, samples were spotted onto Silica-G TLC plates (Analtech, Newark, N.J., USA) and incubated for ~ 45 min with petroleum ether, ethyl ether and acetic acid (80:20:1, v/v/v). PL and TG lipid bands were collected

into separate tubes and methylated at 100 °C for 1.5 hrs. All samples were analyzed using an Agilent Technologies 7890A GC system (Agilent Technologies, Mississauga, ON, Canada) with flame ionization detector. Peaks were identified by comparison to a panel of 49 FA methyl ester standards suspended in hexane (ranging from 8:0 to C24:1n-9). Relative FA values were calculated as a % of total peak area. Absolute FA values were calculated by comparison of individual FA peaks to the internal standard C17:0. As such, individual FA values (for both total and fractionated analyses) are reported as relative percentage (% FA $\pm$ SEM) and/or absolute (μg / 100 μL of serum) values.

Statistical analysis. Anthropometric, bioclinical, and inflammatory parameters were analyzed with Prism 5 software (GraphPad, La Jolla, CA, USA). First, a non-parametric ANOVA Kruskal-Wallis test was used to measure significance between the three groups ($P < 0.05$). Second, a post-hoc non-parametric Mann-Whitney-Wilcoxon test was used for pairwise group comparisons in cases when the initial ANOVA was statistically significant.

An Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) was used to distinguish the three phenotypes based on their FA profiles (mean centred and scaled using Pareto variance) (SIMCA v13.0.3.0, Umetrics AB, Umeå, Sweden). Three principal components were generated and cross-validated 7 times, where 1/7th of the data was randomly left out for each round of validation. Analysis of variance of cross-validated residuals (CV-ANOVA) was performed for each OPLS-DA in order to assess the reliability of the predictive model. FAs with the greatest variability between the three groups were identified using Variables of Importance in Projection (VIP) > 1 [277]. FAs meeting our VIP cut-off were then individually assessed between the three groups using a non-parametric

ANOVA Kruskal-Wallis test ($P < 0.05$). When significance was observed for a FA, a post-hoc Mann-Whitney-Wilcoxon test was subsequently used for pairwise group comparisons (JMP Genomics v5.1, SAS Institute, Cary, NC, USA). FAs meeting our VIP cut-off were also examined by linear regression (adjusted for sex) with measures of adiposity (BMI and body fat %) using JMP Genomics software.

5.3. Results

Characteristics of the groups. General characteristics of the three groups are outlined in **Table 5.1**. Briefly, the three groups (LH, MHO, and MUO) were matched for age, while the obese groups were matched for BMI, waist-to-hip circumference, and body fat (both % and kg). The MHO and LH groups had significantly lower circulating levels of Total-c and LDL-c compared to the MUO group. In contrast, no differences were seen between the MHO and MUO groups for HDL-c and TG levels. Consequently, the MHO group had a Total-c/HDL-c ratio intermediate to that of LH and MUO groups. The MHO and MUO groups were not different for fasted glucose levels or HbA1c. However, the MHO group had fasted insulin and HOMA-IR values intermediate to that of LH and MUO ($P = 0.09$ for both parameters), suggesting a trend for higher insulin sensitivity in MHO individuals.

Inflammatory Marker Analysis. **Table 5.2** outlines the concentrations of circulating inflammatory markers measured in the three groups of individuals. The MHO and LH groups had hsCRP and IL-6 levels significantly lower than the MUO group (**Figure 5.1**), while the MHO group had PDGF- $\beta\beta$ levels intermediate to that of the LH and MUO groups. A similar trend was also seen for IP-10 levels ($P = 0.07$). In contrast, HMW adiponectin levels

were significantly lower in MHO and MUO groups compared to the LH group. No differences were seen in IL-1Ra, IFN- γ , and RANTES levels between the three groups, while TNF- α , MCP-1 and IL-10 levels were not consistently detected due to low circulating levels in several of the study participants.

Fatty Acid Analysis. We first examined total FAs in serum from LH, MHO, and MUO individuals (**Tables 5.3A, B**). Only FAs that were consistently detected across all participants were included in these analyses. Total FA abundance differed significantly between the three groups (**Table 5.3B**, $P = 0.0082$). In alignment with our clinical and inflammatory data, total FA abundance in MHO individuals was intermediate to LH and MUO individuals, where LH individuals had the lowest concentration of serum FAs ($201.4 \pm 5.7 \mu\text{g}/100 \mu\text{L}$), followed by MHO ($278.5 \pm 43.9 \mu\text{g}/100 \mu\text{L}$) and MUO ($406.0 \pm 58.0 \mu\text{g}/100 \mu\text{L}$) individuals. Furthermore, 10 individual FAs (myristic acid, myristoleic acid, palmitoleic acid, heptadecenoic acid, stearic acid, oleic acid, linoleic acid, γ -linolenic acid, α -linolenic acid, and arachidonic acid) were found to consistently differ between the three groups when data was expressed in both relative % and absolute values (**Tables 5.3A, B**); indicating high concordance between the two approaches used to report FA levels. Subsequent fractionation of serum lipids revealed that 8 of the 10 aforementioned FAs were also significantly different in the PL and TG fractions (data not shown); indicating good agreement between total and fractionated FA analyses.

We next examined whether total FA profiles (expressed as either relative % or absolute values), as well as FA profiles from PL and TG fractions (expressed as either relative % or absolute values), could be used to distinguish the three groups. OPLS-DA

modelling indicated that the FA profile from PL and TG fractions expressed as relative % values provided the best combination of fit ($R^2Y_{\text{cum}} = 0.58$) and predictive ability ($Q^2Y_{\text{cum}} = 0.32$, CV-ANOVA = 0.05) to distinguish the three groups (**Table 5.4.**). As seen in Figure 2, OPLS-DA was able to clearly discriminate LH from the MHO and MUO groups, while the MHO and MUO groups showed a small degree of overlap. Subsequent analysis identified a panel of nine FAs from the PL and TG fractions (i.e., VIP > 1) that could discriminate the three groups (**Table 5.5.**). The nine FAs included: PL-linoleic acid, PL-dihomo- γ -linolenic acid, PL-arachidonic acid, PL-erucic acid, TG-myristic acid, TG-palmitic acid, TG-stearic acid, TG-oleic acid, and TG-erucic acid. These nine FA may have potential to serve, collectively, as a biomarker to distinguish MHO from MUO groups.

Examining these nine FAs individually revealed that seven of them differed significantly between the three groups (**Figure 5.3**). The MHO and LH groups had significantly lower levels of serum TG-myristic acid compared to the MUO group. MHO and MUO groups had higher levels of TG-oleic acid and PL-dihomo- γ -linolenic acid and lower levels of TG- and PL-erucic acid compared to the LH group. MHO individuals had a level of TG-stearic acid that was intermediate to that of the LH and MUO groups. Finally, the MHO group had significantly greater levels of PL-arachidonic acid compared to both the LH and MUO groups. No changes were detected between the three groups for PL-linoleic acid and TG-palmitic acid.

Finally, we used linear regression to examine the relationships between the aforementioned seven FAs and measures of adiposity status (BMI and body fat %). PL-DGLA was positively associated with both BMI ($r^2 = 0.252$, $p = 0.006$) and body fat % ($r^2 = 0.231$, $p = 0.008$), while TG-oleic acid was only positively associated with BMI ($r^2 = 0.194$, p

= 0.030). In contrast, TG- and PL-erucic acid were inversely associated with both BMI ($r^2 = 0.326$, $p = 0.001$ and $r^2 = 0.531$, $p < 0.001$; respectively) and body fat % ($r^2 = 0.249$, $p = 0.006$ and $r^2 = 0.350$, $p = 0.001$; respectively), while TG-stearic acid was only inversely associated with BMI ($r^2 = 0.191$, $p = 0.018$). No significant associations were observed between markers of adiposity and PL-arachidonic acid or TG-myristic acid.

Table 5.1.- Study population characteristics

Parameters	LH	MHO	MUO	ANOVA (P-value)	Post-hoc Mann-Whitney-Wilcoxon group comparison (P-values)		
	mean ± SEM	mean ± SEM	mean ± SEM		LH vs. MHO	LH vs. MUO	MHO vs. MUO
Anthropometric measurements							
Number of subjects	10 (3 men, 7 women)	10 (3 men, 7 women)	10 (3 men, 7 women)				
Age (yrs)	51 ± 3	50 ± 4	48 ± 2	0.8418			
Weight (kg)	61.9 ± 2.8	86.2 ± 3.4	92.7 ± 6.2	0.0004	0.0001	0.0003	0.5288
Height (cm)	167 ± 3	168 ± 3	167 ± 3	0.9299			
BMI (kg/m²)	22.1 ± 0.6	30.6 ± 1.1	33.0 ± 1.9	<0.0001	<0.0001	<0.0001	0.4813
Waist circumference (cm)	77 ± 3	98 ± 3	104 ± 5	0.0002	0.0007	0.0003	0.4359
Hip circumference (cm)	96 ± 1	109 ± 2	113 ± 4	0.0002	0.0003	0.0006	0.5960
Waist-to-hip ratio	0.80 ± 0.02	0.90 ± 0.03	0.92 ± 0.02	0.0075	0.0172	0.0046	0.5787
Fat mass (%)	27.4 ± 2.7	39.8 ± 2.4	39.3 ± 2.4	0.0086	0.0039	0.0115	0.9397
Fat mass (kg)	16.7 ± 1.6	34.1 ± 2.4	36.4 ± 3.4	<0.0001	<0.0001	<0.0001	0.7959
Fat-free mass (%)	72.6 ± 2.7	60.2 ± 2.4	60.8 ± 2.4	0.0086	0.0039	0.0115	0.9397
Fat-free mass (kg)	45.1 ± 3.1	52.0 ± 3.2	56.2 ± 4.4	0.1071			
Bioclinical measurements							
Systolic BP (mmHg)	118 ± 4	128 ± 5	128 ± 4	0.1063			
Diastolic BP (mmHg)	75 ± 3	82 ± 2	82 ± 2	0.0664			
Total-c (mmol/L)	4.43 ± 0.30	4.26 ± 0.32	5.34 ± 0.23	0.0169	0.7054	0.0355	0.0073
LDL-c (mmol/L)	2.52 ± 0.25	2.39 ± 0.30	3.27 ± 0.19	0.0401	0.6842	0.0433	0.0232
HDL-c (mmol/L)	1.57 ± 0.08	1.17 ± 0.12	1.04 ± 0.05	0.0018	0.0256	0.0004	0.3634
Total-c/HDL ratio	2.85 ± 0.16	3.81 ± 0.22	5.17 ± 0.18	<0.0001	0.0073	0.0002	0.0010
TG (mmol/L)	0.77 ± 0.05	1.54 ± 0.33	2.26 ± 0.23	0.0004	0.0311	0.0002	0.0524
Fasting glucose (mmol/L)	4.5 ± 0.2	5.0 ± 0.1	5.3 ± 0.2	0.0243	0.0335	0.0171	0.5178
Fasting insulin (pmol/L)	37 ± 17	64 ± 9	118 ± 22	0.0025	0.0129	0.0030	0.0887
HbA1c (%)	5.37 ± 0.07	5.72 ± 0.08	5.73 ± 0.08	0.0051	0.0055	0.0066	0.8775
HOMA-IR	0.65 ± 0.28	1.18 ± 0.16	2.19 ± 0.42	0.0024	0.0143	0.0015	0.0892
HOMA%B	93.7 ± 33.1	105.9 ± 10.1	140.3 ± 17.9	0.0130	0.0337	0.0076	0.2176

Data represented as mean $\pm$ SEM. LH, lean healthy; MHO, metabolically healthy obese; MUO, metabolically unhealthy obese; BMI, body mass index; BP, blood pressure; Total-c, total-cholesterol; LDL-c, low-density lipoprotein; HDL-c, high-density lipoprotein; TG, triglycerides; HbA1c, glycosylated haemoglobin; HOMA-IR, homeostatic model assessment for insulin resistance; HOMA%B, homeostatic model assessment for β -cell function. A non-parametric ANOVA Kruskal-Wallis test followed by a post-hoc Mann-Whitney-Wilcoxon test was used to determine significance between groups ($P < 0.05$).

Table 5.2. - Mean circulating concentration of inflammatory markers

Circulating Marker	LH mean ± SEM	MHO mean ± SEM	MUO mean ± SEM	ANOVA (P-value)	Post-hoc Mann-Whitney-Wilcoxon group comparison (P-values)		
					LH vs. MHO	LH vs. MUO	MHO vs. MUO
Pro-Inflammatory markers							
hsCRP (mg/L)	0.76 ± 0.19	1.75 ± 0.45	5.35 ± 1.99	0.0018	0.1296	0.0009	0.0282
IL-6 (pg/mL)	1.04 ± 0.17	1.37 ± 0.31	2.52 ± 0.53	0.0324	0.7023	0.0169	0.0489
IFN-γ (pg/mL)	402.4 ± 14.3	446.1 ± 17.8	434.6 ± 20.3	0.2611			
IP-10 (pg/mL)	1489 ± 243	1961 ± 311	2363 ± 269	0.0741			
PDGF-ββ (pg/mL)	7373 ± 621	8751 ± 793	9933 ± 468	0.0309	0.1431	0.0089	0.2567
RANTES (pg/mL)	8803 ± 453	8825 ± 433	8292 ± 503	0.9351			
Anti-Inflammatory markers							
HMW adiponectin (ng/mL)	6914 ± 1382	2794 ± 412	2752 ± 529	0.0347	0.0288	0.0288	0.5288
IL-1Ra (pg/mL)	675.1 ± 32.0	756.8 ± 40.6	759.1 ± 29.0	0.1978			

Data represented as mean $\pm$ SEM. LH, lean healthy; MHO, metabolically healthy obese; MUO, metabolically unhealthy obese; hsCRP, high sensitivity C-reactive protein; IL-6, interleukin-6, IFN- γ , interferon γ ; IP-10, interferon- γ inducible protein 10; PDGF- $\beta\beta$, platelet-derived growth factor $\beta\beta$; RANTES, regulated upon activation normal T-cell expressed and secreted; HMW adiponectin, high molecular weight adiponectin; IL-1Ra, interleukin-1 receptor antagonist. A non-parametric ANOVA Kruskal-Wallis test followed by a post-hoc Mann-Whitney-Wilcoxon test was used to determine significance between groups ($P < 0.05$).

Table 5.3. - Mean relative percentage and absolute values of total fatty acids in serum.

(A)

Fatty Acids	LH % ± SEM	MHO % ± SEM	MUO % ± SEM	ANOVA (P-value)	Post-hoc Mann-Whitney-Wilcoxon group comparison (P-values)		
					LH vs. MHO	LH vs. MUO	MHO vs. MUO
14:0							
<i>Myristic Acid</i>	0.625 ± 0.040	0.845 ± 0.081	1.204 ± 0.109	0.0011	0.0756	0.0007	0.0257
15:0							
<i>Pentadecanoic Acid</i>	0.230 ± 0.015	0.209 ± 0.010	0.238 ± 0.010	0.1579	0.2730	0.6774	0.0493
16:0							
<i>Palmitic Acid</i>	20.25 ± 0.39	21.22 ± 0.63	22.25 ± 0.65	0.0539	0.2729	0.0211	0.1857
18:0							
<i>Stearic Acid</i>	7.718 ± 0.183	7.505 ± 0.278	6.730 ± 0.189	0.0119	0.5205	0.0058	0.0312
<i>Nonadecanoic Acid</i>	0.056 ± 0.006	0.065 ± 0.012	0.054 ± 0.006	0.0799	0.9296	0.2697	0.3268
20:0							
<i>Arachidic Acid</i>	0.291 ± 0.033	0.206 ± 0.022	0.191 ± 0.013	0.0437	0.0890	0.0172	0.5706
<i>Behenic Acid</i>	0.461 ± 0.031	0.399 ± 0.059	0.355 ± 0.044	0.3119	0.5205	0.1041	0.6232
24:0							
<i>Lignoceric Acid</i>	2.658 ± 0.312	2.373 ± 0.312	1.804 ± 0.100	0.0344	0.4274	0.0091	0.1405
14:1n-5							
<i>Myristoleic Acid</i>	0.056 ± 0.011	0.086 ± 0.014	0.112 ± 0.016	0.0092	0.0100	0.0113	0.3506
16:1n-7							
<i>Palmitoleic Acid</i>	1.565 ± 0.129	2.160 ± 0.250	2.337 ± 0.147	0.0137	0.1212	0.0022	0.4274
17:1n-7							
<i>Heptadecenoic Acid</i>	0.146 ± 0.011	0.199 ± 0.018	0.200 ± 0.009	0.0118	0.0257	0.0051	1.0000
18:1n-9							
<i>Oleic Acid</i>	19.56 ± 0.55	21.75 ± 1.25	23.76 ± 0.44	0.0052	0.3075	0.0006	0.1212
<i>Vaccenic Acid</i>	1.811 ± 0.072	1.888 ± 0.068	1.769 ± 0.048	0.6116	0.7913	0.7337	0.2730
<i>cis-Nonadecanoic Acid</i>	0.282 ± 0.057	0.275 ± 0.040	0.157 ± 0.011	0.0799	0.7913	0.0962	0.0376
22:1n-9							
<i>Erucic Acid</i>	0.561 ± 0.062	0.585 ± 0.078	0.369 ± 0.033	0.0190	1.0000	0.0172	0.0172
24:1n-9							
<i>Nervonic Acid</i>	0.072 ± 0.006	0.066 ± 0.010	0.043 ± 0.003	0.0028	0.2563	0.0015	0.0190
18:3n-3							
<i>α-Linolenic Acid</i>	0.705 ± 0.049	0.715 ± 0.056	0.953 ± 0.054	0.0057	0.9097	0.0046	0.0091
20:5n-3							
<i>Eicosapentaenoic Acid (EPA)</i>	0.955 ± 0.165	0.912 ± 0.098	0.854 ± 0.114	0.7442	0.9698	0.7913	0.3447
22:3n-3							
<i>Docosatrienoic Acid</i>	0.218 ± 0.017	0.234 ± 0.014	0.208 ± 0.013	0.4339	0.6232	0.4495	0.2263
22:5n-3							
<i>Docosapentaenoic Acid</i>	0.698 ± 0.036	0.641 ± 0.052	0.591 ± 0.029	0.2104	0.6500	0.0376	0.6232
22:6n-3							
<i>Docosahexaenoic Acid (DHA)</i>	0.775 ± 0.042	0.662 ± 0.079	0.526 ± 0.061	0.0373	0.4274	0.0091	0.1620
18:2n-6							
<i>Linoleic Acid</i>	30.46 ± 0.76	25.75 ± 0.95	26.53 ± 1.00	0.0030	0.0036	0.0046	0.6232
18:3n-6							
<i>γ-Linolenic Acid</i>	0.364 ± 0.049	0.603 ± 0.064	0.553 ± 0.041	0.0162	0.0173	0.0140	0.7054
20:2n-6							
<i>Eicosadienoic Acid</i>	0.165 ± 0.020	0.131 ± 0.016	0.127 ± 0.012	0.2470	0.1508	0.1735	0.9698
20:3n-6							
<i>Dihomo-γ-Linolenic Acid (DGLA)</i>	1.476 ± 0.141	1.795 ± 0.095	1.647 ± 0.118	0.2151	0.0890	0.4274	0.3847
20:4n-6							
<i>Arachidonic Acid (AA)</i>	7.383 ± 0.529	8.312 ± 0.662	6.076 ± 0.362	0.0242	0.3075	0.0757	0.0113
22:4n-6							
<i>Adrenic Acid</i>	0.229 ± 0.020	0.208 ± 0.027	0.154 ± 0.021	0.0964	0.5708	0.0376	0.1620
22:5n-6							
<i>Docosapentaenoic Acid</i>	0.214 ± 0.017	0.209 ± 0.014	0.183 ± 0.015	0.4921	1.0000	0.2730	0.3843

(B)

Fatty Acids	LH μg / 100 μl ± SEM	MHO μg / 100 μl ± SEM	MUO μg / 100 μl ± SEM	ANOVA (P-value)	Post-hoc Mann-Whitney-Wilcoxon group comparison (P-values)		
					LH vs. MHO	LH vs. MUO	MHO vs. MUO
14:0							
15:0							
16:0							
18:0							
19:0							
20:0							
22:0							
24:0							
14:1n-5							
16:1n-7							
17:1n-7							
18:1n-9							
18:1n-7							
19:1n-9							
22:1n-9							
24:1n-9							
18:3n-3							
20:5n-3							
22:3n-3							
22:5n-3							
22:6n-3							
18:2n-6							
18:3n-6							
20:2n-6							
20:3n-6							
20:4n-6							
22:4n-6							
22:5n-6							

Total fatty acids (FAs) are reported as (A) relative % and (B) absolute (μg / 100 μL of serum) values in lean healthy (LH), metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO) groups. A non-parametric ANOVA Kruskal-Wallis test followed by a post-hoc Mann-Whitney-Wilcoxon test was used to determine significance between groups. FAs in bold font were significant in the ANOVA test (P < 0.05).

Table 5.4 - Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) of Fatty Acid Profiles.

Dataset used for OPLS-DA	OPLS-DA Parameter			
	R^2X_{cum}	R^2Y_{cum}	Q^2Y_{cum}	CV-ANOVA
<i>Total Fatty Acids</i>				
Absolute (μg / 100 μL of serum)	0.849	0.158	0.103	0.54
Relative (%)	0.743	0.463	0.252	0.09
<i>Fatty Acids from Phospholipid and Triglyceride Fractions Only</i>				
Absolute (μg / 100 μL of serum)	0.666	0.608	0.235	0.40
Relative (%)	0.506	0.579	0.321	0.05

To distinguish the three groups, OPLS-DA analyses were conducted using different fatty acid (FA) datasets corresponding to either total serum FA profiles (expressed as either relative % or absolute values), or FA profiles from phospholipid and triglyceride fractions (expressed as either relative % or absolute values). Values for R^2X_{cum} and R^2Y_{cum} indicate the variation in the X (i.e., FAs) and Y (i.e., the three groups: LH, MHO, and MUO) parameters that are explained by the model. Q^2Y_{cum} represents the model's ability to reliably predict the Y parameter. CV-ANOVA = Analysis of Variance of Cross Validated residuals.

Table 5.5. - Mean relative percentage of phospholipid and triglyceride fatty acids in serum.

Fatty Acids		LH % ± SEM	MHO % ± SEM	MUO % ± SEM	VIP
<i>Phospholipids (PL)</i>					
14:0	<i>Myristic Acid</i>	0.35 ± 0.02	0.41 ± 0.02	0.47 ± 0.03	0.39
15:0	<i>Pentadecanoic Acid</i>	0.25 ± 0.02	0.23 ± 0.01	0.23 ± 0.01	0.10
16:0	<i>Palmitic Acid</i>	28.26 ± 0.45	28.76 ± 0.44	28.97 ± 0.27	0.54
18:0	<i>Stearic Acid</i>	15.66 ± 0.44	16.39 ± 0.44	16.52 ± 0.29	0.80
20:0	<i>Arachidic Acid</i>	0.46 ± 0.03	0.39 ± 0.02	0.35 ± 0.01	0.25
22:0	<i>Behenic Acid</i>	1.37 ± 0.06	1.05 ± 0.05	1.00 ± 0.06	0.58
24:0	<i>Lignoceric Acid</i>	1.03 ± 0.07	0.82 ± 0.08	0.74 ± 0.06	0.42
16:1n-7	<i>Palmitoleic Acid</i>	0.50 ± 0.04	0.42 ± 0.08	0.59 ± 0.06	0.47
17:1n-7	<i>Heptadecenoic Acid</i>	0.11 ± 0.03	0.10 ± 0.01	0.07 ± 0.00	0.14
18:1n-9	<i>Oleic Acid</i>	8.94 ± 0.39	8.6 ± 0.33	8.94 ± 0.31	0.33
18:1n-7	<i>Vaccenic Acid</i>	1.73 ± 0.11	1.85 ± 0.08	1.68 ± 0.08	0.46
22:1n-9	<i>Erucic Acid</i>	1.89 ± 0.21	1.04 ± 0.15	0.83 ± 0.10	1.28
24:1n-9	<i>Nervonic Acid</i>	1.50 ± 0.19	1.5 ± 0.12	1.22 ± 0.11	0.27
18:3n-3	<i>α-Linolenic Acid</i>	0.72 ± 0.09	0.49 ± 0.04	0.63 ± 0.08	0.43
20:5n-3	<i>Eicosapentaenoic Acid (EPA)</i>	0.88 ± 0.16	0.93 ± 0.07	1.21 ± 0.20	0.58
22:3n-3	<i>Docosatrienoic Acid</i>	0.33 ± 0.02	0.35 ± 0.02	0.33 ± 0.02	0.13
22:5n-3	<i>Docosapentaenoic Acid</i>	0.95 ± 0.05	0.91 ± 0.09	0.96 ± 0.05	0.15
22:6n-3	<i>Docosahexaenoic Acid (DHA)</i>	3.74 ± 0.60	3.26 ± 0.54	3.08 ± 0.35	0.51
18:2n-6	<i>Linoleic Acid</i>	18.91 ± 0.46	16.72 ± 0.71	18.44 ± 0.76	1.66
20:3n-6	<i>Dihomo-γ-Linolenic acid (DGLA)</i>	2.41 ± 0.21	3.42 ± 0.22	3.37 ± 0.20	1.12
20:4n-6	<i>Arachidonic Acid (AA)</i>	9.28 ± 0.58	11.71 ± 0.52	9.77 ± 0.54	1.97
22:4n-6	<i>Adrenic Acid</i>	0.51 ± 0.03	0.46 ± 0.02	0.38 ± 0.03	0.27
22:5n-6	<i>Docosapentaenoic Acid</i>	0.23 ± 0.02	0.22 ± 0.02	0.23 ± 0.02	0.09
<i>Triglycerides (TG)</i>					
14:0	<i>Myristic Acid</i>	1.61 ± 0.12	1.74 ± 0.14	2.39 ± 0.18	1.30
16:0	<i>Palmitic Acid</i>	27.06 ± 0.64	29.21 ± 0.67	29.70 ± 1.00	1.72
18:0	<i>Stearic Acid</i>	9.15 ± 0.64	7.86 ± 1.04	6.32 ± 0.40	1.78
16:1n-7	<i>Palmitoleic Acid</i>	3.18 ± 0.23	3.13 ± 0.49	3.20 ± 0.28	0.09
18:1n-9	<i>Oleic Acid</i>	32.16 ± 0.96	35.76 ± 0.99	35.24 ± 0.69	2.11
18:1n-7	<i>Vaccenic Acid</i>	2.23 ± 0.09	2.90 ± 0.12	2.68 ± 0.13	0.96
22:1n-9	<i>Erucic Acid</i>	5.66 ± 0.88	1.74 ± 0.36	1.94 ± 0.17	2.42
18:3n-3	<i>α-Linolenic Acid</i>	2.14 ± 0.36	1.47 ± 0.12	1.99 ± 0.13	0.87
18:2n-6	<i>Linoleic Acid</i>	14.56 ± 0.94	14.5 ± 0.59	14.88 ± 0.64	0.43
20:4n-6	<i>Arachidonic Acid (AA)</i>	1.78 ± 0.25	1.39 ± 0.17	1.28 ± 0.20	0.56

Relative fatty acid (FA) values were calculated as a % of all FAs detected and are reported as relative % FA ± SEM. The FAs in bold font have a Variable of Importance in Projection (VIP) greater than 1 (as determined with an OPLS-DA), which indicates that they are of importance when distinguishing the lean healthy (LH), metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO) groups.

Figure 5.1 - Comparison of mean fasting concentrations of inflammatory markers between groups.

(A) high sensitivity C-reactive protein (hsCRP, mg/mL), **(B)** interleukin-6 (IL-6, pg/mL), **(C)** platelet-derived growth factor $\beta\beta$ (PDGF- $\beta\beta$, pg/mL), and **(D)** high molecular weight adiponectin (HMW adiponectin, ng/mL) in lean healthy (LH), metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO) groups ($n = 10/\text{group}$). A non-parametric ANOVA Kruskal-Wallis test followed by a post-hoc Mann-Whitney-Wilcoxon test was used to determine differences between groups. Bars not sharing the same letter are statistically different ($P < 0.05$). White bars = LH; grey bars = MHO; and black bars = MUO.

Figure 5.2. - Orthogonal Projections to Latent Structures-Discriminatory Analysis (OPLS-DA) corresponding to the fatty acid profile from serum phospholipid and triglyceride fractions.

The fatty acid (FA) profiles from serum phospholipid (PL) and triglyceride (TG) fractions (expressed as relative % values) were analyzed for their ability to distinguish lean healthy (LH), metabolically healthy obese (MHO), and metabolically unhealthy obese (MUO) individuals ($n = 10/\text{group}$). The OPLS-DA parameters obtained revealed 58% of inter-group variability and 32% of prediction ability ($R^2Y_{\text{cum}}=0.58$, $Q^2Y_{\text{cum}}=0.32$, CV-ANOVA = 0.05).

Figure 5.3. - Mean relative percentage values of fatty acids identified in the serum phospholipid and triglyceride fraction.

Fatty acids (FAs) meeting a VIP > 1 in our OPLS-DA model were individually assessed between the three groups using a non-parametric ANOVA Kruskal-Wallis test followed by a post-hoc Mann-Whitney-Wilcoxon test. Bars not sharing the same letter are statistically different ($P < 0.05$). White bars = LH; grey bars = MHO; and black bars = MUO.

5.4. Discussion

The current study makes an important contribution to the growing field of research aimed at better understanding the clinical and molecular basis underlying the MHO phenotype. We have demonstrated that MHO individuals have an inflammatory state comparable to that of LH individuals. Furthermore, underlying this reduced inflammation is a distinct FA profile comprised of saturated, monounsaturated and n-6 polyunsaturated fats. Together, our results demonstrate that MHO individuals are metabolically healthier than their MUO counterparts and that the reduced inflammatory state may stem from a more favourable FA profile.

There is currently no consensus for classifying individuals as MHO [278]. We used the classification criteria initially proposed by Karelis *et al.* [276] with some minor modifications to account for known sex differences. First, we used newly proposed sex-specific cut-off values for BMI, where obesity is defined as $\geq 28 \text{ kg/m}^2$ in men and $\geq 24 \text{ kg/m}^2$ in women, rather than a general cut-off of 30 kg/m^2 [275]. These new cut-offs were found to correlate better with body fat % and thus provide a more accurate assessment of adiposity status. Second, we accounted for known sex-differences in HDL-c, where $> 1.0 \text{ mmol/L}$ for males and $> 1.3 \text{ mmol/L}$ for females were used to assess metabolic health. Finally, we ensured that study participants were not taking medications to normalize hypertriglyceridemia or type 2 diabetes, as this would have created a significant confounder. Using these classification criteria we were able to successfully distinguish LH, MHO, and MUO groups, as seen by group differences in anthropometric and biochemical measurements. Notably, the MHO group had levels of Total-c and LDL-c comparable to the LH group. Further, we found a trend for reduced measures of fasted insulin and HOMA-IR

in MHO versus MUO individuals, suggesting that the MHO group was more insulin sensitive compared to their MUO counterparts. This agrees with past studies in which glucose clamps were used to assess insulin sensitivity in these groups [272]. Collectively, these results suggest that our classification criteria successfully identified MHO individuals from within the DRA cohort.

A number of circulating anti- and pro-inflammatory markers were measured in our three groups in order to determine differences in inflammatory status. We found that circulating levels of hsCRP and IL-6 were significantly reduced in the MHO and LH groups compared to the MUO group; thus, agreeing with previous reports in the area [264,266,267]. Further, MHO subjects had intermediate levels of PDGF- $\beta\beta$ compared to the LH and MUO groups. The same trend was observed for the pro-inflammatory marker IP-10, where MHO individuals had an intermediate level compared to the LH and MUO groups. Together, our results demonstrate that MHO individuals have a reduced inflammatory status compared to their MUO counterparts. The discovery of PDGF- $\beta\beta$ is intriguing due to its suspected contribution to the development of atherogenesis [279]. This aligns with the fact that MHO and MUO subjects in our cohort were strongly distinguished by their lipid profiles (i.e., Total-c and LDL-c), which are routinely used to assess an individual's risk for atherogenesis [280]. It is therefore not surprising that PDGF- $\beta\beta$ levels could distinguish the MHO from MUO group. Further, our findings suggest that subsequent investigations of the MHO phenotype should assess hepatic function. Indeed, hsCRP is primarily produced by the liver and regulated by IL-6 [7], while this organ also plays a central role in lipoprotein production [281]. Thus, our results corroborate recent findings suggesting that studying the liver may provide further clues to help unravel the MHO phenotype [282].

Given that the MHO group was found to be more insulin sensitive (as suggested by fasted insulin and HOMA-IR values) compared to the MUO group, it was somewhat surprising to find that HMW adiponectin levels were comparable between these two groups. Adiponectin is a protein produced by adipose tissue and has known insulin-sensitizing [283] and anti-inflammatory [7] properties. Our adiponectin results are similar to those reported by Telle-Hansen *et al.*, who had an equivalently sized cohort and used similar classification criteria to identify MHO individuals [273]. However, our findings do not agree with those of other studies showing that MHO individuals had higher adiponectin levels compared to MUO individuals [284-288]. This suggests that the role of adiponectin in MHO individuals remains unclear.

Considerable evidence has demonstrated a strong relationship between FAs and inflammation [268,289]. Specifically, saturated [290] and trans [291] fats tend to be positively associated with inflammation, while monounsaturated [292] and polyunsaturated [229] fats tend to be inversely associated; however, recent evidence suggests this is overly simplified. For example, we and others have reported distinct relationships between saturated fats and inflammation, where myristic acid and palmitic acid were positively associated with inflammation, while stearic acid was inversely associated with inflammation [167,293]. In light of these recent findings we aimed to examine whether the reduced inflammatory state seen in MHO individuals compared to MUO individuals could be related to underlying differences in FA profiles. This was accomplished in two steps: (1) we analyzed total serum FA profiles, as well as FA profiles in PL and TG fractions, by discriminant analysis modelling to determine whether we could identify a panel of FAs that could differentiate the three groups; and (2) FAs that had

significant contributions (i.e., $VIP > 1$) in the discriminant analysis were examined individually to determine group differences.

In alignment with our clinical and inflammatory data, we found that MHO individuals had an intermediate level of total FAs compared to LH and MUO individuals. This reinforces the relevance of studying FA profiles in MHO individuals, as this may well contribute to their healthier metabolic phenotype. Discriminant analysis modelling was conducted to determine if serum FA profiles (either total or fractionated) could distinguish LH, MHO, and MUO individuals. The OPLS-DA scatter plot (**Figure 5.2**) revealed that the FA profile from PL and TG fractions (expressed as relative %) could distinguish the three groups better than total serum FAs. Specifically, FAs in the PL and TG fractions could explain 58% of the variability (R^2Y_{cum}) between the three groups, while the predictability of the model (Q^2Y_{cum}) was found to be 32%. This aligns with previous work by Fernández-Real *et al.*, which reported that FAs expressed as relative % values distinguished lean and obese individuals more strongly than when FAs were expressed as absolute values [270]. The outcome of our discriminant analysis modelling was also similar to that previously shown by Donovan *et al.*, who reported a similar scatter plot distribution when using FA profiling to distinguish lean from morbidly obese individuals [294]. Of interest, it was noted that while the LH group in our study was clearly distinct from both of the obese groups, the classification of MHO and MUO individuals showed some overlap. This suggests that despite having a distinct FA profile, some specific FA characteristics are shared between the MHO and MUO groups. For example, Petersson and colleagues demonstrated that a number of FAs (such as oleic acid and DGLA) associated more strongly with adiposity than inflammation [295,296]. This aligns with the results from our regression analyses,

which showed that PL-DGLA, PL-erucic acid, TG-stearic acid, TG-oleic acid, and TG-erucic acid were all associated with measures of adiposity (BMI and body fat %). Given that our MHO and MUO groups are matched for various measures of adiposity (e.g., BMI, % fat, etc), it is therefore not surprising to see a degree of overlap in their FA profiles. Nevertheless, our modelling approach led to the identification of a panel of nine FAs that was able to differentiate the three groups. This panel consisted of TG-myristic acid, TG-palmitic acid, TG-stearic acid, TG-oleic acid, TG-erucic acid, PL-linoleic acid, PL-DGLA, PL-arachidonic acid (AA), and PL-erucic acid. It is important to note that subjects were fasted for 12 hours prior to sample collection, thereby minimizing the possibility that our results reflect acute differences in dietary habits. Since the panel of nine FAs was able to distinguish the three groups, we next examined these FAs individually in order to determine if a pattern existed. Intriguingly, our data revealed that MHO individuals have a more favourable saturated FA profile compared to MUO individuals. It does not appear that monounsaturated fats play a key role in distinguishing MHO from MUO, while twenty-carbon n-6 polyunsaturated fats had a tendency to be higher in MHO compared to MUO. Together, our findings suggest that FAs may indeed be associated with the distinct inflammatory status of the MHO and MUO groups.

Concerning saturated FAs (SFA), the MHO and LH groups had similar levels of TG-myristic acid, which were significantly lower than those observed in the MUO group. This is intriguing in light of previous work by Fernández-Real *et al.* who reported a positive association between fasting serum myristic acid and IL-6 in a group of 232 adults [270]. Further, a reduction in the level of plasma TG enriched with myristic acid was previously shown to be strongly correlated with improvements in insulin sensitivity in obese subjects

during a weight-loss intervention [252]. Interestingly, we found no evidence of an association between TG-myristic acid and measures of adiposity status; suggesting that lower levels of TG-myristic acid may have a causal role in both the reduced inflammatory status and improved insulin sensitivity observed in MHO individuals. Conversely, the levels of TG-stearic acid in the LH and MHO were elevated compared to the MUO group. This agrees with previous work from our lab in which we reported an inverse relationship between circulating stearic acid levels and markers of inflammation in young lean female adults from the Toronto Nutrigenomics and Health Study cohort [239]. Taken together, the lower levels of myristic acid and the elevated levels of stearic acid in MHO individuals (compared to MUO individuals) coincide with a reduced inflammatory state and suggest that the distinct pattern of SFA seen in MHO versus MUO individuals may provide an explanation for the inflammatory characteristics of these two groups.

In MHO individuals, circulating levels of monounsaturated FAs (MUFA) TG-oleic, TG-erucic, and PL-erucic acids were similar to that observed in MUO individuals, but different from LH individuals. Oleic acid was previously shown to be elevated in obese individuals and those at increased risk of metabolic syndrome [297,298]. Moreover, detecting increased oleic acid in the TG fraction (as opposed to the PL fraction) of obese individuals agrees with previous work by Gil-Campos et al. [298]. In comparison, the similar levels of erucic acid detected in MHO and MUO individuals were surprising in light of a recent study showing that elevated plasma levels of PL-erucic acid were associated with an increased risk of heart failure [299]. However, investigations of erucic acid in humans are limited [300] and it is, therefore, difficult to explain our data showing significant reductions in erucic acid (in both the TG and PL fractions) in MHO and MUO individuals compared to LH

individuals. Considering the improved lipid status of MHO individuals compared to MUO individuals, we would have expected a difference in erucic acid levels that would reflect the varying risk for cardiovascular complications. This suggests that further studies examining erucic acid are required, as current evidence is scarce and we presently possess only a limited understanding of its role in metabolism and obesity.

The levels of DGLA and AA, two n-6 polyunsaturated FAs (PUFA), were found to differ in the PL fraction between the three groups, with LH individuals having the lowest levels of both. The higher level of PL-DGLA seen in both obese groups agrees with recent work showing a positive association between BMI and DGLA [301]. Interestingly, we also found that MHO individuals had significantly higher levels of PL-AA compared to both LH and MUO individuals. While the elevated level of PL-AA in the MHO group coincides with past work demonstrating a positive association between AA and BMI, the equivalent levels of AA in the LH and MUO were surprising and warrant further investigation [301]. Moreover, the reason for the distinct pattern of these two n-6 PUFA is unclear; however, it is tempting to speculate that this may alter the balance of pro- and anti-inflammatory eicosanoids. Indeed, while DGLA is a precursor for anti-inflammatory series-1 eicosanoids, AA is a precursor for pro-inflammatory series-2 eicosanoids [302]. Future work will help clarify how this distinct pattern of n-6 PUFA influences eicosanoid biosynthesis, and whether this is an additional mechanism by which inflammation is reduced in MHO individuals.

Our understanding of the metabolic basis for MHO remains in its infancy. However, the current study has shed light on this complex area by showing that this distinct subgroup of obese individuals has a more favourable FA profile compared to their MUO

counterparts. While the current study has focused on the two dominant lipid fractions in serum (i.e., PL and TG), the interesting results presented here lend strong support to conduct a more comprehensive lipidomic analysis to examine other lipid fractions (e.g., non-esterified fatty acids, cholesterol esters, eicosanoids, etc.) that may provide additional clues to help unravel the MHO phenotype. Due to the known links between FAs and inflammation, it is intriguing to postulate that tailoring recommendations regarding dietary fat intake (e.g., increasing the consumption of foods rich in stearic acid) may help to prevent the downstream metabolic consequences associated with obesity-related inflammation. This work reinforces that continued efforts in this area are necessary in order to elucidate how the distinct FA profile of MHO individuals contributes to their reduced inflammatory status.

5.5. Acknowledgements

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Chapter 6. Integrative Conclusion

Current methods to predict T2D risk use total blood TG levels as a measure of metabolic health. However, recent studies suggest that a more accurate means to predict individuals at risk of T2D might be gained by investigating distinct FAs within the TG fraction rather than total TGs [10,303]. This may be explained, in part, by evidence which suggests that the strength of the association between total TG levels and markers of IR varies between individuals of different sex and ethnicity [304]. As such, the overall hypothesis of this thesis was that distinct FAs within specific blood lipid fractions are more robustly associated with markers of IR compared to total blood FAs and TG levels. This was seen as an important first step towards uncovering a panel of FAs that could be positioned as potential biomarkers to more accurately predict T2D risk. Over the course of three studies, the overall hypothesis of this thesis was supported and demonstrated that a more accurate method to predict risk of T2D can be obtained by investigating individual FAs in specific blood lipid fractions.

6.1. Research Summary

Over the course of three studies, I have demonstrated that the strength of associations between markers of IR and total lipid concentrations, in particular total TG, differs between groups of individuals. Accordingly, the work presented in Chapter 3 demonstrated, for the first time, that when ethnicity and sex are examined in tandem that each can independently influence associations between individual FA levels and markers of IR. Indeed, associations between distinct FAs and markers of IR were uncovered that were not reflected in other cohorts. For example, the association between *trans*-vaccenic acid

(18:1 t11) and HOMA-IR, while seemingly valuable for predicting IR risk in Caucasians, may not be as relevant in T2D predictive models for East and South Asians. This is important because it encourages future FA investigations to account for ethnicity and sex, and suggests that models aimed at predicting T2D risk should do the same. However, it must be acknowledged that this population was composed of young healthy individuals that show a limited degree of variability in insulin sensitivity relative to individuals who would be considered “pre-diabetics”. Therefore, despite this study demonstrating that ethnicity and sex influence FA associations with markers of IR, it must be acknowledged that subsequent studies should be undertaken that investigate the FA profiles of multiple ethnicities across a larger range of IR. In doing so, additional associations may be uncovered that did not surface in our healthy population. Accordingly, the study presented in Chapter 4 acknowledged these limitations and aimed to investigate FA associations with IR in a cohort comprised of individuals varying in glucose homeostasis and of a single sex and ethnicity.

A cohort comprised of individuals from one ethnicity and sex (i.e., Caucasian men) were investigated. Furthermore, this population presented a greater degree of variability with regards to IR parameters; thereby allowing for a comparison of total lipid and FA profiles in healthy and “at-risk” individuals. Since total blood TG levels are routinely used to assess metabolic health [1], the TG fraction was selected as the lipid of interest and the abundance of individual FAs in TGs were determined. Despite the inability of total TG levels to distinguish NGI from HGI individuals, 18:1c11 in the TG fraction was significantly and positively associated with both fasting insulin and HOMA-IR values. This discovery was interesting for two reasons. First, although the present study did not distinguish *cis* and

trans FA isoforms, it does support the findings from Chapter 3, which uncovered a significant association between 18:1t11 and HOMA-IR. Since the population sizes studied in Chapters 3 ($n = 461$) and 4 ($n = 20$) were quite different, it is difficult to conclude if the stronger association uncovered in Chapter 3 between HOMA-IR and 18:1t11 ($P = 0.0006$), relative to Chapter 4 ($P = 0.010$), is a reflection of the larger sample size or if HOMA-IR is simply more strongly associated with the 18:1c11. As such, a future study should consider comparing the associations between total 18:1c/t11, 18:1c11, and 18:1t11 with HOMA-IR in order to determine if any one associates more strongly with markers of IR. The second, and key finding of this chapter, was that an individual FA (specifically, 18:1c11) associated with markers of IR (i.e., HOMA-IR and fasting insulin) more strongly than total lipid concentrations (i.e., TG). This confirmed the second hypothesis of the thesis and, in doing so, elected a single FA within the TG fraction as a key FA of interest for distinguishing NGI and HGI individuals varying in insulin sensitivity.

Having supported my first two hypotheses, my final hypothesis required: (1) a direct comparison of the two FA extraction methods used in the previous chapters (i.e., total FA and fractionated FA) and (2) an examination of individuals ranging in insulin sensitivity. To accomplish this, a new cohort was established to permit the comparison of FA profiles between distinct groups of individuals varying in adiposity and metabolic health. Over the course of three years, the Diabetes Risk Assessment (DRA) study was conducted and a final study population of 75 participants was categorized and investigated. In light of the associations between adiposity, inflammation, and metabolic health, 3 study groups were defined that permitted me to examine the ability of fasted serum FAs to distinguish individuals differing in adiposity and metabolic health: lean healthy (LH, $n=10$),

metabolically healthy obese (MHO, n = 10), and the metabolically unhealthy obese (MUO, n = 10). A comparison of total and fractionated FA profiles, composed of FAs measured in PL and TG fractions, demonstrated that the fractionated FA profile distinguished the three groups more robustly than total FAs. This demonstrated that FAs within specific lipid fractions (i.e., PL and TG) can better distinguish metabolically healthy and unhealthy individuals than total FAs. Upon doing so, final hypothesis was confirmed. Moreover, the findings of Chapter 5 suggest that FA profiles of the PL and TG lipid fractions might provide a viable means by which to assess and predict an individual's risk of impaired metabolic health (i.e., IR and T2D). Although the present study targeted the FA profiles of the PL and TG fractions due to their abundance in blood, and well-documented association with IR and T2D, future investigations should consider examining other lipid fractions as well as they may provide additional information to better distinguish metabolically healthy from unhealthy individuals.

6.2. Future Directions

The findings of this thesis are encouraging as they suggest that a better understanding of the dietary habits and biological mechanisms contributing to T2D can be obtained by investigating distinct FAs within lipid fractions. Furthermore, this suggests that a more accurate and informative diagnostic method for assessing an individual's risk of T2D can be developed.

The development of T2D is known to involve a number of tissues, such as adipose, liver, and skeletal muscle. Moreover, it has become increasingly apparent that circulating lipids, such as TGs, are associated with T2D risk. However, while total TG levels are

associated with T2D risk, this thesis demonstrated that an even greater degree of understanding can be obtained by investigating the FAs within the TG and PL lipid fractions. FA profiling can provide a wealth of information and allow researchers to uncover stronger associations between FAs and T2D and obtain a better understanding of the mechanisms by which FAs influence IR and T2D. Indeed, the role of 18:1 c/t11 in the TG fraction was underlined in the present thesis as a FA of particular interest; and yet, the influence of 18:1 c/t11 towards IR is currently poorly understood. This suggests that future studies using lipidomic and FA profiling techniques could uncover other unique relationships between FAs and markers of IR, and reveal novel areas of research, so that we can ultimately better understand the progression of IR and T2D.

Current clinical practice requires the use of numerous diagnostic criteria in order to predict an individual's progression towards T2D; however, assessing these diagnostic criteria can be a time-consuming (e.g., OGTT and repeated measures) and, therefore, costly procedure. However, a number of FAs uncovered by lipidomics and FA profiling investigations have demonstrated a robust ability to distinguish individuals varying in adiposity, IR-status, and glycaemic and insulinaemic response to a meal challenge. This suggests that by harnessing distinct FAs as clinical biomarkers that an alternative method can be developed that will provide a more accurate method of detection and reduce the need for costly and time-consuming clinical examinations. Furthermore, despite the ability of current clinical methods to classify an individual as either healthy or T2D, these assessments do not present additional information that could be used to tailor an individual's method of treatment. For example, elevated fasting glucose levels can indicate an individual's risk of developing T2D; however, it cannot suggest if the elevated glucose

levels are a result of reduced skeletal muscle metabolism or adipose tissue inflammation, or a combination of both. Conversely, the contribution of numerous FAs towards T2D appears to provide specific information regarding a particular pathway within tissues. Such an understanding could be used to develop a targeted treatment option for at-risk individuals that are designed to address the specific pathways underlying their elevated risk. This could ultimately lead to an improvement in T2D prevention and treatment success.

6.3. Concluding Remarks

In closing, this thesis has demonstrated that distinct FAs within specific lipid fractions of fasted serum are more robustly associated with IR than measures of total FAs or total TG levels. Additionally, the work undertaken in the final study demonstrated that FA profiles can distinguish between individuals at varying degrees of IR and T2D risk. This is encouraging, as it suggests that FA profiles may be used in the future to not only monitor an individual's progression towards T2D, but also their response to treatment. Collectively, these studies and their findings are anticipated to encourage investigators to examine individual FAs within circulating lipid fractions in order to gain a better understanding of how differences in circulating FA levels are associated with and contribute to IR and T2D.

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Appendix A. Rationale and Objective

In preparation for the development of the study cohort presented in chapter 5 (i.e., the DRA study cohort), which aimed to use FA profiling techniques to uncover associations between FAs and markers of metabolic health, a literary search was conducted. The purpose of the search was to gain an appreciation of potential technical confounders that could inadvertently influence and skew sensitive metabolomics- and lipidomics-based data in human nutritional research studies. By having a better understanding of these confounders, I was better prepared to organize and manage the DRA study such that the influence of confounders were minimized (e.g., standardized pre-study meals). In doing so, the biological integrity of study samples was maintained while the influences from latent technical variables were reduced. This allowed for the biological associations of interest (e.g., FAs and markers of IR) to be more readily recognized. A summary of the literary search was written and published with the intent of assisting other investigators aiming to harness metabolomics- and lipidomics-based methods in nutritional research.

Appendix A: Harnessing Metabolomics for Nutritional Research

Zulyniak MA* and Mutch DM. Harnessing Metabolomics for Nutritional Research. *Current Pharmaceutical Biotechnology*. 2011, 12(7). 1005-1015.

Abstract

Comprehensive analytical technologies are rapidly becoming a cornerstone of modern nutritional sciences. Two of these technologies, mass spectrometry (MS) and nuclear magnetic resonance (NMR), have proven highly informative for the global analysis of metabolites, commonly referred to as metabolomics. Metabolomics provides a powerful approach to study small molecules in order to better understand the implications and subtle perturbations in metabolism triggered by nutrients. By studying how dietary molecules can modulate the metabolome, researchers have begun to elucidate the molecular pathways by which nutrients affect health and disease, expand the current state of knowledge regarding how inter-individual variability contributes to differences in nutrient metabolism, and develop novel avenues of research for nutritional sciences. Although metabolomics has been more commonly used to study disease states, its use in the nutritional sciences is gaining momentum. The current review is written for the clinical researcher wishing to incorporate metabolomics in dietary intervention studies. This review will highlight the importance and benefit of identifying biomarkers that accurately reflect changes in nutrient intake and metabolism, and present numerous issues that can introduce variability into a dataset and confound a study's biological interpretation, including: sample population demographics, the biological specimen selected, diurnal variation, collection methods, and sample storage parameters. Considering these important

areas at the experimental design stage will ensure that metabolomics provides a comprehensive and accurate assessment of the molecular impact of a dietary intervention.

Introduction

The field of nutritional sciences has embraced the technologies of the post-genomic era, leading to pioneering avenues of research that have begun to delineate how diet can influence health and disease. While the notion that diet affects health is long-standing, it is only in the past twenty years that diet has been comprehensively investigated at the molecular level. Indeed, the impact of nutrients has been studied at all levels of biological complexity, from genes to proteins to metabolites, in order to better understand their effect on phenotype. Several distinct models have been proposed to describe the various relationships that exist between diet and genes, and their ability to modulate disease risk factors; thereby highlighting the complexity that exists when studying the influence of dietary interventions on phenotype [305]. The recognized importance of diet-gene interactions has positioned nutrigenomics as a critical facet of modern nutritional sciences. It is now widely accepted that diet-gene interactions can be bi-directional: genetic variants can affect nutrient metabolism (often referred to as *nutrigenetics*), and nutrients/diets can alter gene expression, protein translation, and metabolism (commonly referred to as *nutrigenomics*) [306-311]. Each 'direction' contributes to the expressed phenotype and underlies the inter-individual variability that challenges effective health management.

The decreased costs of the analytical technologies used for the global analysis of genes (genomics), gene expression (transcriptomics), proteins (proteomics), and metabolites (metabolomics) coupled with our improved ability to manage and interpret the

large datasets generated by these tools has had several important implications for modern nutritional research. Firstly, intervention studies can now incorporate 'omic technologies to improve our understanding of how macronutrients and micronutrients can affect cell and tissue biochemistry. Secondly, the size of study cohorts can expand from tens of subjects to hundreds and thousands of participants. Using these larger study cohorts subsequently leads to more robust and accurate data regarding the impact of a given dietary intervention. Thirdly, population cohorts can now be segregated *a priori*, using genetic and/or molecular information, in order to better understand the different responses that people have to a particular intervention. Such advances have enabled unique nutrient-phenotype differences within defined populations (i.e. different ethnicities, diseases vs. healthy, treated vs. untreated, etc.) to be recognized, and has fueled optimism that the concept of personalized nutrition to optimize health management may be attainable. Although our understanding of the biological complexity underlying nutrient absorption, distribution, metabolism, and the numerous gene-gene / gene-environment interactions influencing these parameters remains far from complete, important progress has been made. For example, Vitamin C is an essential nutrient linked to cardiovascular disease and cancer prevention that presents considerable variability in absorption between populations and only modest correlations between individuals within a population [312-315]. In other words, nutrient absorption is not predictable and the many inter-individual factors (e.g. genetics, diet, and environment) associated with ethnic- and geographically-distinct groups can influence this process. Understanding these population differences often demands a global context of study, as the human body is an extensive and complex biological network.

While genomics and transcriptomics have been widely used to improve our understanding of nutrient bioactivity, metabolomics is an established platform that has been, to date, used comparatively less within the nutritional sciences [9]. The global study of metabolites provides unique information in the quest to unravel how diet affects human health and disease [316-318]. Metabolites represent a metabolic endpoint of gene and protein function; hence perturbations that affect gene and protein function can alter metabolite abundance. Much like a genome, a metabolome is thought to be species-specific and personally unique [319]. Alterations at the metabolite level integrate and reflect changes in genes and proteins, as well as numerous influences generally considered as ‘environmental factors’ (e.g. cultural, geographical, and lifestyle) [320]. Holmes and colleagues reinforced this concept by demonstrating that the metabolic phenotype, i.e. the metabotype, of four distinct populations could be differentiated following the analysis of urine metabolites [321]. For the field of nutrition, metabolomics provides a powerful approach to understand how cultural and geographical differences in dietary habits can affect the metabotype, and subsequently disease risk. Emerging research suggests that the study of metabolite profiles can provide important basic information related to a particular physiological state while simultaneously identifying novel biomarkers of potential clinical relevance [320,322,323]. Because diet is a complex mixture that varies in macronutrient and micronutrient content both within and between populations, the ability to identify metabolite markers that either directly or indirectly provide accurate information regarding nutrient intake is desirable, yet highly challenging. If we consider our previous example regarding Vitamin C, the correlation between vitamin C intake and its metabolite concentrations varies considerably in individuals within a given population ($r \sim 0.40$) and

between different populations (India and Spain present r -values of 0.12 and 0.53, respectively) [315]. Thus overcoming challenges such as this will be paramount for understanding the role that nutrients have on an individual's health and risk for disease.

The current review is directed to clinical researchers and nutritionists who are considering using metabolomics to expand and develop future areas of exploration and discovery in their nutrition programs. The goal of this article is not to review the analytical technologies and bioinformatics that are integral to metabolomics, but rather to focus on the numerous sources of variability that can influence nutrition-based research prior to analysis and interpretation. Several areas of particular relevance will be addressed, including issues related to population demographics, selection of biological fluid specimen, diurnal variation, collection methods and sample storage. Knowledge and appreciation of these concepts prior to commencing a large-scale diet intervention study will help ensure that metabolomics provides clinical researchers with robust and relevant information.

I. Identification of Nutritional Biomarkers

The relationship between diet and health has long been recognized; however, it is only recently that the field of nutritional sciences has incorporated modern molecular biology and biochemistry technologies to decipher the molecular mechanisms by which nutrients affect health. Dietary recommendations and prevention strategies for most diseases are, by and large, homogenous: increased fruit, vegetable, whole-grain, and fibre consumption and limited intake of salt, alcohol, fats, and refined foods [324-327]. Although invaluable for improving mass-population health (e.g. as seen with the *Canada Food Guide* and *Dietary Guidelines for Americans*), understanding the molecular mechanisms influenced

by these dietary interventions will be constructive for individuals and population subsets with unique health and dietary considerations [328-331]. Metabolomics provides one approach that will help guide improvements for the personalization of health maintenance and disease prevention in accordance to individual- and/or ethnic-specific requirements. Metabolite analysis is typically undertaken using one of two investigative approaches: *metabolic fingerprinting* or *targeted metabolite analysis* (**Figure 1**). *Metabolic fingerprinting* typically refers to the global unbiased scan of the metabolome with the purpose of discovering a metabolite profile (i.e. metabotype) that distinguishes a group of interest (e.g. diseased vs. healthy, treated vs. untreated, etc.) from another [319,332,333]. An alternative approach for metabolite investigation is *targeted analysis*, which focuses on the analysis of a defined subset of metabolites. These metabolites may share a common biochemical pathway or be chemically related (e.g. lipids, carbohydrates, etc.). Such targeted analyses provide a means to acquire a deeper understanding of the molecular events that underlie changes in phenotype [319,332,333]. Although these approaches will provide distinct molecular information, they are both capable of identifying potential biomarkers of clinical and/or diagnostic relevance.

The goal for the discovery of a valid, relevant, and informative biomarker is that it provides insight that integrates numerous biological and environmental determinants. For the field of nutrition, biomarker discovery is challenged in particular because of diet complexity and geographic differences in foods. As such, it is entirely possible that a biomarker validated in one population may not work in another. For example, carotenoids are used as a blood biomarker for dietary fruit and vegetable consumption; however, in order to detect carotenoids in blood, they must be solubilized and absorbed from the

Appendix A, Figure 1 - A metabolomic approach of study: targeted profiling and untargeted global analysis. Targeted metabolite profiling sets out to gain a greater understanding of a biological pathway or class by quantifying a distinct selection of metabolites. Alternatively, untargeted metabolite analysis is used to identify a metabolic fingerprint that distinguishes two groups and which may eventually be implemented in clinical diagnosis.

intestine [334]. It has been noted that carotenoids range in absorption efficiencies and bioavailability between individuals by 10-50%. This is due to numerous factors, such as total dietary fat content and composition (which can affect the solubilization of foods), genetic variability in genes coding for gut transporters, and menstrual and hormonal cyclical activity [335-337]. Unlu *et al.* found that foods with a greater lipid content, such as

avocados, can alter carotenoid absorption [338]; suggesting that blood carotenoid levels may provide an indication of an individual's dietary habits and provide a relative estimate of diet lipid content. Therefore, an accurate and reproducible means by which to monitor dietary habits, food preferences, and different absorption efficiencies between individuals and ethnicities represents a highly desirable goal that will assist in our study of the relationship between diet and health. Metabolomics provides one approach by which biomarkers reflecting one or more of these endpoints can be identified. With thousands of metabolites already identified in the Human Metabolite Database (HMDB) and an estimated 200,000 more potentially ingested as plant matter [339], one should be optimistic that biomarkers can and will be identified in order to obtain objective information about an individual's dietary habits. Food frequency questionnaires (FFQ) are routinely used in large-scale intervention studies to gather information regarding an individual's lifestyle; however, ensuring objectivity with FFQs is difficult and there is a considerable degree of subjectivity when one assesses their own lifestyle habits. Incorporating nutritional biomarkers into intervention studies will provide an objective mean by which to more accurately define an individual's baseline prior to commencing a dietary intervention, as well as monitor compliance throughout a study.

II. Analytical Platforms

The global analysis of metabolites requires analytical platforms of high sensitivity and discriminatory qualities. The two technologies most commonly used for metabolite detection and studies are: (i) nuclear magnetic resonance (NMR) and (ii) mass spectrometry (MS) coupled with chromatography - commonly gas (GC) or high-

performance liquid chromatography (HPLC). While these methods are both integral for metabolomics-based research, they vary considerably with regards to sample preparation, data collection and metabolite identification capabilities. As such, each platform produces information that is complementary to the other. The choice of analytical platform is entirely dependent on the study goals and the type of information (i.e. metabolic fingerprint vs. pathway or related metabolites) desired by the researcher. Excellent reviews already exist outlining the strengths and applications of each analytical platform [340,341]. As such, we will only provide a brief overview of the two platforms so that researchers new to metabolomics can appreciate the studies discussed in the next section and recognize the sensitivity and strengths of these analytical techniques.

NMR involves subjecting metabolites within a magnetic field to radio frequency pulses, thereby inducing an atomic nuclear spin that emits a distinct quantity of energy corresponding to a particular analyte within a sample [342,343]. Predominantly used for the detection of abundant metabolites ($\geq 1\mu\text{g/mL}$), which corresponds to $\sim 10\%$ of the human metabolome [344,345], NMR analysis can identify up to several hundred metabolites in a single sample. Moreover, NMR is typically used to discover a metabolite fingerprint that distinguishes two experimental conditions or populations [316]. Perhaps one of the most significant advantages of NMR is that biological fluids do not need to be processed (e.g. deproteinized) prior to analysis [344,346,347], which minimizes sample manipulation and provides less opportunity for human error.

MS coupled with GC or HPLC is an alternate analytical method for the study of metabolite profiles [343]. This method requires that biological samples undergo deproteinization, derivatization and volatilization (GC) prior to their analysis. It is

important to recognize that various methods exist for sample preparation and that each method will alter both the number and identity of detected metabolites [348,349]. Preceded by chromatographic separation, MS can discern molecules less than 1 Da with an accuracy of less than 1 part per million (ppm) [342,350-352]. A high accuracy for minute masses is required to ensure definitive detection and quantification of metabolites, both large (>1000 Da) and small (< 50 Da), and any changes in concentration [352,353]. In contrast with NMR, MS is capable of identifying several thousand metabolites in a single biological sample [348].

NMR and MS are complimentary tools for metabolomics: NMR allows researchers to construct metabolite fingerprints of common biological fluids with little preparation while MS can quantify a vast number of targeted metabolites of varying size and abundance. Because each platform provides distinct information, the notion of using both analytical platforms in a single study is attractive. A recent example in nutrition coupled NMR and MS in order to study the effects of a short-term, daily consumption of chocolate [354]. The authors used NMR to identify distinct metabolic fingerprints between control and chocolate-consuming individuals and MS provided quantitative data for 148 metabolites [354]. This is but one example demonstrating that using both analytical approaches within a single study can increase the number of detected and quantified metabolites, as well as provide a more comprehensive understanding of the metabolic response to a particular intervention.

Finally, it is paramount to recognize that statistics and bioinformatics are a crucial component of metabolomics research and will vary depending on the analytical platform utilized; however, a thorough discussion of the various tools is beyond the scope of the

present manuscript. Readers interested in learning more about the bioinformatics tools employed in metabolomics research are encouraged to read recent articles by Wishart [355] and Issaq et al. [340].

III. Sources of Variability Relevant for Nutrition-based Metabolomics

In recent years, metabolomics has garnered more attention in nutritional sciences. Examples now exist in areas related to food and metabolism, such as the study of nutrient bioactivity [356,357], food preferences [358], food authenticity [359,360], and dietary interventions [354,361]. The emergence of nutritional metabolomics has benefited from initiatives to standardize study designs, sample acquisition, data analysis, and statistical analyses [362,363]. Establishing these initiatives has coincided with our improved understanding of the numerous sources of variability that can influence metabolomic datasets. For example, factors such as population demographics, the biological specimen selected, the time of day when samples are collected, sample collection methods, and sample storage temperatures are all able to independently affect biofluid metabolite concentrations; thereby leading to increased data variability. While impossible to account for all variables (i.e. many variables may yet be unknown or "uncontrollable") it remains important to recognize and consider these variables when establishing an experimental design. Failure to do so can influence results and potentially lead to an inappropriate interpretation of the data [313,364]. Therefore, the following sections will highlight recent knowledge that is pertinent for nutritional metabolomics (summarized in **Table 1**).

Appendix A, Table 1 - Identified procedural events with potential for inducing unintended variability amongst samples

Procedure	Source Of Variability	Consideration	References
Population Demographics	Geography, ethnicity, and lifestyle	Geographic and ethnic homogeneous study participants.	[365]
Standardized Diets	Varying dietary habits	24-hour standardized diet prior to sample collection.	[366]
Diurnal Variation	Circadian rhythm and heterogeneous metabolic rates	Definitive collection time point and protocols that are standardized.	[367,368]
Sample Selection	Selection of biofluid for analysis	If chronic intervention (i.e. > 7 days), plasma or serum are typically used. If acute (i.e. ≤ 7 days), urine may be ideal for metabolite detection.	[369-371]
Sample Collection	Preservatives and non-homogenous anticoagulants and vacutainers	Preservatives assist with urine metabolite preservation. Collect blood samples in vacutainers from a single batch and manufacturer.	[365,372-374]
Storage	Temperature, duration, freeze-thaw cycling and exposure to non-storage conditions	Store samples at temperatures less than -20°C. Minimize freeze-thaw cycling. Randomized Autosampler positioning.	[364,372,375]

IIIa. Population Demographics

The inter-individual variability that exists in metabolite profiles is a result of genetic and environmental factors. This inter-individual variability was found to be the most important factor to affect metabolomic datasets, in comparison to the numerous influences related to sample collection, treatment, and storage (concepts discussed in the following

sections) [365]. This implies that lifestyle habits are most likely driving this variability. Studies by Holmes *et al.* and Assfalg *et al.* further demonstrated that metabolic phenotypes differed amongst populations [321] and between individuals [376], respectively.

Holmes *et al.* recruited 4630 participants from across four populations (China, Japan, United Kingdom, and the United States) and profiled metabolites with ^1H -NMR and 24-hour urine specimens [321]. The authors were able to distinguish each of these four populations. Moreover, they were able to differentiate native Japanese participants from those who had immigrated to the United States. The metabolites that distinguished these various groups, such as amino acids, creatine and trimethylamine-N-oxide, are predominantly of dietary origin; suggesting that dietary habits have a dominant effect on the daily urine metabolome over genetic factors. The identification of these metabolites provides important information that can be used to unravel how lifestyle differences between populations may alter disease susceptibility.

Taking this concept one step further, Assfalg *et al.* demonstrated that as few as 12 metabolites could be used to characterize and distinguish individuals from one another [376]. Recruiting 22 healthy subjects from the same work environment in Italy, the authors used ^1H -NMR to profile urine metabolites over a three-month period. They demonstrated that each participant was defined by a distinct metabolite fingerprint, and suggested that such information could be used to develop more personalized interventions. Furthermore, this individual metabolite fingerprint was found to be stable during the study period. While it is important to note that the authors based their conclusions on data from a small study (only 22 subjects), their results nevertheless suggest that it may be possible to distinguish individuals in large population studies; however, it will most likely require a more

extensive panel of metabolites to do so. Recently, Bernini and colleagues followed up this study with a long-term analysis and revealed that an individual's metabolite fingerprint can be divided into two fractions: a stable core that appears constant and a dynamic core that reflects alterations in lifestyle [377]. Analyzing urine metabolite profiles from adults at various time points over a three-year period was sufficient to accurately and consistently distinguish participants, suggesting that the adult metabolome may be relatively stable. However, the authors also demonstrated that ^1H -NMR profiles can reveal short-term changes in the metabolome. These changes are most likely attributed to lifestyle alterations, such as diet and exercise, as well as alterations to gut microbiota. Indeed, recent evidence demonstrates that gut bacteria have a significant impact on the host metabolome [378].

Taken together, these studies indicate important caveats for nutritional metabolomics research. Although inter-individual variability in metabolite profiles will continue to confound future metabolomics-based research, accounting for geographic location and ethnicity will help to homogenize the dataset by minimizing the influence of distinct dietary habits. Furthermore, recognizing the existence of stable metabolites, resistant to change over time, is intriguing. The challenge, however, will be to distinguish these stable metabolites from those that are sensitive to dietary intervention. As such, a single metabolomic “snap-shot” may be insufficient to confidently assess the influence of a dietary intervention on whole-body metabolism. Rather, assessing a particular diet will require the analysis of multiple samples taken throughout the experimental period; thus enabling the researcher to reduce the number of false-positives and differentiate the stable core of metabolites from those that are sensitive to an intervention.

IIIb. Standardizing Diet

Although accounting for geographic location and ethnicity will help to minimize variability in metabolomic datasets, it is evident that the dietary habits of any two individuals from the same region can vary considerably. As such, these inter-individual differences in lifestyle will generate significant noise in metabolomic datasets that may mask changes associated with a dietary intervention. In order to circumvent this important confounder, it is highly advantageous to standardize the diet prior to commencing a study protocol.

One of the first examples of diet standardization in nutritional metabolomics was performed by Solanky *et al.* prior to the study of soy isoflavones [366]. Over two complete menstrual cycles, diet and exercise were closely monitored in premenopausal women. During the second month, all meals were prepared and provided for each subject, thereby ensuring that all subjects had a similar intake of dietary soy. Urine samples were collected over a 24-hour period and analyzed by ^1H -NMR. It was found that dietary soy affects the abundance of metabolites in several biological pathways, including energy metabolism. While the authors did not examine the impact of diet standardization *per se*, this study suggests that standardizing the diet may help the researcher identify subtle influences of dietary molecules that are often masked by inter-individual variability.

Examining the metabolite profiles of serum, urine, and saliva following diet standardization has demonstrated a reduction in the variability seen between study participants. Walsh and colleagues recruited 30 healthy individuals to provide plasma, urine, and saliva samples on four separate mornings [379]. The goal of this study was to demonstrate whether diet standardization would reduce intra- and inter-individual

variability in various biofluids. For the first two visits, participants were asked to carry out their normal daily routine. On study visit 3, subjects were asked to reproduce exactly what they did prior to study 2, thereby assessing *intra*-individual variability and in preparation for study visit 4, participants were provided with a standardized diet for the 24-period preceding the visit, thereby assessing *inter*-individual variability. The authors showed that intra-individual variability was consistently lower than inter-individual variability. Surprisingly, only the urine metabolite profile responded to diet standardization. Inter-individual variability in the urine metabolome was reduced following diet standardization, whereas that of plasma and saliva was not. This suggests that the urine metabolome may be the most appropriate biofluid to examine following an acute dietary intervention.

Another study, by Winnike *et al.*, demonstrated that prolonged diet standardization terms (2 weeks) did not further reduce inter-individual variability in serum and urine samples [380]. The authors did not detect a benefit of diet standardization on the urine metabolome; however, it should be noted that the two studies differed in urine collection timing - Winnike *et al.* collected over a 24-hour period while Walsh *et al.* collected urine after an overnight fast. This suggests that a 24-hour collection period may introduce noise into urinary metabolite profiles that can dilute the subtle effects of an acute dietary intervention. Furthermore, Winnike *et al.* found an effect of diet standardization in serum samples for approximately half of their participants, which contrasts with the findings by Walsh *et al.* A further difference between the two studies was that Winnike *et al.* used an inpatient study design in contrast to the free-living model used by Walsh *et al.* It is possible that an inpatient study design diminished the influence of certain currently unrecognized environmental variables. Furthermore, Winnike *et al.* permitted study participants to

drink water, tea, and decaffeinated coffee *ad libitum*. While details of beverage consumption in the Walsh *et al.* study are not clearly indicated, this may have also contributed to the discrepancies between the two studies. Indeed, a previous report showed that water, tea, and coffee have differential effects on the human urine metabolite profile [381].

Taken together, these studies demonstrate the benefit of diet standardization on metabolite profiles and, importantly, suggest that there is no additional benefit when standardizing diet for more than 24 hours. This is highly advantageous for future dietary intervention studies, since the ability to standardize diets and ensure that participants adhere to experimental protocols represents a major challenge. Participants are more likely to adhere to a standardized protocol that lasts only 24 hours versus a longer term. This will ultimately lead to improvements in participant compliance and data quality. Such knowledge is of particular importance as the prevalence of large-scale intervention studies (such as the European Framework Programs NUGENOB and DiOGenes) begin to incorporate metabolomics for the study of dietary interventions.

IIIc. Diurnal Variation

An additional source of variation that is highly relevant for intervention studies is diurnal variation. Park *et al.* identified three phases in plasma metabolite profiles that corresponded to morning, afternoon, and night [382]. Furthermore, the inter-individual differences in digestion, nutrient absorption, and metabolism introduced additional variability related to these three phases, in which lipid and amino acid levels were found to vary significantly.

To study the influence of circadian rhythm on the metabolome, Minami *et al.* analyzed mice with varied genetic background, sex, age, meal times, light conditions and those subjected to "jet lagging" conditions [367]. Capillary electrophoresis and MS analysis identified 28 metabolites oscillating in significant accordance with time of day and provided a predictability of chronological time upwards of 0.6 ± 0.29 hours [367]. Maher *et al.* noted that human urine samples collected from a single subject less than 2 hours apart varied significantly in the abundance of glucose, insulin, lipid and free-radical levels [368]. As such, Maher *et al.* recommended collecting samples at a well-defined time-point or precise event (e.g. 10 minutes after completing breakfast) to assist in minimizing the influences of circadian rhythm and unintended metabolome variance [368]. Slupsky *et al.* also found that metabolite profiles change from morning to afternoon and reported that gender had little influence on the magnitude of diurnal variation [383].

As metabolomic studies become larger in scale, defining standard operating procedures to account for diurnal variation when collecting samples is paramount to reduce the overall impact of this confounder on a metabolomic dataset. It is recognized that inter-individual genetic variability will modify metabolite profiles that vary diurnally; however, defining precise collection protocols that are standardized and maintained will help to minimize the impact.

IIId. Sample Selection

While the study of tissue metabolite profiles will provide important knowledge regarding the molecular mechanisms triggered by nutrients, the analysis of biofluids remains highly advantageous because of the relative ease of obtaining these samples.

Moreover, using biofluids to measure metabolites linked with a particular phenotype can be more rapidly implemented as a routine diagnostic tool. Therefore, this section will focus on the use of biofluids for nutritional metabolomics research.

The biofluids commonly used for nutritional metabolomics are serum, plasma and urine. Published literature suggests that each biofluid provides unique information and that the appropriate study sample will depend on the biological question asked [313]. Perhaps the most important determinant is whether the primary goal of the research is to study an acute or a chronic nutritional response. A past study by Bertram *et al.* suggests that blood samples provide information stemming from prolonged nutrient exposure, while urine reflects acute dietary modifications. This study examined the biochemical differences following the short-term intake of milk and meat proteins in prepubertal boys [369]. Serum was collected following an overnight fast and urine was collected over a 24-hour period at two time points during the study: baseline and 7 days post-intervention. The authors reported that the analysis of urine samples clearly distinguished before and after the 7-day consumption of milk and meat protein; however, no changes were observed in serum after the 7-day consumption of meat protein and only minor changes observed following milk protein consumption. These results suggest that serum metabolites are more tightly regulated than urine metabolites, and may fail to capture subtle changes in the metabolome following an acute dietary intervention. Furthermore, in light of the previously mentioned studies, it is possible that changes to the urine metabolome were diluted by using a 24-hour collection protocol.

The ability to use metabolomics to monitor nutrient intake can serve to assess compliance in large intervention studies. Typically food intake is monitored by FFQs;

however, this is prone to inaccuracies because it is entirely dependent on the honesty and recollection of the participant. As such, identifying a biomarker to determine food intake will provide an objective method to assess dietary habits and improve study reproducibility. For example, an association between the intake of refined sugars and various cancers has been found; although not all studies have replicated these associations. Kuhnle *et al.* recently determined that monitoring urinary sucrose levels by liquid chromatography-mass spectrometry is a suitable indicator of acute sugar intake [371]. Joosen *et al.* subsequently determined that body mass index does not influence the association between dietary sugar intake and urinary sucrose levels [370].

Taken together, biofluids are appropriate for monitoring both food intake as well as the biochemical perturbations triggered with a dietary intervention. However, it appears that urine best reflects acute dietary interventions, whereas blood best reflects more long-term changes in dietary habits. Furthermore, urine may also be used to provide an unbiased indication of an individual's compliance during a study protocol.

IIIe. Sample Collection

Sample selection is crucial in order to appropriately address the biological question, but the methods used to collect biofluids are important to consider. The use of preservatives with urine samples does not appear necessary if samples are stored at -20°C; however, should a preservative be used then it appears the ideal choice is NaN₃ because of its limited interference in NMR spectra [372]. A recent study examining the stability of several routine clinical markers also found no benefit for including preservatives in a 24-hour urine collection period [384].

When collecting serum, Teahan *et al.* found that a variable time of coagulation could influence metabolite profiles between samples [365]. When samples were coagulated for >35 minutes, lactate levels increased as a result of a continued cellular glucose metabolism [365]. To reduce lactate production, Teahan *et al.* recommended placing samples on ice immediately following venupuncture and then maintaining a uniform clot-time of 30 minutes before centrifugation; thereby permitting complete clot-formation while minimizing lactate production [365].

The anticoagulants used to obtain plasma can be a source of variability in measured metabolite profiles [373,374]. Studies by Drake *et al.* and Bowen *et al.* found that polymer-leaching and an uneven anticoagulant coating between vacutainers can influence intra- and inter-individual analyte profile variability in MS and NMR studies [373,374]. Bowen *et al.* found that concentrations of free fatty acids and triiodothyronine (thyroid hormone) were influenced by the choice of collection tube, which led to clinically significant differences [374,385]. Drake *et al.* analyzed sterile saline solution placed into different brands of vacutainers and detected manufacturer-distinct MS peaks for 2 brands of vacutainers that had no relation to the sample [373].

While the variability introduced by the collection tubes is difficult to control, it is important that standardized procedures are used during an intervention study in order to minimize the variability associated with sample collection. Using tubes from a single batch and manufacturer and consistent sample preparation are two means by which this variability can be reduced.

III.f. Sample Storage

Following sample collection, preventing metabolite degradation is of the utmost importance. Long-term dietary intervention studies may last from weeks to months, and participant recruitment into a study protocol may be staggered over months and years. As such, it is imperative that samples destined for metabolomic-based research are preserved in optimal conditions to avoid the influence of factors such as storage temperature, freeze-thaw cycles, and light conditions. Failure to avoid these confounders may mask data that is biologically relevant and impede meaningful data from being obtained.

Research indicates that sample storage temperatures are relevant for both NMR and MS. Lauridison *et al.* found that human urine samples stored at -25°C or colder prevented changes in ¹H-NMR metabolite fingerprints over a period of 26 weeks [372]. The formation of acetate (a byproduct of bacterial glucose fermentation) in some urine samples was observed when stored at 4°C; suggesting the presence of a bacterial contamination and potential influence for metabolite profiles. Incorporating preservatives such as NaF and NaN₃ helped prevent other shifts in urinary metabolites when samples were stored at 4°C; however, there was no advantage of using preservatives when samples were stored at -25°C or colder. Gika *et al.* found similar results when they assessed the stability of MS metabolite profiles in urine following a 6-month storage period [386]. While these results suggest that metabolite fingerprints are stable if conserved at cold temperatures, evidence reveals that long-term storage of samples even at cold temperatures can only limit and not fully prevent metabolite degradation. For example, vitamin C concentrations in plasma samples stored at -70°C decline by approximately 25% per annum [387]. These losses were only prevented when samples were stored at ultra-low temperatures (-196°C); at which

vitamin C concentrations were stable for up to 11 years [387]. Interestingly, Jenab *et al.* also noted a correlation between metabolite degradation and sex. More specifically, plasma samples collected from women showed a greater loss of vitamin C than those collected from men when stored identically; suggesting that perhaps hormonal or proteolytic differences between sexes may affect metabolite degradation [387].

Light and room temperature conditions during sample analysis may also affect metabolite preservation. Deprez *et al.* investigated biases in autosampler positioning, which can affect the amount of time a sample is exposed to room temperature and lighting conditions prior to analysis [375]. An analysis delay of 3 hours reflected coefficient variations of less than 1%; however, the same sample at 15 and 24 hours provided significant peak intensity changes, most notably for tyrosine, phenylalanine and glycerol [375]. In dietary intervention studies, such changes could lead to inaccurate conclusions regarding food habits and nutrient bioavailability. Thus it is important to both randomize sample positioning and run shorter autosampler trials (i.e. less than 3 hour). Furthermore, a randomized replication of the autosampler trial will also help to minimize the influences associated with room temperature and lighting conditions.

Investigating the influence of freeze-thaw cycling and temperature storage on lipid profiles, Zivkovic *et al.* found that samples exposed to multiple freeze-thaw cycles had little impact on the lipid profile ($\leq 4\%$ variability); however, diacylglycerol concentrations in particular decreased by 19% after one week in samples stored at -20°C compared to -80°C [364]. Therefore, to maintain metabolite integrity and minimize degradation, samples should be stored at temperatures less than -20°C , especially over prolonged periods of time, and limited in temperature fluctuation exposure. However, as demonstrated by

diacylglycerol, it is possible that metabolite degradation is not uniform and even storage temperatures of -80°C may not prevent variable metabolite degradation.

The use of isotope-labeled internal standards that are spiked into biofluid samples at the time of collection offer an attractive method for monitoring the preservation of samples over time [388]. Alternatively, it is possible to statistically adjust metabolite measurements for degradation, as demonstrated by Mutch *et al.* [353]. However, it is ideal to prepare and store multiple aliquots appropriately in order to avoid freeze-thaw cycles and minimize degradation.

IV. Concluding Remarks

One of the most critical aspects for biomarker discovery is the notion of reproducibility. As exemplified in the previous sections, numerous sources of variability can impact both the metabolite profile detected and the abundance of these metabolites. As many potential biomarkers are constituents of the diet, a number of issues related to their bioavailability must be appreciated before one can consider them as a useful and accurate reflection of an individual's response to the dietary intervention. This means that distinguishing a differentiating metabolite from a candidate biomarker is of paramount importance. The definition of a candidate biomarker should be reserved for those metabolites that demonstrate a reproducible discriminatory quality between phenotypes [313,350,389,390]. This is in contrast to differentiating metabolites, which are non-validated observations of concentration variation between two defined groups [391]. The importance of biomarker validation is to ensure that the identified metabolite response is categorical of the phenotype, and not simply related to an unaccounted source of variability

or statistical anomaly applicable only to the studied population. Biomarker validation is typically overlooked in previously published metabolomics literature. To the authors' knowledge, only a few studies have performed rigorous validation protocols before proposing a candidate biomarker [322,392,393]. This is predominantly because of the long and arduous work required to identify, validate, and characterize a new biomarker.

Nevertheless, metabolomics is well positioned to provide a wealth of complementary information that will enable a better understanding of the biological impact of a particular micro- or macro-nutrient. For example, metabolite data will provide an alternate (to FFQs) and objective manner by which to assess an individual's lifestyle; thereby enabling inter-individual differences in lifestyle to be better accounted for. Knowledge of ethnic differences will lead to an improved normalization of metabolomics data generated from large multi-ethnic study cohorts; suggesting that subjects within these cohorts can be more accurately stratified. Measuring accepted nutritional biomarkers will lead to a dramatic improvement evaluating a participant's compliance during a study, thereby minimizing one of the most important confounders in a dietary intervention protocol. Finally, appreciating the numerous sources (both biological and technical) that can influence the robustness of a metabolomics dataset will assist in unraveling the inter-individual variability in response to a dietary intervention. As our appreciation of the numerous sources of variation capable of influencing metabolite profiles continues to improve, metabolomics is well positioned to play an important role for unraveling the influence of diet on health and disease.

Participants Needed For Diabetes Risk Assessment Study

A new study is being conducted by the Department of Human Health and Nutritional Sciences at the University of Guelph in collaboration with Dr. Susan Stephenson (Guelph Family Health Team). We're looking for **adults 35-70 years old** to examine the differences in metabolism among people varying in body weight and diabetes risk.

Participants will be required to attend 1 study visit (< 4 hours):

- Body Composition Analysis
- Lifestyle Questionnaire
- Provide up to 6 Blood Samples
- Fat Tissue Sample

Breakfast Provided

Financial Compensation

If you are interested in participating in this study, please let us know by email at DRAstudy@uoguelph.ca.

This study has been reviewed and approved by the University of Guelph Human Research Ethics Board (REB # 10AP033)

Appendix C: Diabetes Risk Assessment (DRA) study consent form

CONSENT TO PARTICIPATE IN RESEARCH

New and Innovative Bioanalytical Tools to Assess Lifestyle Recommendations for Managing Type-2 Diabetes

You are asked to participate in a collaborative research study conducted by Dr. Susan L Stephenson (M.D.) from the Guelph Family Health Team, and Professors David M Mutch and Alison M Duncan from the Department of Human Health and Nutritional Sciences at the University of Guelph.

If you have any questions or concerns about the research, please feel free to contact:

- **Susan L. Stephenson**, M.D., Guelph Family Health Team, 21 Yarmouth Street, Guelph, 519-822-7831, drsue@xplornet.ca.
- **David M. Mutch**, Ph.D., Assistant Professor, Department of Human Health & Nutritional Sciences, University of Guelph, 519-824-4120 ext. 53322, dmutch@uoguelph.ca.
- **Alison M. Duncan**, Ph.D., R.D., Associate Professor, Department of Human Health and Nutritional Sciences, University of Guelph, 519-824-4120 ext. 53416, amduncan@uoguelph.ca.

PURPOSE OF THE STUDY

Diabetes is one of the fastest growing diseases in Canada; however, lifestyle changes (for example, changes in diet and physical activity) can prevent or postpone the development of this disease. This research project aims to better understand the metabolic characteristics of diabetic and obese persons by using transcriptomics and metabolomics methods. Humans are incredibly complex at the molecular level. The human genome consists of approximately 26,000 genes, where each gene codes for at least one protein. These proteins then act to regulate the abundance of metabolites, which are small molecules that regulate all body functions. It is important to recognize that not all genes, proteins, and metabolites are expressed in each tissue or organ. Therefore the goal of this study is to better understand those genes and metabolites expressed in subcutaneous adipose tissue and blood, respectively. Transcriptomics describes a technique used to analyze global gene expression. In this study we will be looking at differences in fat samples collected from the abdomen of lean, obese, lean/diabetic, and obese/diabetic individuals. This will help us to understand how gene activity differs in fat tissue between obese and diabetic patients. The second method, metabolomics, is a field of research interested in the body's production of small molecules, such as amino acids (i.e. the building blocks of proteins), carbohydrates (e.g. sugars and starches), fatty acids (e.g. saturated and unsaturated fats), and vitamins. In this study, we will use metabolomics to measure these small molecules in blood samples in order to better understand the relationship between fat tissue gene expression and blood metabolite profiles between lean, obese, and diabetic people. A better understanding of these metabolic conditions will have value in predicting cases, preventing downstream complications, and personalizing therapeutic and lifestyle interventions to improve health management. Please note that this study is purely a research study and not intended for therapeutic or diagnostic purposes.

PARTICIPANT RECRUITMENT

Individuals will indicate their interest to participate in this research study to either Dr. Stephenson or to her staff at the Yarmouth Medical Centre in Guelph, ON. Dr. Stephenson will then examine the individual's electronic health record to determine whether they are eligible to participate in the research study. Furthermore, they will be asked to complete a Fat Tissue Collection Screening Form to ensure that they are eligible for fat tissue collection (this will be assessed by Dr. Stephenson prior to fat collection). A document outlining Fat Tissue Collection Information is included with this letter. The interested individual will have to meet the requirements for one of the following groups in Table 1, as well as not have evidence of acute or chronic inflammatory disease, infectious diseases, viral infection, cancer and/or known alcohol consumption (i.e. more than 2 drinks/day, where 1 drink = 10 g alcohol). The participant must also be comfortable consuming a standardized McDonald's breakfast consisting of 2 Sausage McMuffin with Egg Sandwiches, 1 apple turnover, and 1 medium orange juice (266 mL). Confidential medical information will not be distributed by Dr. Stephenson to anyone outside of the Yarmouth Medical Centre in Guelph. The only information that will be relayed to all investigators involved in the study will be the following: gender, ethnicity, age, fasting blood glucose, fasting insulin, Hb1Ac %, body mass index, body weight (kg) and waist circumference.

Table 1. Selection Criteria for Participant Classification				
Variable	Lean (Healthy)	Lean/Diabetic	Obese	Obese/Diabetic
Fasting blood glucose (mmol/L)	< 6.9	≥ 7.0	< 6.9	≥ 7.0
Hb1Ac (%)	4.0 – 5.9	≥ 6.0	4.0 – 5.9	≥ 6.0
Body mass index (kg/m ²)	20 – 25	20 – 25	30 – 40	30 – 40

PROCEDURES

If you accept to participate in this study, you will be asked to attend a single pre-scheduled study visit at the Human Nutraceutical Research Unit (HNRU), which is located in room 144 of the Food Science-Guelph Food Technology Centre Building, 88 McGilvray Street at the University of Guelph.

Prior to the study visit:

The night before the study visit, participants are required to consume a meal that has been deemed acceptable by the study organizers. Furthermore, the participant will be expected to purchase this meal independently. Participants will be instructed to:

- For 12 hours prior: avoid food and beverages (except water, which will be encouraged both the night before and morning of).
- For 48 hours prior: avoid rigorous exercise, alcohol, over-the-counter medications, dietary supplements, and vitamins (only prescribed medications are acceptable).
- For the dinner before the study day visit, participants will consume a standardized meal (vegetarian option available): 1 single-serving frozen meal (from PC, Stouffers, Lean Cuisine, etc); 1 dinner roll (whole wheat or white); 1 side vegetable of their choice (corn, peas, broccoli, carrots, squash, zucchini, or green beans); 1 fruit (apple, orange, banana, peach, grapes, or melon); and 1 bottled water (500 mL). With dinner and during the overnight fast participants will be instructed to only drink water (no tea or coffee permitted). Participants will need to purchase/coordinate this meal themselves in order to avoid the need for an extra study visit.

- No gum, sweets, cough candies, or juice can be consumed prior to and during your study visit.

These restrictions are meant to minimize the influence of individual lifestyle and dietary habits, which will help to reduce measurement variability in blood metabolites and adipose tissue gene expression.

At your study day visit, the following will occur:

1. A study researcher will review the study consent form with you and if you are comfortable, you will sign the study consent form.
2. Your body weight and height will be measured.
3. Your body composition will be measured using a BOD POD, which uses air displacement to measure your body's density (See Figure for a visual representation of the BOD POD).
4. Next, a catheter will be placed into your forearm and you will provide a blood sample of approximately 30 ml.
5. Next, you will provide a small fat tissue sample which will be obtained from an anaesthetized area on your abdomen – please refer to the Fat Tissue Collection Information Sheet that is appended to this letter for complete details about the procedure and its risks.
6. Subsequently you will be provided a standardized McDonald's breakfast, which will include 2 Sausage McMuffin with Egg Sandwiches, 1 apple turnover, and 1 medium orange juice (266 mL).
7. Once you have consumed your breakfast, five blood samples (~ 10 mL/each) will be collected over the subsequent 2-hour period.
8. You will complete a short lifestyle questionnaire (~ 20 questions) on your general health and dietary habits.

Blood samples will be analyzed for molecules (e.g. fatty acids, sugars, etc.) and fat tissue samples will be analyzed for gene expression in order to identify new markers that differentiate individuals according to their diabetes risk. Blood and fat tissue samples will be stored at -80 °C for 5 years, at which point the samples will be disposed of accordingly.

POTENTIAL RISKS AND DISCOMFORTS

Body Composition: The risks associated with the body composition measurement, using a BOD POD, are discussed in detail the BOD POD Information sheet.

Briefly, measuring body composition generates no risk of physical harm to the participant; however, individuals may experience a feeling of embarrassment, as it requires a personal bathing suit to be worn for a short period of time; hospital gowns will be available and worn immediately before and after the test. Also, there is a very low risk of claustrophobia. This risk is minimized due to a large, clear Plexiglas window at the front of the instrument. Should

subjects feel uncomfortable at any time, there is “stop test” button located under the left knee. Pressing this button automatically stops the test and disengages the door for easy exiting. Alternatively, patients can communicate with the Investigator through the Plexiglas window that they would like the test stopped.

Blood Collection: Potential risks include a small risk of infection, slight discomfort, and mild bruising. These risks will be minimized by having only medically approved personnel collect your blood from a disinfected area of your arm using sterilized equipment. Also, you will be encouraged to drink water the night before and the morning of the sampling to ensure proper hydration, which can help alleviate any potential discomfort associated with blood sampling.

Fat Tissue Collection: The risks associated with Fat Collection have been outlined in the Fat Tissue Collection Information Sheet (attached), which you are expected to read. Also, the Fat Collection Screening Form will assist in minimizing the associated risks by “flagging” potential areas of concern. Here we provide a brief outline of possible risks:

A rash may develop if you are allergic to iodine (antiseptic); however, if known in advance (via Screening Form) then only alcohol will be used. From the incision, there is a low risk of a blot clot and infection. This will be minimized by ensuring proper cleaning of the incision area before and after the procedure.

The anaesthetic may result in a burning feeling at the site of injection. This will last only 5 – 10 seconds. There is an extremely low risk of allergic reaction to the local injection (reported to be 1 in 1 million). If this occurs, we will not continue with the fat sample collection because of the risk of bleeding. The redness of the skin will disappear after a while.

Subjects may experience local soreness and stiffness in the abdominal area of the incision. There is a very low risk of internal bleeding at the collection site which can result in prolonged pain and stiffness in the leg.

On occasion, a small lump of scar tissue may form under the site of the incision, but this normally disappears within 2-3 months, or within a few weeks if massaged. A small visible scar may remain following the incision; however, the likelihood of this occurring is very low.

There is the possibility of a small area of numbness (about the size of a two-dollar coin) around the collection site. This usually resolves over 5 – 6 months. There is a very low risk (estimated at less than 1/5000) of damage to a small nerve branch to the muscle. This would result in partial weakness of the individual abdominal muscle and would likely have no impact on day-to-day activities. Nerve injuries like this usually resolve in 8 – 12 months.

Following the procedure, we advise that you not perform any physical activity (e.g. bending or carrying heavy objects) for 1-2 days following the study day visit. This will ensure minimal bruising in the area where the fat tissue sample was obtained. Bruising may occur and last for up to 2-3 weeks. To ensure proper care, a follow-up appointment with your general physician (Dr. Susan Stephenson) will be scheduled 7 days following your study visit and fat tissue collection.

POTENTIAL BENEFITS TO PARTICIPANTS AND/OR TO SOCIETY

You will receive a summary of your body composition and clinical results. Furthermore, this research study has the potential to benefit multiple sectors in the diabetes health care delivery system (from medical doctors to the individual patient) by demonstrating that knowledge of an individual's metabolic phenotype can complement and improve the methods currently used for monitoring the impact of lifestyle interventions used for diabetes and obesity management.

PAYMENT FOR PARTICIPATION

As a token of our appreciation for your participation in this research project, you will be compensated. We recognize that you may not wish to participate in all aspects of this study. Therefore we have established a pro-rated reimbursement table in order to compensate you according to your degree of participation (Table 2). Participants that have agreed to a study activity (e.g. fat collection) but are unable to complete the activity for reasons beyond their control (e.g. anaesthetic allergy) will be fully compensated.

Table 2. The compensation is arranged to ensure every patient a minimum amount of \$50, with a potential maximum of \$250.

Study Activity	Amount of Compensation		
	\$50	\$150	\$250
Questionnaire	•	•	•
Body Composition Analysis	•	•	•
Fasting Blood Sample	•	•	•
Postprandial Blood Collection		•	•
Fat Tissue Sample			•

CONFIDENTIALITY

Every effort will be made to maintain confidentiality in all aspects of this research. All participants will be assigned a unique numerical identifier and any data reported in a publication or presentation will be overall group data. Blood and fat tissue samples will be stored in a locked -80°C freezer that is accessible only by the principal researchers and graduate students involved in the project. Both samples will only be used to measure metabolites and gene expression. Following 5 years of storage at -80 °C, the samples will be disposed of accordingly. Your name will not appear in any report, publication or presentation resulting from this study. All documents or computer files related to the study will be stored in a locked office and on a password-protected computer. Data collected from this study will be used as part of a graduate student thesis and may be published in a journal article. The data will be retained for a period of 5 years after publication.

PARTICIPATION AND WITHDRAWAL

You can choose whether to be in this study or not. If you volunteer to be in this study, you may withdraw at any time without consequences of any kind. You may also refuse to answer any questions in the lifestyle questionnaire that you don't want to answer and still remain eligible for the study.

RIGHTS OF RESEARCH PARTICIPANTS

You may withdraw your consent at any time and discontinue participation without penalty. You are not waiving any legal claims, rights or remedies because of your participation in this research study. This study has been reviewed and received ethics clearance through the University of Guelph Research Ethics Board. If you have questions regarding your rights as a research participant, contact:

Research Ethics Coordinator
University of Guelph
437 University Centre
Guelph, ON N1G 2W1

Telephone: (519) 824-4120, ext. 56606
E-mail: sauld@uoguelph.ca
Fax: (519) 821-5236

SIGNATURE OF RESEARCH PARTICIPANT/LEGAL REPRESENTATIVE

I have read the information provided for the study “**New and Innovative Bioanalytical Tools to Assess Lifestyle Recommendations for Managing Type-2 Diabetes**”, including the Fat Tissue Collection Information sheet, as described herein. My questions have been answered to my satisfaction, and I agree to participate in this study. I have been given a copy of this form.

_____
Name of Participant (please print)

_____
Signature of Participant

_____
Date

SIGNATURE OF WITNESS

_____
Name of Witness (please print)

_____
Signature of Witness

_____
Date