Investigating the role of sex and genetic variation in the *FADS1/2* genes on lipid and fatty acid profiles and the potential impact on response to EPA and DHA supplementation

by

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A Thesis

presented to

The University of Guelph

In partial fulfilment of requirements
for the degree of

Doctor of Philosophy

in

Human Health and Nutritional Sciences

Guelph, Ontario, Canada

ABSTRACT

INVESTIGATING THE ROLE OF SEX AND GENETIC VARIATION IN THE *FADS1/2* GENES ON LIPID AND FATTY ACID PROFILES, AND THE POTENTIAL IMPACT ON RESPOSNE TO EPA AND DHA SUPPLEMENTATION

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University of Guelph, 2019

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Dr. David Mutch

Cardiovascular disease (CVD) is the number one cause of death globally, with well-established risk factors and biomarkers. This thesis aimed to understand whether genetic variants and biological sex impact CVD risk factors and if they modify an individual's response to treatment with the omega-3 polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In Study 1, it was found that sex differences in blood lipid and fatty acid (FA) levels in humans are not associated with variants in lipid metabolism genes, including the rs174537 single nucleotide polymorphism (SNP) in fatty acid desaturase 1 (*FADS1*). However, as a whole, major alleles for this SNP had higher circulating levels of arachidonic acid (AA) and lower dihomo-γ-linoleic acid (DGLA). Given that the *FADS1* genotype was found to influence circulating FA profiles in a sex-independent manner, we next hypothesized that this same SNP may also influence FA profiles in adipose tissue. Thus, in Study 2, it was demonstrated that *FADS1* genotype is associated with different subcutaneous adipose tissue (SAT) FA profiles. Specifically, SAT levels of AA and DGLA explain ~19% of the variance between genotype. As both Study 1 and 2 were static and assessed the impact of genetic variance and sex on CVD risk factors, Study 3 investigated if these same factors influence response to n-3 FA treatment. Using a randomized double-blind placebo-controlled study, the

independent effects of 12 weeks of treatment with EPA or DHA on blood lipid and FA levels was examined in healthy men and women. Results showed that DHA treatment reduced circulating triglyceride (TAG) levels, while EPA did not. This effect was more efficacious in individuals with higher TAG levels at baseline but was not dependent on biological sex or *FADS1* genotype. In totality, this thesis provides further understanding of the role that genetic variance and sex have in the regulation of blood FA, blood lipids, and adipose tissue FA, while also adding novel insights into how these factors influence an individual's response to n-3 PUFA treatment.

ACKNOWLEDGEMENTS

To my advisor, Dr. David Mutch. Thank you for seeing the potential in a masters student who had little wet lab experience, but enormous passion for clinical work. Your passion for your craft shows through every idea, project, and paper completed in your lab. I am appreciative of our talks on self-improvement, goal-development and project-planning, I will be sure to employ these methods to keep developing these skills in my future career.

To my committee members, Dr. Bruce Holub, Dr. Jess Haines, and Dr. Lindsay Robinson: thank you so much for your advice and wisdom throughout my thesis. Your ideas and suggestions always took my projects to the next level, and helped to create projects that I could be truly proud of. There is a reason you are so frequently chosen as committee members, and I am so thankful you were able to make the time to be a part of mine.

Lindsay, from the time I was in undergrad, I heard innumerable positive things about you. I can honestly say, every single one of them is true. I cannot explain how much it meant to me to be able to seek out your help throughout my PhD, you were elemental to the completion of this. I wouldn't be where I am without your encouragement, support and everlasting positivity. Thank you for seeing my weaknesses as my strengths, and for always imploring me to dream big.

Dr. Amanda Wright: I cannot say enough about how brilliant, kind and charismatic you are. I was not your student, but you took me under your wing and taught me so much all the same. Thank you for always being the voice of reason, when I needed some help finding my way; supplying a ridiculously quick and witty joke, when I really needed a laugh; and for showing me

by example how to be an exemplary human. Thank you for being one of the strongest mentors to me throughout my graduate career.

A huge thank you to Dr. Graham Holloway for always providing sound advice, scientific expertise, and incredible support throughout the academic process. Your meticulous review of my work was instrumental to the completion of this thesis. This wouldn't have been possible without you, and "thank you" just doesn't seem quite enough.

Thank you to the ladies of HHNS (Becky, Andra, Anne and Ann) that keep everything running, while also always offering a kind smile, and a warm laugh everyday. Lyn thank you for your help in the GC lab, and for all of the hilarious conversation throughout.

Shannon Pare, you made my clinical trial experience in my PhD the most positive, educating, and enjoyable during your time as manager of the HNRU. Your open door attitude, and willingness to answer any question was invaluable to the completion of the trial. An additional thank you to Dr. Amy Tucker for her help within the HNRU throughout my PhD.

Thank you to the Millar and Spriet lab for your collaboration throughout TGIF. I looked forward to waking up ridiculously early, just so I could see your smiling faces. Sebastian, you are an incredible human, full of kindness, empathy, and awful jokes. Thank you for your counsel and advice throughout the past 4 years. You are destined for amazing things: I am so happy I got to work with you, and gain a wonderful friend.

I would like to thank members of the Mutch lab, past and present, for making this place feel like home. Dr. Kaitlin Roke, Steve Dragos, Dr. Ousseynou Sarr, Dr. Fernanda Fernandes, Katie Suitor, Cristina Tache, Peri Fenwick, Alex Rajna, Dana Lowry, Barbora Hucik, and Kira Wang..

You have all created lasting memories that I will take with me forever, and I am so grateful to have met you all.

To my friends Elie, Adrian, and Nicole, you guys became the heart of my graduate world, and were there through all the trials and tribulations. I can't explain the love and admiration I have for you guys, and proud to say you are like family to me. Your futures are so bright, and I cannot wait to see what life has in store for you!

Kaity, you were my fairy godmother of the lab, and truly did show me everything that I know. Without you I would have been unable to merge so well with the PhD lifestyle, and transition to the higher standards of productivity, independence and understanding. So, thank you! Steve, your laugh, to me, will always be one of the most contagious, and I considered it a productive day if I could make one happen. You are such a warm and wonderful human, and every minute shared in the lab was a treasure. Suits, you quite honestly are one of the funniest people I have ever met, and have a direct line to my funny bone. I am fairly certain the two of us just fed off each other, like two babies giggling at one another. Your brilliant, quick witted and have an incredible work ethic, and you always have a friend in me.

Alex (and Kayleigh), Dana and Barb, I didn't think by halfway through my PhD I would be making new friends, but you three became fast friends, and practically family. Your humor and bright dispositions helped to paint everyday with a new light, and it was impossible to tear down the smile pasted on my face after every day.

To my parents, thank you for always supporting my (sometimes crazy) ideas, and always giving me a safe place to land. I am the cumulation of all of the countless hours you spent encouraging and fostering my creativity and critical thinking. Sometimes I think that you all truly understand

me better than I know myself, and can say honestly that you guys have saved my life. I hope to someday be just 1/10th of the phenomenal humans you are.

To my sisters, the other two parts of my whole, I hope there will be a day when I can repay you for all of the love, support and positivity you have given me. “Thank you” seems so inadequate.. but here goes. Thank you for reminding me that I am loved, that happiness is within my grasp, and that life is larger than the bad days. Thank you for the reminder that progress is not always linear, but to focus on the positive line that best fits. Life moves so fast, that it is important to have best friends that can slow it down, remind you of your priorities, and set you on your way (with a little pat on the butt). I am so proud to call you both my sisters, and will forever be your #1 fan.

Rob and Dan, I never knew how much I was missing not having brothers, until I got two. You two provided the best distraction from grad school when I needed it most. Because of you two, I always felt like I was “living the dream”.

Willem, I truly understand what people mean by saying “I cannot put into words how I feel” because all the words I would choose fall flat of the shine and effervescence you bring to my life. The clichés you inspire me, make me want to be a better person, and I am SO proud of you, all fit exceptionally well… but I would add that you will never know how often the smallest gestures made the biggest difference. Thank you for being the light I couldn’t always see on days when getting out of bed was too much to bear. Keep on smiling.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AI	Adequate intake
ALA	α-linolenic acid
ANGPTL4	Angiopoeitin-like protein 4
ANOVA	Analysis of variance
APOA5	Apolipoprotein A-V
APOC3	Apolipoprotein C3
AT	Adipose Tissue
AUC	Area under the curve
BF3MeOH	Boron trifluoride-methanol
BMI	Body mass index
CD36	Cluster of differentiation-36
CETP	Cholesteryl ester transfer protein
CVD	Cardiovascular disease
D5D	Delta-5-Desaturase
D6D	Delta-6-Desaturase
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DiOGenes	The Diet, Obesity and Genes Study
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
EE	Ethyl ester
ELOVL2	Elongase 2
ELOVL5	Elongase 5
ELOVL6	Elongase 6
EPA	Eicosapentaenoic acid
FA	Fatty acid
FABP-2	Fatty acid binding protein-2
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
FAME	Fatty acid methyl esters
FAO	Food and Agricultural Organization
FASN	Fatty Acid Synthase

FFA	Free fatty acid
GC	Gas chromatography
GOLDN	The Genetics of Lipid Lowering Drugs and Diet Network
GPR120	G-protein coupled receptor 120
GWAS	Genome-Wide Association Study
H^+	Hydrogen Ion
H_2O	Water
HDL-c	High-density lipoprotein-cholesterol
hsCRP	High sensitivity C-reactive protein
HSD	Honest significant difference
HSL	Hormone sensitive lipase
HWE	Hardy–Weinberg equilibrium
IL-6	Interleukin-6
KCAL	Kilocalorie
LA	Linoleic acid
LD	Linkage disequilibrium
LDL-c	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein lipase receptor
LPL	Lipoprotein Lipase
MAF	Minor allele frequency
MaR1	Maresin 1
MaR1	Maresin 2
MUFA	Monounsaturated fatty acid
n-3 PUFA	Omega-3 polyunsaturated fatty acid
n-6 PUFA	Omega-6 polyunsaturated fatty acid
NADH	Nicotinamide adenine dinucleotide (reduced)
NAD+	Nicotinamide adenine dinucleotide (oxidized)
NF-κB	Nuclear factor-kappa B
NPD1	Neuroprotectin D1
O_2	Oxygen
PCSK9	Proprotein convertase subtilisin/kexin type 9
PL	Phospholipids
PPARα	Peroxisome proliferator-activated receptor alpha
PUFA	Polyunsaturated fatty acids
QRT-PCR	Quantitative reverse transcription polymerase chain reaction

RBC	Red blood cells
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RvD1	Resolvin D1
RvE1	Resolvin E1
SCD-1	Stearoyl-CoA desaturase-1
SEM	Standard error of the mean
SFA	Saturated fatty acids
SNP	Single nucleotide polymorphism
SREBP-1c	Sterol regulatory element-binding protein-1c
T2D	Type-2 diabetes
TAG	Triglycerides
TC	Total cholesterol
TFA	Trans fatty acids
TNF-α	Tumor necrosis factor-alpha
VAT	Visceral adipose tissue
VLDL	Very low-density lipoprotein
WHO	World Health Organization
YRS	Years

1 Review of the Literature

1.1 Overview

The world is facing an epidemic, marked by increased incidence and prevalence of numerous chronic diseases such as obesity and cardiovascular disease (CVD) [1; 2]. Frontline strategies for prevention and treatment are often pharmaceutical or surgical, however, these are expensive and often have undesirable side effects [3]. As a result, mounting interest has developed for use of lifestyle based strategies such as nutrition, that can be implemented throughout the lifespan [3]. One such nutritional strategy is the use of omega-3 polyunsaturated fatty acids (n-3 PUFA) and other dietary fatty acids [4; 5]. N-3 PUFA are found naturally in the diet through sources such as fatty fish, or they can be incorporated into food products or consumed as supplements via capsules or oils [5]. Importantly, n-3 PUFA have been shown to i) lower plasma triglyceride (TAG) concentrations, ii) exhibit potent anti-inflammatory and immunomodulatory actions, and iii) improve vascular function [6; 7; 8]. Therefore, n-3 PUFA supplementation offers an easy and cost-effective strategy to improve cardiovascular health. However, recent literature has suggested that not all individuals respond similarly to n-3 PUFA supplementation, which emphasizes the importance of considering what factors play a role in determining an individual's response [9; 10; 11]. While n-3 PUFA are well characterized to improve health, the interaction between genetics, hormone environment (sex) and different n-3 PUFA remain poorly defined. Therefore, the overarching objective of this thesis is to investigate how genetics, sex, and n-3 PUFA formulation impact risk factors for CVD.

1.1.1 Epidemiology and Etiology of CVD

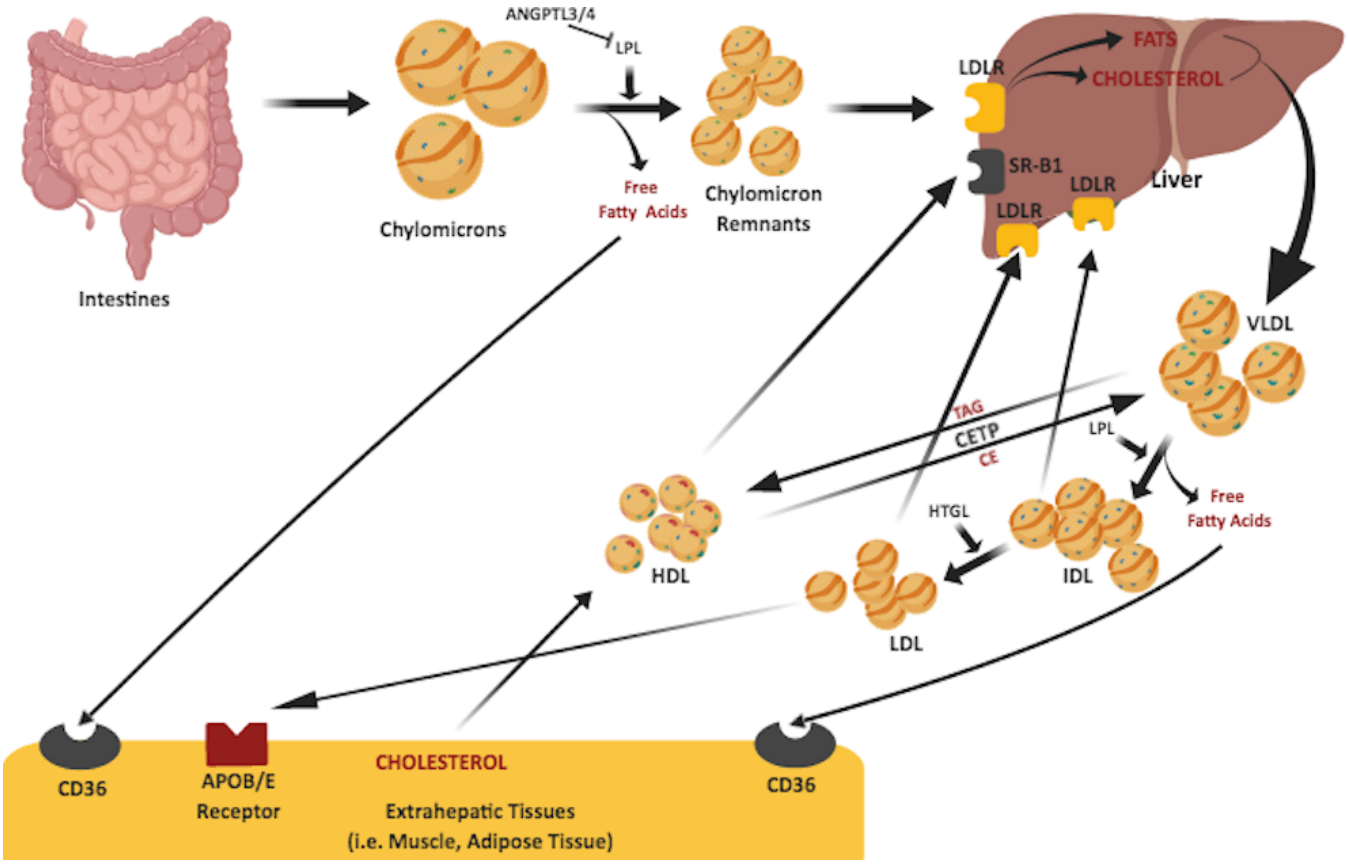
Approximately 17.9 million people die each year from CVD, accounting for 31% of global deaths[2], making it the number one case of death globally [1]. CVD is defined as the group of disorders of the heart and blood vessels, and includes, but is not limited to, hypertension, coronary heart disease, cerebrovascular disease and heart failure [12]. The progression of CVD often has a slow onset that begins with vascular dysfunction, leading to impaired endothelial function, followed by inflammation within the vessel wall [13]. This often leads to pathological conditions such as atherosclerosis, thrombosis or high blood pressure, which can subsequently cause myocardial infarction and stroke [13; 14]. Dyslipidemia, characterized as increased circulating levels of low-density lipoprotein-cholesterol (LDL-c) and TAG, but decreased high-density lipoprotein-cholesterol (HDL-c) levels, is one of the strongest risk factors for the pathological onset of CVD [15]. Because of this, understanding how circulating lipids and lipoproteins play a critical role in CVD onset is important to identify.

1.1.2 Circulating Lipoproteins and Lipids

The physiology behind circulating lipids and lipoproteins is a pertinent subject of research in the area of CVD, and involves an interplay between numerous organs and tissues [16; 17] (**Figure 1-1)**. Plasma cholesterol is also a known indicator of CVD risk, and is transported in the bloodstream in four different classes of lipoprotein particles: chylomicrons, very low-density lipoproteins (VLDL), LDL-c, and HDL-c [18]. Chylomicrons aid in the transport of dietary derived TAGs and cholesterol from the gut, and into the lymphatic system for distribution around the body [18]. VLDL are synthesized in the liver and are predominately responsible for exporting endogenous TAGs (from plasma free fatty acids, FFA) and cholesterol to peripheral

tissues [16; 19]. Therefore, VLDL levels climb higher with increases in intrahepatic FFA, such as in the case of high fat diet consumption [16; 18; 19]. LDL are created from circulating VLDL that have returned back to the liver [20]. LDL are essential for carrying blood cholesterol, with approximately 70% of circulating levels of cholesterol being found in LDL [20]. LDL particles can range in size from large to small, but it is the smaller and more-dense molecules that are responsible for the metabolic perturbations and increased CVD risk, associated with circulating LDL [20]. This is due to the decreased affinity for the liver LDL-receptor, ultimately leading to an increased time within the circulation [20]. Finally, HDL is necessary for collecting cholesterol from the periphery (tissues) and from other lipoproteins, to bring it back to the liver for clearance [21]. Previous research suggests that the LDL-cholesterol/HDL-cholesterol ratio (LDL-c/HDL-c) could be a useful clinical indicator for relative CVD risk, with a smaller ratio being optimal [22]. One such study evaluated the prospective association of LDL-c/HDL-c and the risk of sudden cardiac death, which was responsible for 50% of CVD related deaths in a sample of 2,616 healthy men from eastern Finland [23]. After a median follow up time of 23 years, there was approximately a two-fold increase in the risk of sudden cardiac death (hazard ratio: 1.94, 95%, confidence interval: 1.21-3.11; p=0.006) when comparing the top ($>$4.22) versus bottom ($\leq$2.30) quintile of serum LDL-c/HDL-c ratio [23]. Together, when these lipoproteins (e.g. chylomicrons, VLDL, LDL-c, and HDL-c) are elevated in circulation, they can accumulate in the arteries and, over time, lead to plaque formation [24]. This plaque hardens, narrows the arteries, and can ultimately lead to potentially severe ischemia or thrombotic occlusion of critical conduit arteries serving important tissues (heart, brain, limbs, etc.) [24]. The development of these atherosclerotic plaques is often due to TAGs released from adipose tissue (AT) [24], which are elevated in situations such as obesity.

Figure 1-1: Lipid and Lipoprotein Metabolism. Shown is a depiction of how lipid and lipoprotein metabolism revolve within the body. *Figure created with BioRender.



1.1.3 Obesity and Adipose Tissue as a Modifier of CVD Risk

Obesity is associated with an increased risk of health complications, in part, due to expansion of AT depots and associated AT dysfunction that has long been an understood risk factor for CVD [25]. AT is made up of many cell types, such as adipocytes, macrophages, endothelial cells, pericytes and stem cells, allowing it to exhibit very dynamic functions [25]. Adipocytes can contain multiple lipid droplets, such as in the case of brown adipose tissue, or a single lipid droplet, which is found in white adipose tissue [25]. Lipids are able to be stored in this droplet during times of increased availability (i.e. postprandial), and can be released during times of energy demand [25; 26]. However, during the development of obesity, AT can undergo hypertrophy (preexistent adipocytes increase in volume) and/or hyperplasia (recruitment of new preadipocytes) [26]. In obesity, there is an accumulation of subcutaneous (waist, subscapular, gluteal, and femoral regions) and visceral (perirenal, gonadal, epicardial, retroperitoneal, omental, and mesenteric regions) AT via hyperplasia and hypertrophy, respectively [26]. Moreover, obesity can lead to inflammation of AT (particularly in visceral regions) [26], which results in the increased infiltration of immune cells as well as an increased secretion of proinflammatory cytokines [27]. Following excessive lipid accumulation, reactive oxygen species (ROS) are postulated to result in increased production of proinflammatory cytokines such as MCP-1, IL-6 (interleukin-6) and TNF-α (tumor necrosis factor-alpha), which lead to the recruitment of macrophages and inflammatory molecules [28]. As these adipocytes and resident macrophages secrete cytokines, they can act in both paracrine and autocrine ways, resulting ultimately in a state of systemic inflammation [29]. This state of inflammation can be deleterious to health, as it leads to the increased lipolytic activity and the release of FFA from AT into the circulation [30]. Once released from AT, hepatocytes take up the FFA and, following excessive

lipid accumulation, this leads to decreased insulin sensitivity and the onset of insulin resistance and increased glucose production [30]. Together, these events further propagate the inflammatory response and lead to chronic, and pathological, inflammation [29]. This chronic state of systemic inflammation has been reported to exist prior to, and significantly increase the risk for, CVD [29]. Although the relationship between AT and the onset of CVD is strong, there are a number of modifiable risk factors that can aid in risk reduction.

1.2 Modifiable Risk Factors for Obesity and CVD

An easily modifiable risk factor in the prevention and treatment of obesity and CVD is diet. The typical Western diet has a high intake of fat, high salt consumption, and elevated levels of refined sugars; therefore, it is suggested that reducing the intake of these dietary components is beneficial to health [31]. However, in the case of dietary fat, it is important to consider not only the amount of fat, but also the types of fat consumed as dietary fat type may be either beneficial or detrimental to overall disease risk.

1.2.1 Dietary Fats

Dietary fats are essential for many different structural components of cells and physiological processes within the body, including cell and tissue metabolism, as well as function and response to hormones and other signals [32]. Additionally, fats are an important source of energy for the body, and aid in the absorption of fat-soluble vitamins and carotenoids [32]. Current recommendations for daily energy intake suggest approximately 20-35% of caloric consumption should be from fats [32]. There are five types of dietary fats: trans (TFA), saturated

(SFA), monounsaturated (MUFA), omega-6 polyunsaturated (n-6 PUFA) and n-3 PUFA, and they differ in chemical structure as well as physical properties [33].

Trans fats have a long hydrocarbon chain with one or more double bonds in the *trans* (as opposed to *cis*) configuration [33; 34]. Trans fats can be found naturally in ruminant animal-based foods (beef, lamb, dairy products, etc.)[33; 34]. SFA have hydrocarbon chains that contain only single bonds [33; 34]. This type of fat can be found largely in dairy products and animal based foods (beef, chicken, pork, etc.), but is also found in some oils, such as palm or coconut [33; 34]. Fatty acids with one double bond in the cis configuration of the hydrocarbon chain are known as MUFA, and they can be found in nuts and seeds, as well as a number of vegetable oils (e.g. olive, canola, and peanut) [33; 34]. Finally, hydrocarbon chains with two or more double bonds are known as polyunsaturated fatty acids (PUFA) [33]. N-3 and n-6 PUFA differ due to the placement of the first double bond on the hydrocarbon chain (third vs. the sixth carbon). PUFAs have long been associated with circulating lipid and lipoprotein levels, as well as tissue composition, consequently playing an important role in CVD risk.

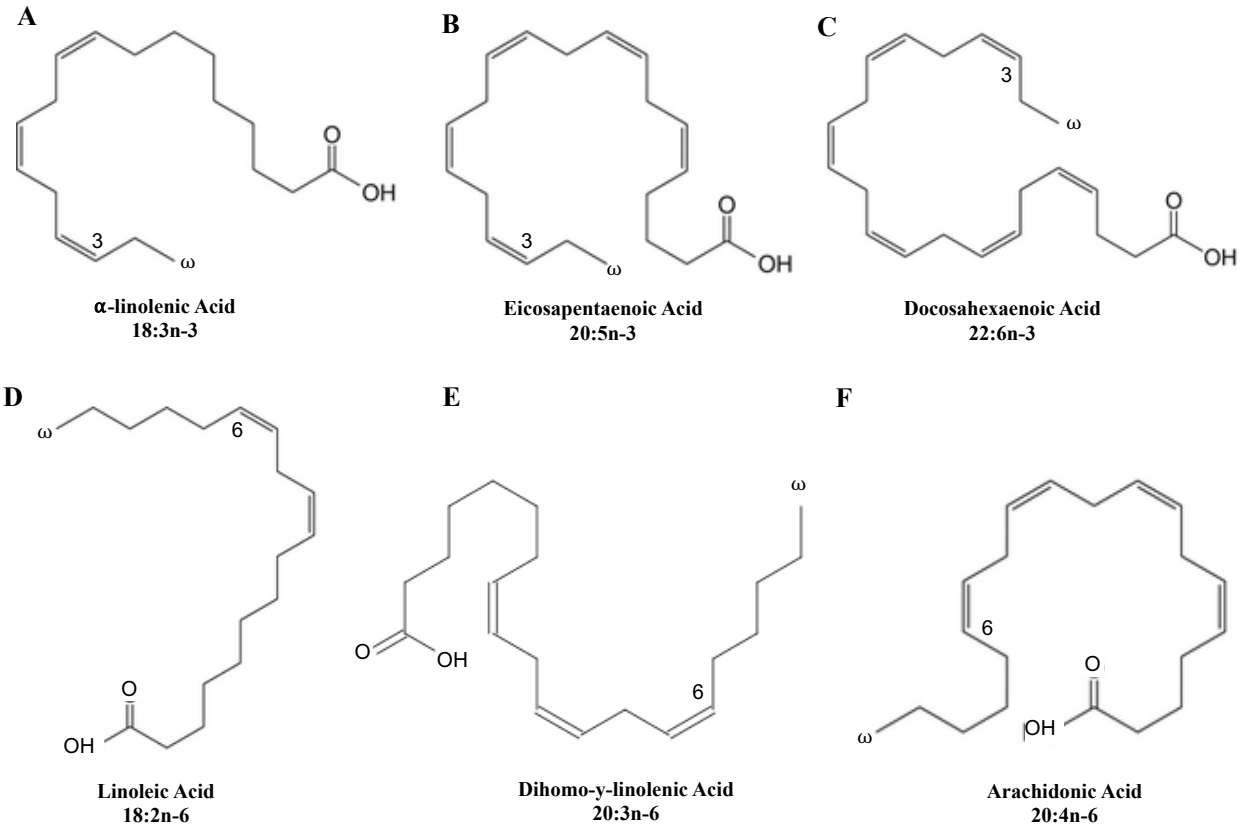
1.2.2 N-3 Polyunsaturated Fatty Acids

1.2.2.1 Chemical Structure and Nomenclature

Structurally, PUFA contain an acyl chain with a carboxyl group at one end (-COOH) and a methyl group ($-CH_3$) at the other [35; 36]. It is this carboxyl group that easily forms ester bonds with alcohol groups to create acylglycerols; the most important of which being triacylglycerols and phospholipids (PL) [36]. N-3 PUFA have the first double bond on the third carbon from the methyl group, whereas n-6 PUFA have the first double bond on the sixth carbon from the methyl end (**Figure 1-2**) [35; 36]. Mammals are unable to synthesize n-3 linoleic acid (LA, 18:2n-6) and α-

linolenic acid (ALA, 18:3n-3) *de novo*, and therefore these fatty acids must be obtained from the diet [36]. Because of this, LA and ALA are known as dietary essential fatty acids [35; 36]. Eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and di-homo-γ-linolenic acid (DGLA, 20:3n-6) and arachidonic acid (AA, 20:4n-6), are long chain PUFA produced from the enzymatic elongation and desaturation of ALA and LA, respectively.

Figure 1-2: Biochemical structures of n-3 PUFA ALA, EPA, DHA, and n-6 PUFA LA, DGLA, and AA. *Adapted from U.S National Library of Medicine.



1.2.2.2 Sources and Reference Intakes

N-3 PUFA are found in a number of dietary sources and these sources differ for ALA, EPA, and DHA. ALA is commonly found in green leaves, seeds, seed oils and nuts [37; 38]. Flaxseed is one of the most abundant sources of ALA, with approximately 45-55% of the fatty acids being ALA, while soybean oil and canola oil contains significantly lower amounts (5-10%) as ALA. In contrast, when considering dietary sources of longer chain PUFA as EPA and DHA, seafood is the greatest dietary source [38] with fatty fish (e.g. mackerel, salmon, tuna, etc.) being the best source of n-3 PUFA. In regard to plant sources of EPA and DHA, seaweed and algae are sources with the highest levels [39]. Because of this, canola has recently been genetically modified with the genes from microalgae, enabling the canola to produce oil rich in DHA [40].

In order to ensure that nutritional needs are met in the Canadian population, dietary reference intakes are set for many micro- and macro-nutrients. With respect to ALA, there is an adequate intake (AI) set at 1.6 g/day for males and 1.1 g/day for females [42; 43], but no set upper limit [43]. The average North American consumption of dietary ALA is between 0.5-2 grams per day [43]. Regarding EPA and DHA, there are a number of different recommendations for daily consumption depending on the institution (**Table 1**), however, there are currently no dietary reference intakes [41]. The consensus among health organizations within Canada, as well as internationally, is a recommended minimum consumption of 0.25-0.50 g/day of combined EPA and DHA for healthy adults. In cases of adverse health conditions, such as CVD, this recommendation may be higher [44]. A single serving of a "lean" fish might only contain 0.2-0.3 grams of long chain PUFA, whereas fatty fish can contain 1.5-3 grams per serving [43].

Unfortunately, current estimates as to the amount of EPA + DHA consumed daily in Canada is approximately 0.10-0.20 grams [45], which is well below the recommended intake.

Evidence has shown that different forms of long chain n-3 PUFA possess different bioavailability and kinetics. This has been shown in human trials where a specific dose of EPA and /or DHA was supplemented over a defined period of time, with the biomarker outcome being increases in blood levels of EPA and/or DHA [46]. Long chain PUFA can be in one of five supplemental forms: TAG, ethyl ester (EE), phospholipid, FFA, and partial glyceride [46]. TAG forms are the most common and are also the naturally occurring form found in our food supply (i.e. seafood). Because of this, research has found that supplemental forms of EPA and/or DHA as TAG have similar bioavailability to that of seafood sources [47; 48; 49]. EE forms are created through the use of molecular distillation to connect EPA/DHA fatty acids to ethanol, in order to create a more "concentrated" form [49]. However, the majority of the scientific evidence has concluded that TAG forms are more bioavailable than the EE form [49].

Unlike TAG, in the phospholipid form, EPA, DHA and other fatty acids are connected to a glycerol backbone with a polar head group (phosphorylated base moiety), as in the case of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. This particular form has increased in popularity in recent years over claims of increased bioavailability [46]. As krill oil contains EPA and DHA predominantly in a phospholipid form, trials have looked at the comparison between krill oil and TAG formulations [46]. Contrary to marketing claims, phospholipid formulations are not more bioavailable than TAG forms, but in fact, any increases in bioavailability in krill oil are a result of higher EPA or DHA [46]. Finally, EPA or DHA as FFA as well as partial glycerides are the least common of the supplemental forms [46]. All of these

supplemental forms aid in increasing the n-3 PUFA within membranes and lipid-rich tissues and allow for facilitation of many beneficial biological functions in the human body [50].

Table 1-1: Omega-3 fatty acid consumption recommendations by organization.

Organization	Target Population	Recommendation
World Health Organization (WHO) (Global) [51]	Adult Population	1-2% of energy/day n-3 PUFA
International Society for the Study of Fatty Acids and Lipids (ISSFAL) (Global) [52; 53]	Adult Population, cardiovascular health	~500 mg/day EPA+DHA
	Pregnant/Lactating Women	200 mg/day DHA
European Food Safety Authority (Europe) [54]	Adult Population	250 mg/day EPA+DHA
	Pregnant/Lactating Women	Adult recommendation + 100-200mg/day DHA
	2-18 years	250 mg/day EPA+DHA
	7-24 months	100 mg/day DHA
American Heart Association (United States of America) [44]	Adults without Chronic Heart Disease	Eat fish 2x/week (3oz cooked/meal)
	Adults with Chronic Heart Disease	1 g/day EPA+DHA (preferably from oily fish sources)
	Adults with high TAG	2-4 g/day EPA+DHA
Academy of Nutrition and Dietetics (American Dietetics Association) (United States of America) [42]	Adult Population	500 mg/day EPA+DHA
Minister of National Health and Welfare (Canada) [55]	Adult Population	1.2-1.6 g/day total n-3 PUFA (ALA, EPA, DHA)
Dietitians of Canada (Canada) [43]	Adult Population	300-450 mg/day n-3 PUFA
	Men 19+	1.6 g/day ALA
	Women 19+	1.1 g/day ALA
	Pregnant/Lactating Women	1.3-1.4 g/day ALA

1.2.2.3 CVD Risk Factors: Mechanism of Action and Biological Function

When n-3 PUFA consumption is increased, there is a subsequent increase in the n-3 PUFA content in cells and tissues [50]. These changes are able to alter cellular function in a number of different ways [50]. To begin, n-3 PUFA compete with n-6 PUFA for incorporation into cell membranes; ultimately leading to increased membrane fluidity and raft structure [56]. These rafts are critical for intracellular signaling and allow for increased activity of transporters and receptors [56]. In addition, increased n-3 PUFA availability allows for the production of potent bioactive and anti-inflammatory lipid metabolites (termed eicosanoids), and decreased production of pro-inflammatory eicosanoids that are produced from n-6 PUFA [4; 57; 58; 59]. This potential role of n-3 PUFA in reducing inflammation is important, as epidemiological evidence shows that individuals with increased markers of systemic inflammation have a markedly increased risk for the onset of CVD [60]. Specifically, an updated meta-analysis found that 1 standard deviation above baseline levels for IL-6, IL-18 and TNF-α resulted in a 10-25% risk increase in myocardial infarction, as well death resulting from coronary heart disease [60]. Thus, increasing n-3 PUFA consumption may mediate protection against CVD through a reduction in systemic inflammation.

This n-3 PUFA mediated improvement in inflammation is accomplished by eicosanoids derived from EPA and DHA, including resolvins, protectins, and maresins, which have an overall anti-inflammatory effect [4; 58; 59; 61]. Specifically, they do not function to prevent the onset of inflammation, but instead aid in the timely resolution of the inflammatory response [4; 58; 59]. To date, there have been nine resolvins (RvE1-RvE3, and RvD1-RvD6), two protectins (neuroprotectin D1 (NPD1) and AT-PD1) and two maresins (MaR1 and MaR2) characterised [4;

[59; 61]. The D series resolvins, as well as both neuroprotectins and maresins, are pro-resolving lipid mediators that are synthesized from DHA, whereas only the E series resolvins are produced from EPA [4; 59; 61]. When looking at the bioactive metabolites produced from n-6 PUFAs, there are both pro- and anti-inflammatory molecules created [62]. More specifically, the n-6 PUFA AA is a precursor for the production of both pro-inflammatory eicosanoids, i.e. prostaglandins, thromboxanes and leukotrienes, as well as anti-inflammatory eicosanoids, i.e. lipoxins and asprin-triggered lipoxins [62]. Aside from the production of inflammation-resolving bioactive molecules, n-3 PUFA can inhibit the production of eicosanoids derived from n-6 PUFA [4; 58; 59; 61]. This inhibitory effect is due to the shared enzymatic steps in the biosynthetic pathways, and the competition between n-6 and n-3 PUFA for conversion into their longer chain forms [63; 64]. The higher n-3 PUFA concentration getting pushed through the pathway causes a bottleneck in the enzymatic process, and ultimately leads to decreased availability of enzymes for n-6 PUFA intermediates to be converted into their longer chain forms [63; 64]. Thus, less pro-inflammatory eicosanoids subsequently produced [63; 64]. This reinforces the essentiality of a better n-6:n-3 PUFA ratio. This topic is discussed further, and in greater detail within section **1.5.3**. It is the physiological equilibrium of inflammatory responses, and a timely resolution, that prevents chronic inflammation and associated cardiometabolic risk [59; 61; 62].

The association of n-6 PUFA with CVD risk has been a long-scrutinized debate within the scientific community. Classically, increased consumption of n-6 PUFA was established to be a strong risk factor for many cardiometabolic and CVD [65], largely due to increased production of AA-derived prostaglandins, thromboxanes and leukotrienes [65; 66]. These molecules, created via the cyclooxygenase and lipoxygenase pathways, are potent promoters of acute and chronic inflammation, in addition to increasing platelet aggregation, vascular permeability and

bronchoconstriction [65; 67]. These effects can ultimately lead to the phenotype commonly seen in a number of human diseases, such as asthma, arthritis and CVD [67].

While an increase in n-6 PUFA is considered detrimental to health, in contrast increases in n-3 PUFA have been associated with reductions in circulating levels of pro-inflammatory cytokines [68]. Nuclear factor-kappa B (NF-κB) is an inducible transcription factor responsible for the production of cytokines and chemokines, including TNF-α, IL-1B, and IL-6, which are markers of inflammation and closely linked to the pathogenesis of CVD [68; 69]. Two studies to date have investigated the specific differential effects of EPA versus DHA on markers of inflammation in humans. Allaire et al. found that 10 weeks of supplementation with DHA in individuals with increased abdominal obesity and low grade inflammation resulted in a significant decrease in plasma IL-6 (12%), IL-18 (7%), CRP (C-reactive protein) (8%) and TNF-α (15%) when compared to corn oil, whereas supplementation with EPA only saw a significant decrease in plasma IL-6 (13%) [70]. When directly comparing these two fatty acids head to head, IL-18 decreased significantly more in the DHA as compared to the EPA supplemental group [70]. Conversely, Mori et al. saw that there were no significant differences following EPA or DHA treatment on plasma CRP and IL-6 levels in a shorter study (6-weeks) in hypertensive, type-2 diabetic participants [71]. Therefore, although there are somewhat conflicting results from clinical trials, n-3 PUFA have the potential to act in an anti-inflammatory role.

In addition to reported anti-inflammatory effects, n-3 PUFA have been shown to aid in the treatment of hypertriglyceridemia; another prominent risk factor for CVD [38; 72]. The decreased TAG synthesis as a result of n-3 PUFA consumption is postulated to occur due to reduced substrate availability, increased phospholipid synthesis, or reduced activity of fatty acid and

TAG synthesizing enzymes [72]. The reduced substrate availability, or fatty acids, can be due to an increase in beta-oxidation, a decrease in the delivery of FFA to the liver, decreased acetyl-CoA carboxylase activity or decreased *de novo* synthesis in the liver [72]. A recent meta-analysis of randomized, placebo-controlled trials showed that compared to control, supplementation with EPA and DHA produced significant reductions in TAGs of 0.37 mmol/L [73]. Furthermore, more recent studies have found significant decreases in LDL particle size, as well as plasma LDL-c, which is believed to be due to a decrease in LDL synthesis [69; 74]. As LDL-c reduction often coincides with the reduction in TAGs, it is difficult to discriminate which contributes more to decreasing the risk of CVD. Emerging evidence, however, has shown that supplements containing EPA or DHA have different effects on LDL-c and TAGs [75].

A recent review by Innes and colleagues compared the differential effects of EPA vs. DHA on cardiometabolic risk factors and concluded that, although both FA significantly reduced fasting plasma TAGs by 12-26%, DHA may be more efficacious [75]. This conclusion were similar to two more trials that were not included in this review, due to their stringent selection process. In these, treatment with approximately 5 g/day of EPA or DHA to normolipidemic individuals for four weeks resulted in a 22% reduction in TAGs in the DHA group ($p=0.032$) as compared to the olive oil control, while the EPA group failed to produce a significant decrease ($p=0.258$) [76]. Hansen and colleagues also found in normolipidemic individuals that DHA resulted in greater TAG lowering effects following 4 g/day of EPA or DHA treatment, although this trial was not controlled [77]. In contrast to these beneficial effects, DHA, but not EPA, treatment was found to increase LDL particle size [77]. To date, however, EPA and DHA consumption has only been shown to have moderate effects on circulating HDL-c levels [78; 79; 80]. Taken together, these anti-inflammatory, as well as lipid-decreasing effects, of long-chain n-3

PUFA play a substantial role in the risk reduction for CVD, and highlight potential for possible therapeutic support through diet [81].

1.3 Non-Modifiable Risk Factors for Obesity and CVD

In addition to modifiable risk factors such as diet, the risk of CVD is impacted by a number of non-modifiable risk factors, such as biological sex and genetics. Due to the pervasive inheritability of CVD, it is important to understand the mechanisms by which these non-modifiable factors can alter cardiovascular risk.

1.3.1 Genetic Variation

Single nucleotide polymorphisms, or SNPs, are variations at a single position in a DNA (deoxyribonucleic acid) sequence [82; 83; 84]. SNPs can occur by insertion, deletion, or point genetic mutations [82; 83; 84]. These variations will occur in individuals in the population in >1%, and when these variations occur within a gene, the gene can be described as having more than one allele [82; 83; 84]. On average, SNPs occur once in every 1,000 nucleotides, equating to approximately 4-5 million SNPs within the human genome [85]. When SNPs occur within the sequence of a gene, they are more likely to play a role in disease risk, as they affect the physiological function of the gene [82; 83; 84]. Due to the low mutation rates, it is highly unlikely that the same mutation will occur at the exact same location [82; 83; 84]. It is the study of the frequency with which these genetic variations occur within a population that is of recent interest for its implication in chronic diseases.

1.3.2 Population Genetics and Genetic Heritability of Disease

The study of population genetics involves the observation of genetic variation within populations, as well as the changes in gene or allele frequency over generations [86]. Factors such as population size, mutations, genetic drift, natural selection, environmental diversity, migration and non-random mating patterns, can affect the genetic diversity within a population [87]. The Hardy-Weinberg equilibrium (HWE) calculation allows for the prediction of the frequency in alleles and genotypes within a population with controlled assumptions (large sample size, random mating patterns, no mutations, genetic drift, natural selection, etc.) [87; 88]. Therefore, it is assumed that when a genetic variant is described as within HWE, the allele and genotype frequencies are close to the expected (current allele/genotype frequencies) [88].

When two loci (a fixed position on a chromosome used as a genetic marker) are near one another in the genome, the probability of these loci being inherited together increases [89]. Linkage disequilibrium (LD) measures the probability of these two loci being seen in the same haplotype of a population, whereas LD refers to a situation where haplotype frequencies within a population are the same if the genes at each locus were combined at random [89]. Therefore, linkage equilibrium is when LD is at zero, and vice versa [89].

Within the last decade there have been enormous developments in our knowledge of the genetic heritability of disease. Previously, linkage studies as well as candidate-gene association studies were the best trials available to understand the heritability of disease [90; 91]. Since then, genetic variants have been researched extensively for their association with human disease via a different method. Utilizing specific chip-based microarrays, genome-wide association studies (GWAS) have allowed for many genetic variants to be analyzed for associations with a human

disease in large cohorts [90; 91]. Through the discovery of these disease-associated genetic variants, earlier detection and prevention of the disease is possible. The most common methodology for this is the case-control scenario in which a healthy group is compared to a group affected by the disease [90; 91]. DNA is collected from both groups, and via the aforementioned chip-based microarray technology, approximately 500,00-1,000,000 SNPs are assayed [92]. These SNPs are then analyzed for their association to common disease phenotypes [92]. By this method, one is able to associate genetic markers to a clinical outcome, and thus identify how genetic differences play a role in assessing the risk for CVD.

1.3.3 Sex Differences

Another important non-modifiable risk factor for CVD is sex. Due to the differences in sex chromosomes between male and female, metabolic processes are regulated in a sexually dimorphic way, and result in variable risk for CVD [26]. Whether there are genetic variants prevalent in a sex-specific manner and whether such affects cardiovascular risk had been less explored. Liu and colleagues incorporated biological sex into GWAS analyses to elucidate gene-disease associations that might exist between men and women [93]. After correction for false discovery, they found that the rs7865618 SNP in CDKN2B-AS1, located on chromosome 9p21 is associated to increased incidence of coronary artery disease in males [93], which agrees with prior work [94]. In another study investigating sex-specific gene-disease interactions, Silander and colleagues investigated the gender differences in genetic risk profiles for CVD in two Finnish population cohorts [95]. Interestingly, the rs2069840 SNP in the *IL-6* gene showed strong associations to CVD incidence in men but not women ($p_{interaction}$=0.0004) [95]. Anagnostopoulou et al., was specifically interested in the *CETP* (cholesteryl ester transfer protein) and *LPL*

(lipoprotein lipase) polymorphisms and their sex specific effects on postprandial lipids in familial hypercholesterolemia [96]. Following an oral fat tolerance test, men carrying the B2 allele of the TaqIB polymorphism in *CETP* had significantly higher postprandial TAG peaks, as well as a prolonged return to baseline values as compared to women carrying the same allele [96]. These data show that sex-specific analyses within GWAS studies allow for a more completed understanding on individual disease risk.

An important biological mechanism of these sex differences is the contribution of individual hormones, such as estrogen. Estrogen concentrations have been proposed to elicit effects on the expression of genes involved with fat metabolism [97; 98]. Predominately mediated by the estrogen receptors alpha and beta, biological effects involve the regulation of the expression of estrogen-responsive genes via nuclear estrogen receptors [97; 98], as well as plasma membrane associated receptors [99; 100]. An experiment completed in ovariectomized rats evaluated the effect of a low-LA diet with or without estrogen on the expression of fatty acid conversion enzymes and transcription factors [101]. The low-LA diet and the low-LA+estrogen diet increased the hepatic expressions of *PPAR-α* (peroxisome proliferator-activated receptor-alpha), fatty acid desaturase (*FADS*) *2*, elongase of very long chain fatty acids 2 (*ELOVL2*) and *ELOVL5*. From these results, one can conclude that both the low LA diet and estrogen were able to enhance the conversion of ALA into DHA [101]. Other animal studies investigating the effect of estrogen on fatty acid metabolizing enzymes and n-3 PUFA levels have reported similar results [102; 103] and no significant effects [102]. In agreement with this animal work, these concepts also apply to human models based predominately via analysis of n-3 PUFA levels and not through quantification of expression of fatty acid metabolizing enzymes [104; 105; 106; 107]. Because of these effects of estrogen on the enzymes involved in the synthesis of n-3 PUFA as well as the products

from the biosynthetic pathway, it remains unknown if women would respond differently to n-3 PUFA intervention.

1.4 Fatty Acid-Gene Interactions

It has been long recognized that dietary patterns have a direct causal link to CVD risk. Unfortunately, simply changing the diet has not always proved effective. In the recent decade, GWAS have shed light on the biological mechanisms behind differential responses to foods and nutrients. Through the use of GWAS, genetic variants have been established for their associations with markers of CVD risk. The first wave of GWAS studies to emerge in the research field of CVD was in 2007, which identified a locus on chromosome 9p21 that was highly associated with myocardial infarction (CDKN2B-AS1) [108]. GWAS studies were key for the identification of many more candidate SNPs, and eventually led to the creation of international consortia [109; 110; 111; 112]. This large consortium allowed for exploration of genome-wide variants to be run in up to 180,000 people, with about 50% of these individuals having coronary artery disease [111]. This compilation allowed for more advanced GWAS in larger populations to occur ultimately identifying key genes that are involved in associated lipid and fatty acid metabolism.

1.4.1 GWAS: Genes Involved in Lipid and Fatty Acid Metabolism

LPL, apolipoprotein A-V (*APOA5*), apolipoprotein C3 (*APOC3*), and angiopoietin-like 4 (*ANGPTL4*) are some of the lipid metabolizing genes with alleles identified to affect overall cardiovascular risk [113]. LPL is a critical enzyme attached to the luminal surface of endothelial cells, that hydrolyzes the TAG bound to VLDL or chylomicrons [114]. This activity can be

increased by APOA5, an activator of LPL, or decreased by APOC3 and ANGPTL4 [115]. Because of this, the *APOA5* mutations can increase plasma TAG, whereas loss of function variants in *APOC3* and *ANGPTL4* can decrease TAG levels and subsequent cardiovascular risk [115; 116]. Furthermore, *LPL* has a number of different variants that can additionally alter its activity [114]. In addition to LPL, LDL-c and HDL-c may be impacted by genetics. For example, LDL metabolism has been found to be effected by a number of different genes, and subsequently many variants within those genes [113]. These variants are found to lead to changes in the genes that code for proteins involves in hepatic LDL-c clearance, and among the strongest associations is low-density lipoprotein receptor (*LDLR*), proprotein convertase subtilisin/kexin type 9 (*PCSK9*) and apolipoprotein B (*APOB*) [113]. *LDLR* is a protein coding gene for a cell surface receptor that binds LDL and transports it into the cell via endocytosis [113]. However, *PCSK9* encodes for a hepatic protease that attaches to the LDLR and ultimately leads to its transport into the cell and lysosomal destruction [117]. Finally, the *APOB* gene encodes for a large amphipathic glycoprotein called apolipoprotein B, which is critical in lipoprotein metabolism [118]. One of the two forms of this glycoprotein, apoB-100, is a core structural component of circulating lipoproteins, and is a ligand for receptor-mediated endocytosis of LDL [118]. Over 1000 different variants have been found in the genes coding for *LDLR*, *PCSK9* and *APOB* that lead to familial hypercholesterolemia [119]. Pertaining to HDL-c, CETP is responsible for the transfer of esterified cholesterol from HDL-c to other apolipoprotein B containing lipoproteins, in exchange for TAGs. Variants in the *CETP* gene have been associated with lower CETP mass and activity, ultimately leading to higher HDL-c concentrations, lower LDL-c levels, and overall lower cardiovascular risk [120]. Together, these data suggest that LPL, LDL-c, and HDL-c levels can be impacted by genetic variation in genes encoding their metabolism.

GWAS have also been employed to identify genetic variants that are associated with circulating fatty acid utilization/metabolism and CVD phenotype. These studies first began in serum and plasma to research genetic determinants of circulating fatty acid levels [121; 122; 123; 124]. More recently, this has transitioned to the use of red blood cell (RBC) fatty acids for a more stable indicator of chronic fatty acid levels [125]. Pertaining to SFA and MUFA metabolism, there are three critical genes for these processes: *FASN* (fatty acid synthase), *SCD-1* (Stearoyl-CoA desaturase-1), and elongase 6 (*ELOVL6*). The process begins with FASN, ultimately converting malonyl-CoA into the SFA as palmitate (16:0) [126]. Palmitate can then either be desaturated into palmitoleate (16:1n-7) by the enzymatic action of SCD-1, or elongated into stearate (18:0) by ELOVL6, which can then either be desaturated by SCD-1 (18:0 to 18:1n-9 or 18:1n-6, respectively) or continue via elongation into longer chain SFA. ELOVL6 can additionally elongate palmitoleate to oleate (18:1n-9) or vaccinate (18:1n-7) [126]. Due to the role of *SCD-1* in lipid homeostasis, variants in these genes have been associated with hyperlipidemia and the obese phenotype [127; 128; 129], whereas variants in *ELOVL6* have been associated with type 2 diabetes [130; 131]. Likewise, genetic variants modulating the biosynthesis and metabolism of n-3 and n-6 PUFA have been explored for their role in CVD etiology. Variants in *ELOVL2*, a gene encoding an elongation enzyme in the PUFA biosynthetic pathway, has been demonstrated to lead to increased levels of EPA and docosapentaenoic acid (DPA) but decreased levels of DHA [132; 133]. Al Saleh and colleagues' demonstrated this relationship, with minor allele carriers for 3 SNPs in *ELOVL2* having 30% higher plasma EPA levels than their major allele carrier counterparts following 6 months of supplementation with 1.8 grams of fish oil [134]. This suggests decreased production or activity of the elongase enzyme, and ultimately limits the beneficial impact that DHA can have systemically [134]. Thus, *FASN*, *SCD-1*, and *ELOVL6* are important

genes in fatty acid metabolism; however, the fatty acid desaturate gene (*FADS*) is critical in the biosynthesis of PUFAs.

1.5 Fatty acid desaturase genes (*FADS*)

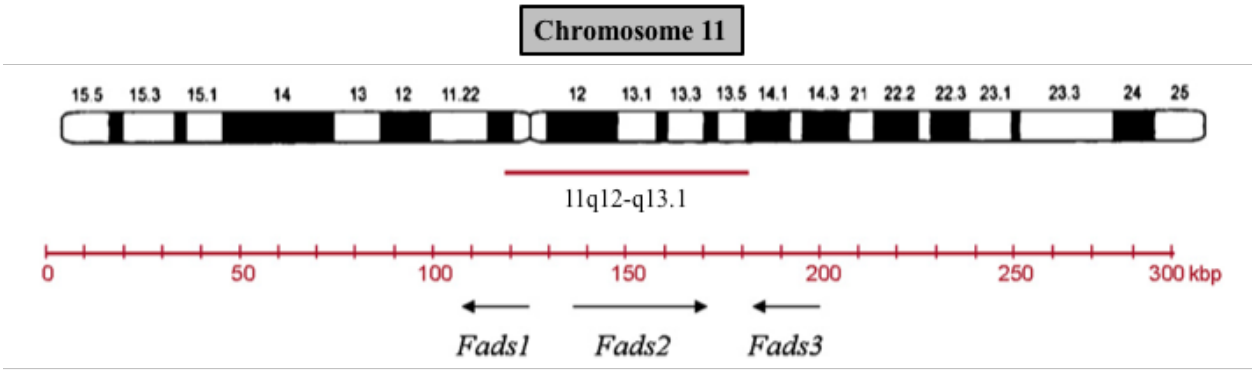
FADS1/2 are genes encoding for nonheme iron-containing enzymes that are responsible for catalyzing the introduction of a double bond into a fatty acid [135]. The *FADS1* and *FADS2* genes are critical in the biosynthesis of PUFAs, and genetic variants identified are associated with decreased long chain PUFA production. Specifically, individuals who are carriers of minor alleles have decreased conversion efficiency from ALA to EPA and DHA [136; 137; 138], such that large multi-ethnic studies have observed higher ALA, and lower EPA and DHA levels in these persons [122]. Similarly, the INCHIANTI GWAS found minor allele carriers for a genetic variant in *FADS1* (rs174537) had 18.6% less AA production, which was confirmed in the GOLDN (The Genetics of Lipid Lowering Drugs and Diet Network) study [139]. These data provide evidence for the role of genetic variants in the PUFA biosynthetic pathway and their importance in cardiovascular risk assessment.

1.5.1 Gene Classification of *FADS*

The *FADS1* and *FADS2* genes are separated by an 11kb region on human chromosome 11 (11q12-q13.1) and contain 12 exons and 11 introns (**Figure 1-4**). *FADS1* and *FADS2* span the 17.2kb and 39.1kb regions, respectively [135; 140]. The *FADS1* and *FADS2* genes encode for the Δ5 and Δ6 desaturases, respectively, which are membrane bound desaturases [135; 140]. The delta-5 desaturase, also known as D5D, is a desaturase that is responsible for desaturation on 20-carbon fatty acids from the methyl end. The delta-6-desaturase (D6D) enzymes also desaturate from the

methyl end, but are responsible for the desaturation of both 18-carbon and 24-carbon fatty acids [141]. Both genes that encode for D5D and D6D are 444 amino acids in length. Although D5D and D6D are enzymes with different functions, they share a 61% amino acid identity and are 75% similar [142]. There is additionally a *FADS3* gene located in the 6.0kb telemetric side of the *FADS2* gene, which encodes 445 amino acids [140; 143]. However, unlike the *FADS1/2* genes which play critical roles in the biosynthesis of long chain PUFA, the function and purpose of the *FADS3* gene still remains to be fully elucidated [140; 143].

Figure 1-3: The fatty acid desaturase (*FADS*) genes on chromosome 11.*adapted from Blanchard et al., 2011[144].

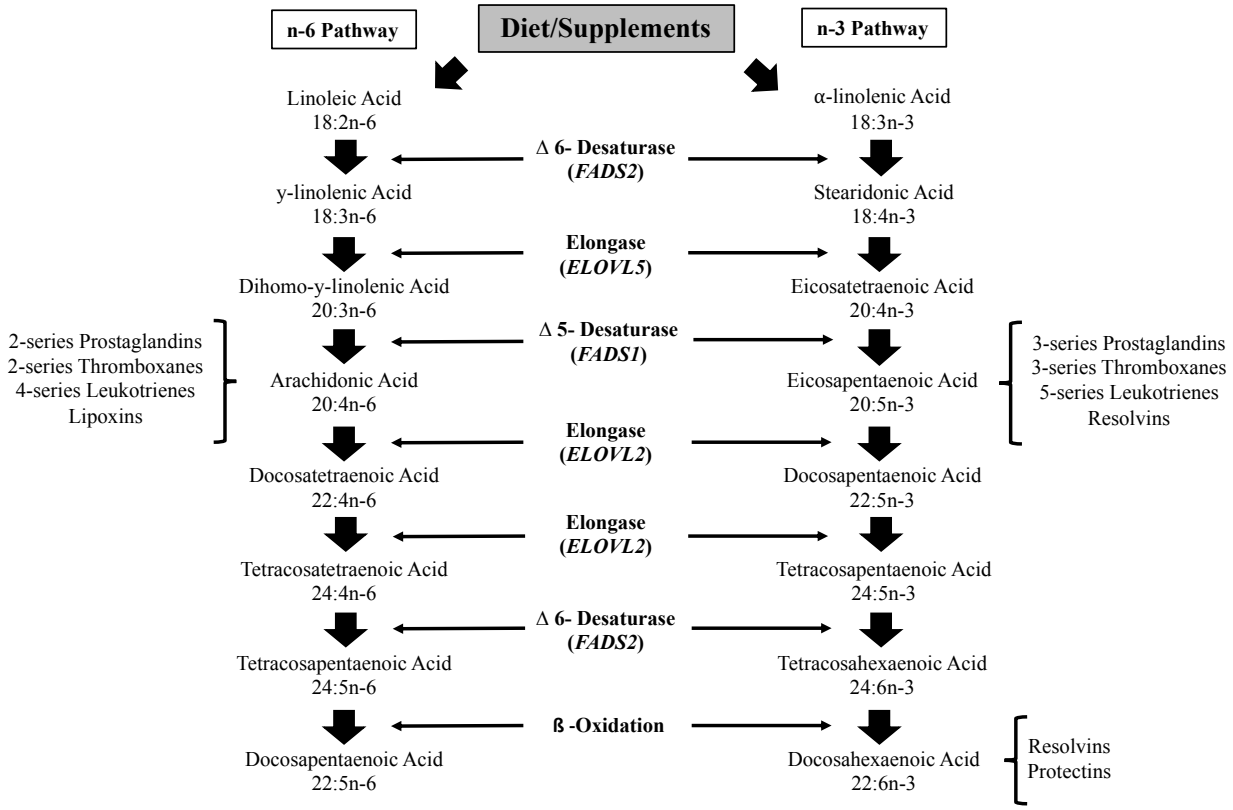


FADS1	FADS2	FADS3
Start: 61,799,625 bp	Start: 61,816,203 bp	Start: 61,873,523 bp
End: 61,817,057 bp	End: 61,867,354 bp	End: 61,892,224 bp

1.5.2 Biosynthesis of Omega-3 and Omega-6 PUFA

FADS1 and *FADS2* are critically important in the biosynthesis of n-3 and n-6 PUFA, which uses a series of elongation and desaturation steps [36; 145]. Briefly, within the endoplasmic reticulum, 18:3n-3 and 18:2n-6 are converted to 18:4n-3 and 18:3n-6 through the action of D6D [36; 145]. These products then undergo elongation by *ELOVL2/5*, in which malonyl-CoA donates a carbon group to create 20:4n-3, 20:3n-6, and CoASH [36; 145]. These two fatty acids are then enzymatically desaturated by D5D, to create 20:5n-3 and 20:4n-6 [36; 145]. Endoplasmic reticulum-mediated elongation occurs again for two successive steps via *ELOVL2/5* to create 22:5n-3 and 22:4n-6, followed by 24:5n-3 and 24:4n-6, respectively [36; 145]. D6D then catalyzes the conversion of 24:5n-3 and 24:4n-6 into 24:6n-3 and 24:5n-6 via the same reaction outlined above [36; 145]. The production of longer chain n-6 PUFA from 18:2n-6 compete with the production of longer chain n-3 PUFA from 18:3n-3, for biosynthetic enzymes within the endoplasmic reticulum [36; 145]. Because of this, n-6 and n-3 PUFA can competitively inhibit one another in times of high enzymatic demand. In the final steps of longer chain PUFA biosynthesis, 24:5n-6 and 24:6n-3 are converted to 22:5n-6 and 22:6n-3, respectively, via peroxisomal ß-oxidation [36; 145].

Figure 1-4: The polyunsaturated fatty acid biosynthetic pathway.



1.5.3 Function of Delta-5 and Delta-6 Desaturases

As mentioned previously, long chain PUFA, such as 20:4n-6, 20:5n-3 or 22:6n-3, are essential for many physiological processes in the body. The dietary essential fatty acids LA (18:2n-6) and ALA (18:3n-3) are the precursors for a pathway that leads to the production of these longer, and more desaturated, fatty acid products [36]. Mammals are able to desaturate fatty acids at positions Δ5, Δ6, and Δ9 [135]. The Δ9 desaturase SCD-1 is responsible for the conversion of SFA into MUFA [126; 146]; while Δ5 and Δ6 desaturases are responsible for desaturating longer chain PUFAs, as mentioned previously. As suggested by the names, the Δ5 desaturase catalyzes the desaturation even between the 5^{th} and 6^{th} carbon, whereas Δ6 desaturase is responsible for the desaturation between the 6^{th} and 7^{th} carbon [135]. In order for a double bond to be inserted into a PUFA, the Δ5 and Δ6 desaturases use O_2 (oxygen), NADH (nicotinamide adenine dinucleotide, reduced) and H^+ (hydrogen ion) as substrates, and produce (2)H_2O (water) and NAD^+ (nicotinamide adenine dinucleotide, oxidized) [135; 141]. Briefly, LA is first converted by D6D into γ-linolenic acid (GLA, 18:3n-6), then elongated to DGLA, and then converted by D5D into AA [135; 141]. Similarly, ALA is desaturated and elongated into EPA (20:5n-3), which can then undergo further conversion into DHA (22:6n-3) [135; 141]. The initial desaturation by D6D is known as the "rate limiting" step of longer chain PUFA production, and can create a bottleneck for the production of both n-6 and n-3 PUFAs [135; 141]. These processes are essential for the production of long chain PUFA; however, this biosynthetic pathway is not 100% effective in the conversion process within humans.

1.5.4 Conversion Efficiency of *FADS1/2*

Originally, stable isotope trials in humans of European decent showed that when defined amounts of 18 carbon PUFAs were consumed (i.e. 18:2n-6, 18:3n3), a small, but uniform, proportion of these were converted to their longer chain PUFA products [147]. This led researchers to believe that the conversion efficiency was uniform among all sexes, ages and ethnicities. Recently, research investigating the variants that exist in the *FADS1/2* cluster have concluded that the conversion efficiency is not in fact uniform, but can vary significantly. In the literature, the conversion efficiency of ALA to EPA varies greatly (0.2-21%), as well as ALA to DHA (0-9%) [148]. Adult males have a lower conversion efficiency than females, with 8% of ALA being converted to EPA, and less than 0.1% to DHA; while pre-menopausal females have approximately 9% conversion efficiency to DHA [149; 150; 151]. The theory behind this high conversion efficiency in women is that there is a decreased ß oxidation of ALA as compared to their male counterparts [149; 150]. Within women, the efficiency can again vary depending on whether they are pregnant or lactating [152; 153]. During this time, there is increased D6D activity due to higher estrogen levels, which results in increased conversion of ALA to DHA [152; 153]. Furthermore, competition for biosynthetic enzymes between dietary n-3 and n-6 essential fatty acids plays a large role in the conversion efficiency [63]. The Western diet has led to a shift in the n-6:n-3 PUFA ratio to a higher proportion of n-6 and lower proportion of n-3. Therefore, some have suggested that the amount of n-3 PUFA needed could be reduced by 10-fold if our consumption of n-6 PUFA were lowered to 2% of our energy intake, based on a 2000 kilocalorie(kcal)/day diet [63]. Thus, given that we can only covert a small portion of ALA into DHA and EPA, understanding how we can maximize this conversion or how it is impacted is of importance.

1.5.5 Implication of *FADS1/2* Genes in Disease Phenotype

Due to the essentiality of the *FADS1/2* genes for production of longer chain n-3 and n-6 PUFA, genetic variation in these genes can ultimately play a role in the disease phenotype [65; 154; 155]. Among the first trials to investigate this relationship was a study completed by Malerba and colleagues that investigated 13 SNPs in the *FADS1/2/3* genes and their association with fatty acid levels in serum (PL) in 658 Italian adults with CVD [138]. Major allele homozygotes in the *FADS1/2* gene were observed to have higher AA levels, a known precursor to pro-inflammatory molecules [138]. In a trial investigating the effects of the *FADS* genes on biosynthetic efficiency of n-3 and n-6 PUFA, Ameur et al. completed genome-wide genotyping on 5 different European cohorts (n=5652) [156]. In this study two common *FADS* haplotypes were observed, which were associated with lower levels of LA and ALA, and higher levels of longer chain PUFAs such as EPA, DGLA, DHA and AA [156]. Similarly, in one case control study investigating the possible role of the *FADS* genes on total cholesterol (TC) and TAG levels, 495 controls were compared to 497 patients with coronary artery disease [136]. Results showed that genetic variants in *FADS2* influence TC in a northern Chinese Han population [136]. LDL-c was also found to be related to the *FADS* genotype, in a study completed by Li et al [137]. In this study completed in 872 type 2 diabetic patients with coronary artery disease, the major allele in the rs1745437 SNP had increased levels of LDL-c, as well as elevated AA [137]. In addition to these findings investigating circulating lipid levels (i.e. plasma, serum, RBC), similar effects have been observed in human tissue. One study completed by Wang and colleagues investigated the lipid composition, and the *FADS* genotypes for 6 SNPs in 154 human liver tissue samples [157]. Their findings showed that minor allele carriers were associated with the accumulation of long chain fatty acids, increased ratios of more saturated to less saturated long chain fatty acids, as well as

overall increased liver fat content [157]. It is from the totality of this evidence, that a relationship can be seen between the *FADS* genotypes and CVD risk.

1.6 Summary

CVD and obesity incidence are increasing substantially. Thus it is important to understand both the modifiable and non-modifiable risk factors in order to fully understand the etiology of these diseases. N-3 PUFA have beneficial biological functions in the body, and therefore are important nutrients that can reduce the risk of these diseases [4; 5; 38]. As there are known genetic variants that can affect the biosynthesis of n-3 PUFA, and considering their low consumption in the Western diet, understanding the *FADS1* and *FADS2* genes, their critical role in this pathway, and factors that might play a role in *FADS1/2* efficiency, is essential [135; 138; 139]. Furthermore, it is important to understand what other genetic variants are associated with bioclinical markers (i.e. TAG, LDL-c, HDL-c) for CVD [112; 115; 116; 118; 120; 126; 134].

2 Thesis Objectives and Rationale

CVD is the number one cause of death globally [1]. Pharmaceutical or surgical strategies for prevention and treatment are expensive and often have undesirable side effects [3]. Interest has grown for factors that can contribute to individual variation in CVD risk, therefore this thesis investigated possible mechanisms that could contribute. Consequently, the overarching aim of this thesis was to understand whether genetic variance and biological sex impact CVD risk and if they can explain the individual response to treatment with the omega-3 polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). To achieve this, three studies were conducted as described below.

2.1 Study 1: Sex differences in blood HDL-c, the total cholesterol / HDL-c ratio, and palmitoleic acid are not associated with variants in common candidate genes.

2.1.1 Objective

To examine potential sex-genotype interactions between a panel of common candidate SNPs previously associated with blood lipids and/or fatty acids, with the lipid / fatty acid profile in two population-based cohorts. More specifically, the aims of Study 1 were to:

- Compare differences in anthropometric, clinical, and RBC FA data between men and women.
- Examine the association of SNPs between lipids and fatty acids via linear regression.
- Analyze genotype-sex interactions where appropriate.

2.1.2 Hypothesis

We hypothesized that blood lipid and fatty acid differences between males and females could be explained by common variants in genes regulating lipid and fatty acid metabolism.

2.2 Study 2: *FADS1* genotype is distinguished by human subcutaneous adipose tissue fatty acids, but not inflammatory gene expression.

2.2.1 Objective

To examine if *FADS1/FADS2* genotypes were associated with SAT fatty acid composition and the expression of obesity-related transcripts involved in inflammation, immune function, lipid metabolism, and cellular differentiation in participants from the DiOGenes (Diet, Obesity and Genes) study. Specifically, the aims of Study 2 were to:

- Examine the association between SNPs and SAT fatty acids via linear regression.
- Assess whether SAT fatty acids or SAT mRNA (messenger ribonucleic acid) levels could predict population sub-groups as defined by the rs174537 genotype of *FADS1* (i.e., major vs. minor allele carriers).
- Assess association between specific fatty acids and gene expression via linear regression.

2.2.2 Hypothesis

We hypothesized that individuals carrying major alleles in the *FADS1/FADS2* gene cluster would show increased fatty acid desaturase pathway activity and increased inflammatory gene expression in SAT.

2.3 Study 3: Treatment with High-Dose DHA, but not High-Dose EPA, is Associated with Reductions in Serum TAG levels in Young Adults.

2.3.1 Objective

To examine the independent effects of EPA and DHA on serum TAG levels, as well as other lipid and fatty acids associated with cardiometabolic risk, in young, healthy adults. Specifically, the aims of Study 3 were to:

- Investigate whether baseline healthy status impacts the supplementation response for serum TAG.
- Analyze change in serum TAG levels and influence of biological sex.
- Analyze change in serum TAG levels and influence of *FADS1* genotype for the rs174537 SNP.

2.3.2 Hypothesis

We hypothesized that both EPA and DHA treatment would affect fatty acid levels by promoting a shift to increased n-3 PUFA incorporation into erythrocytes, as well as decreased TAG levels and improved cardiometabolic risk factors. Furthermore, such decreased TAG levels would be due to biological sex or genetic differences.

3 Sex differences in blood HDL-c, the total cholesterol / HDL-c ratio, and palmitoleic acid are not associated with variants in common candidate genes

Presented as published:

Klingel SL, Roke K, Hidalgo B, Aslibekyan S, Straka RJ, An P, Province MA, Hopkins PN, Arnett DK, Ordovas JM, Lai CQ, Mutch DM (2017) Sex Differences in Blood HDL-c, the Total Cholesterol/HDL-c Ratio, and Palmitoleic Acid are Not Associated with Variants in Common Candidate Genes. *Lipids* **52**, 969-980.

3.1 Abstract

Blood lipids are associated with cardiovascular disease (CVD) risk. Moreover, circulating lipid and fatty acid levels vary between men and women, and evidence demonstrates these traits may be influenced by single nucleotide polymorphisms (SNPs). Sex-genotype interactions related to blood lipids and fatty acids have been poorly investigated and may help elucidate sex differences in CVD risk. The goal of this study was to investigate if the influence of SNPs previously associated with blood lipids and fatty acids varies in a sex-specific manner. Lipids and fatty acids were measured in serum and red blood cells (RBCs), respectively, in 94 adults (18-30 yrs) from the GONE FISHIN' cohort and 118 age-matched individuals from the GOLDN (The Genetics of Lipid Lowering Drugs and Diet Network) cohort. HDL-c (high-density lipoprotein-cholesterol) levels were higher and the TC (total cholesterol)/HDL-c ratio was lower in women versus men ($p<0.01$). RBC palmitoleic acid and the stearoyl-CoA desaturase index were both higher in women ($p<0.01$). Fatty acid desaturase (FADS) pathway activity (estimated using the ratio of eicosapentaenoic acid / alpha-linolenic acid) was higher in men ($p<0.01$). The AA genotype for rs1800775 in *CETP* had a lower TC/HDL-C ratio in men, but not women ($p_{int}=0.03$). Independent of sex, major alleles for rs174537 in *FADS1* (GG) and rs3211956 in *CD36* (TT) had higher arachidonic acid (AA), lower dihomo-γ-linoleic acid (DGLA), and a higher FADS1 activity compared to minor alleles. The current study showed that blood lipid and fatty acid levels vary between healthy young men and women, but that the observed sex differences are not associated with common variants in candidate lipid metabolism genes.

3.2 Introduction

Sex-specific differences in cellular, metabolic, and physiological processes are well recognized and thought to contribute directly to differences in cardiovascular disease (CVD) risk between men and women [158]. Dyslipidemia represents an important determinant of CVD risk [159], and was previously reported to have a stronger association with risk of acute myocardial infarction in men compared to women [160]. Broadly speaking, both blood lipid (e.g., low-density lipoprotein cholesterol, LDL-c; high-density lipoprotein cholesterol, HDL-c; triacylglycerol, TAG) and fatty acid (saturated fatty acids, SFA; monounsaturated fatty acids, MUFA; polyunsaturated fatty acids, PUFA) profiles have been associated with CVD risk and shown to differ between sexes [161; 162]. For example, pre-menopausal women have higher HDL-c levels compared to men [163], while men tend to have higher TAG and LDL-c levels compared to women [164; 165; 166]. Similarly, sex differences have also been reported in the fatty acid composition of blood phospholipids (PL). For example, α-linolenic acid (ALA) and docosahexaenoic acid (DHA) are on average higher in women compared to men [167], while the levels of SFA and MUFA are generally higher in men [167]. A large number of factors (e.g., hormones, body fat composition, age, and physical activity) contribute to the aforementioned sex differences in blood lipids and fatty acids; however, emerging evidence suggests that genetic factors may contribute to these sex differences.

Past genome-wide association studies (GWAS) have identified relationships between single nucleotide polymorphisms (SNPs) and lipids / fatty acids. These studies suggest that genetics may explain a significant proportion of the variation in circulating lipids and fatty acids. Indeed, 95 SNPs were found to explain up to 14% of the variability observed in blood lipids and,

more importantly, these variants correlated with CVD and metabolic risk [168; 169]. Similarly, large-scale genetic investigations reported that SNPs can account for 8-14% of the variance seen in abundant fatty acids in red blood cells (RBCs) [170]. While these past studies accounted for sex as a covariate in their analyses, evidence exists to suggest that genomic variation may underlie sex differences in various lipid and fatty acid traits [93; 171]. Consequently, further examination of the relationship between genetic variants and lipid/fatty acid parameters in a sex-specific manner may reveal helpful clues to better understand differences in CVD risk between men and women. Therefore, the goal of the current study was to examine potential sex-genotype interactions between a panel of common candidate SNPs previously associated with blood lipids and/or fatty acids and the lipid/fatty acid profile in two population-based cohorts.

3.3 Methods

3.3.1 Cohort Characteristics

The GONE FISHIN' cohort comprised 37 men and 57 women between 19-30 years of age, recruited at the University of Guelph between 2012-2016 for various omega-3 polyunsaturated fatty acids (n-3 PUFA) supplementation studies. The present study used only baseline anthropometric, bioclinical, and RBC fatty acid data from these individuals. All participants were deemed healthy and did not report a diagnosed medical condition (e.g., cancer or diabetes mellitus). Individuals using lipid altering medications were excluded. 35 of 57 females reported using oral contraceptives. These studies were approved by the Human Research Ethics Board at the University of Guelph, and written consent was collected for all participants.

The Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study is a part of the NHLBI Family Heart Study. GOLDN recruited participants (n=1,327) from families of European descent at two field centers: Minneapolis, MN and Salt Lake City, UT. The objective of the intervention study was to identify genetic factors that determine lipid responses to a high-fat meal challenge and 3-week treatment of fenofibrate (160mg/per day) [172]. Prior to study visit, participants were requested not to use lipid-lowering medications for at least 4 weeks, fast for at least 8 h, and consume no alcohol for at least 24 h. The study protocol was approved by the Institutional Review Boards at Tufts University, the University of Minnesota, University of Utah, and the University of Alabama at Birmingham. All participants provided written consent for the study. The present study consisted of a subset of age-matched men (n=57) and women (n=61) (i.e., age ≤30 years old) at baseline from the GOLDN study.

3.3.2 Clinical and Anthropometric Measurements

GONE FISHIN': Blood collection occurred at the Human Nutraceutical Research Unit at the University of Guelph (Guelph, Ontario). Blood samples were collected from participants after an overnight fast via venous puncture. The blood collection study visit was scheduled during the follicular phase of the women's menstrual cycle, as lipid measurements were previously reported to be more consistent during this time [173]. Fasted serum samples from each participant were sent to Lifelabs Medical Laboratory Services (Kitchener, Ontario, Canada) for the analysis of TAG, HDL-c, LDL-c, and TC. Anthropometric measures (age, height, body weight, and calculated BMI) were also collected on the day of the visit using standard protocols.

GOLDN: Blood samples were collected from participants after overnight fasting [174]. The lipid profile (e.g., TAG, HDL-c, LDL-c, and TC) was measured using traditional (enzymatic)

method [175]. Height and weight were measured while participants were dressed in an examining gown and wearing no shoes. BMI was calculated as weight in kilograms divided by the square of height in meters [176].

3.3.3 Fatty Acid Analysis of Red Blood Cells

GONE FISHIN': RBCs were separated from plasma following centrifugation at 3,000 rpm for 15 min at 4°C. RBCs were then aliquoted into 1.5mL Eppendorf tubes and stored at -80°C until analyzed. RBC FAs were extracted with chloroform:methanol (2:1, v/v) using the methodology established by Folch et al. [177]. Gas chromatography (GC) was performed as previously described [178]. Fatty acid methyl esters (FAME) were separated by GC using an Agilent 6890B gas chromatograph (Agilent Technologies, California, United States). FA peaks were identified by comparison to retention times of FAME standards. Individual FAs are indicated as a relative percentage (%) of total FAs. Fatty acids contributing to <0.25% of the total FA profile were excluded for the present analysis. Enzyme activities were estimated using established product-to-precursor ratios for stearoyl-CoA desaturase (SCD-16 and SCD-18), elongase 6 (ELOVL6), and fatty acid desaturases 1 and 2 (FADS1 and FADS2), as previously reported [179]

GOLDN: RBC fatty acids were extracted with a mixture of chloroform and methanol (2:1, by volume), collected in heptane, and then injected onto a Varian CP7420 100-m capillary column with a Hewlett-Packard 5890 gas chromatograph equipped with an HP6890A autosampler. To separate fatty acids from 12:0 through 24:1n-9, the initial temperature of 190°C was increased to 240°C over 50 min [180]. Enzyme activities were estimated as described above.

3.3.4 Selection of Genetic Variants

The panel of candidate SNPs used for the present analysis were selected following an extensive review of the literature for gene variants associated with lipid and/or fatty acid metabolism (Table 2). SNPs selected for the present investigation met several criteria, including: 1) associations were identified in large study populations, 2) used participants primarily of European / Caucasian descent, 3) showed a statistically significant association with a lipid and/or fatty acid parameter, and 4) had been replicated in two or more independent studies. Finally, candidate SNPs had to have a minor allele frequency (MAF) of >5% in the European population in 1000 Genomes (build ID 144).

3.3.5 DNA Extraction and Genotyping

GONE FISHIN': DNA was extracted from saliva for female participants using the Oragene DNA collection kit (DNA Genotek Ontario, Canada), and from whole blood for male participants using the Qiagen Paxgene Blood DNA kit (Qiagen, Toronto, Canada), according to manufacturer instructions. Genotyping was performed using the Sequenom MassArray platform at the Sick Kids Genetic Analysis Facility (Toronto, Canada). A total of 94 DNA samples were analyzed, of which no Mendelian errors were detected. Two DNA samples were randomly selected for replication and 100% concordance was achieved.

GOLDN: In this study, data from GOLDN was used to replicate findings from the GONE FISHIN' population. The process of genome-wide genotyping in GOLDN has been described in detail [181; 182]. Briefly, the hybrid genotype data of 10 SNPs was used, among which two SNPs (rs174570 and rs688) were genotyped using the Affymetrix Genome-wide 6.0 Array (Affymetrix, Santa Clara, CA). The remaining eight SNPs (rs174537, rs1799883, rs1800775,

rs2060792, rs2151916, rs3211956, rs6824447, rs953413, with the imputation quality score R Square >0.63) were imputed using MaCH software (Version 1.0.16) with the human genome build 36 as reference, and genotyped SNPs that met the following criteria [183]: call rate >96%, MAF >1%, and Hardy-Weinberg Equilibrium (HWE) test $P > 10^{-6}$.

3.3.6 Statistical Analyses

Prior to data analysis, all bioclinical and anthropometric variables were assessed for normality using the Shapiro Wilk's test. A non-parametric Mann-Whitney U-Test was used to compare differences in anthropometric, clinical and RBC FA data between men and women. Deviations from HWE were tested for each SNP within the total population, as well as within the male and female subgroups, using a χ-square test. This conservative approach ensured that both HWE and MAF were comparable in the total population, as well as within the male and female subgroups. Because of low numbers for homozygote minor alleles for most SNPs, we combined heterozygous and minor homozygous subjects into a single group termed "minor allele carriers". Linear regression was used to examine the associations between SNPs and lipids and/or fatty acids. Models were adjusted for age and BMI. When appropriate, the genotype-sex interaction was assessed with a 2-way analysis of variance (ANOVA, reported as p_{int}), followed by a post-hoc Tukey's HSD (honest significant difference). GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) and JMP 12 Statistical Software (SAS Institute, Cary, NC, USA) were used for all GONE FISHIN' cohort analyses.

For replication in GOLDN, similar covariates were adjusted in the same genetic model (i.e., heterozygous and minor homozygous subjects were combined into a single group termed

"minor allele carriers"). In addition, family relationship was modeled using proc GENMOD in SAS 9.4 (Cary, NC) software.

A $p < 0.05$ was considered statistically significant. A Bonferroni correction was used to account for multiple testing when investigating differences in RBC FAs between men and women. We performed a post-hoc power calculation using values for palmitoleic acid and HDL-c, since these were previously reported to differ between men and women. With a sample size of 37 men and 57 women in the GONE FISHIN' cohort, this study had 99% power to detect differences in fatty acids and blood lipids at a 5% significance level.

3.4 Results

3.4.1 Blood Lipids

All blood lipid values were in the normal healthy range in participants from both cohorts, in accordance with American Heart Association guidelines [184]. Compared to the GONE FISHIN' cohort, values for LDL-c, the TC/HDL-c ratio, and TAG were slightly higher in the GOLDN cohort, while TC and HDL-c levels were slightly lower. As reported in **Table 3-1**, women in both cohorts had higher HDL-c levels ($p<0.01$), while men had a higher TC/HDL-c ratio ($p<0.01$). No other differences in blood lipids were observed between men and women.

Table 3-1: Blood lipid measures in the GONE FISHIN' and GOLDN cohorts. Data is reported as mean ± SEM (Standard error of the mean). Bolded values indicate significant sex differences within each cohort ($p<0.05$). BMI, body mass index; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol.

	GONE FISHIN'				GOLDN			
Parameter	Total (n=94)	Female (n=57)	Male (n=37)	P value	Total (n=118)	Female (n=61)	Male (n=57)	P value
Age (years)	21.99 ± 0.20	21.63 ± 0.20	22.54 ± 0.37	0.08	22.5 ± 0.45	22.71 ± 0.51	22.28 ± 0.76	0.81
BMI (kg/m^2)	24.07 ± 0.35	23.72 ± 0.46	24.61 ± 0.53	0.10	25.64 ± 0.53	26.00 ± 0.82	25.26 ± 0.67	0.78
Total Cholesterol (TC) (mmol/L)	4.30 ± 0.10	4.39 ± 0.14	4.19 ± 0.13	0.17	3.97 ± 0.07	3.97 ± 0.07	3.97 ± 0.07	1.00
LDL-c (mmol/L)	2.32 ± 0.08	2.23 ± 0.10	2.46 ± 0.12	0.13	2.48 ± 0.07	2.42 ± 0.09	2.55 ± 0.09	0.26
HDL-c (mmol/L)	1.60 ± 0.04	1.76 ± 0.05	1.35 ± 0.05	2.49E-7	1.14 ± 0.03	1.22 ± 0.04	1.06 ± 0.03	5.80E-3
TC/HDL-c ratio	2.86 ± 0.08	2.63 ± 0.09	3.21 ± 0.13	2.51E-4	3.68 ± 0.11	3.39 ± 0.12	3.99 ± 0.19	0.02
Triglycerides (mmol/L)	0.96 ± 0.04	1.03 ± 0.06	0.84 ± 0.04	0.08	1.11 ± 0.07	1.04 ± 0.09	1.17 ± 0.10	0.36

3.4.2 RBC fatty acids

Palmitic, stearic, oleic, linoleic and arachidonic acid were the dominant fatty acids in RBCs in both cohorts (**Table 3-2**). Measurements for four fatty acids were not available in the GOLDN cohort (i.e., lignoceric, vaccenic, erucic, and adrenic acid); therefore, these fatty acids were not considered further.

To identify sex differences in RBC FAs, we performed three comparisons that varied in their degree of stringency. First, we investigated which RBC FAs differed significantly between men and women in both cohorts after accounting for multiple testing (i.e., high stringency). Only palmitoleic acid (16:1n-7) met this criterion, with women having higher levels (**Table 3-2**). Second, we next considered FAs that were significantly different between men and women, but for which this difference met our significance threshold corrected for multiple testing in only one of the two cohorts (i.e., medium stringency). As shown in **Table 3-2**, myristic acid (14:0) and DHA (22:6n-3) were higher in RBCs in women, while eicosapentaenoic acid (EPA, 20:5n-3) levels were lower in women. Finally, we also considered those fatty acids that were found to be statistically significantly different between sexes in only one of the two cohorts in the absence of a multiple testing correction (i.e., low stringency). SFA (stearic acid – 18:0; behenic acid – 22:0) were present at higher levels in men, while PUFA (ALA – 18:3n-3; docosapentaenoic acid – 22:5n-3) were lower (**Table 3-2**). No other differences in fatty acids were observed between men and women.

Table 3-2: Red blood cell fatty acid values in men and women in the GONE FISHIN' and GOLDN cohorts. Fatty acids in women and men are reported as relative % of total fatty acids in RBCs. A Mann Whitney U-test was used to determine sex differences ($p \leq 0.05$). Bolded values indicate significance in both cohorts following Bonferroni correction. Data is reported as mean% ± SEM. ND, not determined.

		GONE FISHIN'				GOLDN			
Common Name	Fatty Acid	Total (n=94)	Female (n=57)	Male (n=37)	P value	Total (n=118)	Female (n=61)	Male (n=57)	P value
Saturated									
Myristic Acid	14:0	0.54 ± 0.02	0.62 ± 0.02	0.44 ± 0.03	7.22E-5	0.29 ± 0.01	0.31 ± 0.02	0.26 ± 0.01	0.04
Palmitic Acid	16:0	22.30 ± 0.12	22.28 ± 0.18	22.33 ± 0.16	0.73	22.62 ± 0.11	22.81 ± 0.16	22.42 ± 0.13	0.15
Stearic Acid	18:0	11.95 ± 0.15	11.57 ± 0.15	12.53 ± 0.21	1.61E-3	9.20 ± 0.06	9.12 ± 0.10	9.30 ± 0.07	0.18
Arachidic Acid	20:0	0.47 ± 0.02	0.42 ± 0.01	0.55 ± 0.05	0.02	0.20 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.40
Behenic Acid	22:0	1.31 ± 0.02	1.27 ± 0.02	1.37 ± 0.02	1.89E-3	0.49 ± 0.01	0.51 ± 0.02	0.48 ± 0.02	0.20
Lignoceric Acid	24:0	3.91 ± 0.22	3.98 ± 0.36	3.79 ± 0.07	0.05	ND	ND	ND	ND
Monounsaturated									
Palmitoleic Acid	16:1n-7	0.64 ± 0.03	0.75 ± 0.02	0.44 ± 0.03	5.66E-10	0.36 ± 0.01	0.40 ± 0.01	0.32 ± 0.01	9.97E-4
Oleic Acid	18:1n-9	13.40 ± 0.18	13.45 ± 0.12	13.33 ± 0.41	0.30	13.91 ± 0.08	13.82 ± 0.11	13.99 ± 0.13	0.26
Vaccenic Acid	18:1n-7	1.27 ± 0.04	1.39 ± 0.10	1.10 ± 0.02	0.18	ND	ND	ND	ND
Erucic Acid	22:1n-9	0.59 ± 0.02	0.64 ± 0.02	0.52 ± 0.02	1.96E-4	ND	ND	ND	ND

Nervonic Acid	24:1n-9	3.94 ± 0.06	4.01 ± 0.07	3.83 ± 0.13	0.33	1.13 ± 0.03	1.15 ± 0.04	1.11 ± 0.05	0.22
Polyunsaturated									
Linoleic Acid	18:2n-6	13.79 ± 0.16	13.89 ± 0.19	13.64 ± 0.29	0.40	13.30 ± 0.12	13.14 ± 0.17	13.48 ± 0.17	0.18
α-Linolenic Acid	18:3n-3	0.45 ± 0.12	0.47 ± 0.01	0.44 ± 0.10	0.01	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.60
dihomo-γ-linoleic acid	20:3n-6	1.62 ± 0.04	1.59 ± 0.06	1.65 ± 0.08	0.83	1.76 ± 0.03	1.76 ± 0.03	1.76 ± 0.05	0.38
Arachidonic Acid	20:4n-6	12.52 ± 0.12	12.24 ± 0.13	12.96 ± 0.21	2.49E-3	13.69 ± 0.08	13.67 ± 0.11	13.71 ± 0.13	0.59
Eicosapentaenoic Acid	20:5n-3	0.57 ± 0.02	0.53 ± 0.02	0.64 ± 0.03	1.85E-3	0.42 ± 0.01	0.39 ± 0.01	0.46 ± 0.02	0.01
Adrenic Acid	22:4n-6	2.49 ± 0.09	2.65 ± 0.20	2.24 ± 0.05	0.69	ND	ND	ND	ND
Docosapentaenoic Acid	22:5n-3	2.01 ± 0.03	1.98 ± 0.04	2.06 ± 0.04	0.33	2.07 ± 0.03	1.99 ± 0.04	2.16 ± 0.03	7.76E-3
Docosahexaenoic Acid	22:6n-3	3.33 ± 0.07	3.49 ± 0.09	3.08 ± 0.10	3.04E-3	2.69 ± 0.06	2.88 ± 0.07	2.50 ± 0.08	4.22E-4
Estimates of Enzyme Activity									
SCD-16	16:1n-7/16:0	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	5.47E-11	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	1.81E-3
SCD-18	18:1n-9/18:0	1.12 ± 0.08	1.17 ± 0.12	1.06 ± 0.03	0.02	1.52 ± 0.02	1.53 ± 0.02	1.51 ± 0.02	0.60
ELOVL6	18:0/16:0	0.54 ± 0.01	0.52 ± 0.01	0.56 ± 0.01	7.62E-3	0.41 ± 0.01	0.40 ± 0.01	0.42 ± 0.01	0.05
FADS1	20:4n-6/20:3n-6	7.73 ± 0.63	7.70 ± 0.28	7.85 ± 1.55	0.57	8.08 ± 0.15	7.99 ± 0.19	8.19 ± 0.25	0.49

n-3 FADS pathway activity	20:5n-3/18:3n-3	1.28 ± 0.10	1.15 ± 0.10	1.45 ± 0.18	1.07E-3	3.09 ± 0.10	2.67 ± 0.10	3.53 ± 0.17	1.90E-05
n-6 FADS pathway activity	20:4n-6/18:2n-6	0.91 ± 0.02	0.88 ± 0.02	0.95 ± 0.03	0.01	1.04 ± 0.01	1.05 ± 0.02	1.03 ± 0.02	0.51

3.4.3 Desaturation and Elongation Activities

Estimated enzyme activities were generally comparable between the two cohorts. The only exception was with the estimate for “n-3 FADS pathway activity”, which was noticeably higher in GOLDN participants, driven by their lower levels of ALA. Similar trends were seen for the “n-6 FADS pathway activity” estimate.

Using a similar approach with regards to analysis stringency (as outlined above for individual fatty acids), we first examined estimated enzyme activities that were statistically significant after accounting for multiple testing in both cohorts. The SCD desaturation index (SCD-16) was higher in women, while "n-3 FADS pathway activity" was higher in men (**Table 3-2**). The ELOVL6 estimate was marginally higher in men, but met our criteria for multiple testing in only one of the two cohorts. The remaining estimates (SCD-18, FADS1 and n-6 FADS pathway activity) were not significantly different between men and women in either cohort.

3.4.4 Characteristics of Candidate SNPs

To investigate genotype-sex interactions, we selected 23 candidate SNPs in 17 genes that were previously reported in the literature to be associated with lipid and/or fatty acid metabolism (**Table 3-3**). Specifically, we examined the relationship between SNPs in *FASN* (fatty acid synthase), *SREBP1C* (sterol regulatory element-binding protein-1c), *APOA5 (*apolipoprotein A-V), *CD36* (cluster of differentiation-36), *HSL* (hormone sensitive lipase), *LPL* (lipoprotein lipase), *CETP* (cholesteryl ester transfer protein), and *PPARα* (peroxisome proliferator-activated receptor-alpha) with blood lipids, and the relationship between SNPs in *FADS1* (fatty acid

desaturase 1), *FADS2* (fatty acid desaturase 2), *ELOVL2* (elongase 2), *ELOVL6* (elongase-6), *SCD1, FASN, FABP-2* (Fatty acid binding protein-2), *GPR120* (G-protein coupled receptor 120), and *CD36* with RBC FA. MAFs for all SNPs ranged between 6% and 50% (**Table 3-3**). Four SNPs were not in HWE (rs688, rs2297508, rs61866610, 10883463) and therefore not considered further.

Table 3-3: Candidate SNP panel calculated from EUR population in 1000 Genomes, release build ID 144. SNP, single nucleotide polymorphism; MAF, minor allele frequency; NA, not available; FAs, fatty acids.

			GONE FISHIN'			GOLDN			
SNP	Gene	MAF 1000 Genomes	MAF Total	MAF Males	MAF Females	MAF Total	MAF Females	MAF Males	Investigated SNP associations with Lipid and/or FAs
rs174537 (G/T)	FADS1	0.35	0.29	0.32	0.27	0.38	0.34	0.42	Fatty acids
rs174575 (C/G)	FADS2	0.26	0.28	0.35	0.24	NA	NA	NA	Fatty acids
rs174570 (C/T)	FADS2	0.16	0.15	0.18	0.14	0.16	0.15	0.17	Fatty acids
rs953413 (A/G)	ELOVL2	0.44	0.50	0.55	0.46	0.40	0.39	0.39	Fatty acids
rs9393903 (G/A)	ELOVL2	0.24	0.27	0.28	0.25	NA	NA	NA	Fatty acids
rs6824447 (A/G)	ELOVL6	0.47	0.48	0.42	0.48	0.47	0.44	0.48	Fatty acids
rs2060792 (T/C)	SCD1	0.32	0.27	0.20	0.32	0.26	0.22	0.31	Fatty acids
rs10883463 (T/C)	SCD1	0.05	0.09	0.19	0.08	NA	NA	NA	Fatty acids
rs2229422 (A/G)	FASN	0.31	0.35	0.27	0.39	NA	NA	NA	Lipids & Fatty acids
rs2297508 (A/G)	SREBP-1C	0.41	0.45	0.46	0.44	NA	NA	NA	Lipids
rs662799 (A/G)	APOA-5	0.08	0.07	0.08	0.06	NA	NA	NA	Lipids

rs1799883 (C/T)	FABP-2	0.27	0.30	0.26	0.33	0.25	0.21	0.28	Fatty acids
rs61866610 (C/T)	GPR120	0.04	0.08	0.07	0.09	NA	NA	NA	Fatty acids
rs3211956 (T/G)	CD36	0.08	0.06	0.05	0.06	0.09	0.11	0.07	Lipids & Fatty acids
rs2151916 (T/C)	CD36	0.37	0.35	0.41	0.31	0.44	0.42	0.46	Lipids & Fatty acids
rs1206034 (G/A)	HSL	0.35	0.36	0.32	0.38	NA	NA	NA	Lipids
rs320 (T/G)	LPL	0.29	0.31	0.34	0.30	NA	NA	NA	Lipids
rs328 (C/G)	LPL	0.13	0.14	0.14	0.14	NA	NA	NA	Lipids
rs1800775 (A/C)	CETP	0.49	0.49	0.47	0.49	0.43	0.47	0.40	Lipids
rs688 (C/T)	LDLR	0.44	0.38	0.36	0.39	0.46	0.45	0.47	Lipids
rs1800206 (C/G)	PPAR-α	0.05	0.08	0.08	0.08	NA	NA	NA	Lipids & Fatty acids
rs708272 (G/A)	CETP	0.43	0.41	0.43	0.39	NA	NA	NA	Lipids
rs5882 (A/G)	CETP	0.33	0.37	0.34	0.39	NA	NA	NA	Lipids

3.4.5 Gene-Lipid Associations

We first investigated associations between SNPs and blood lipids in men and women combined in each cohort. Statistically significant associations between rs1800775 in *CETP* with HDL-c levels (p=2.49E-7) and the TC/HDL-c ratio (p=2.51E-4) were observed in the GONE FISHIN' cohort. Specifically, we found that major allele carriers (AA) had significantly higher HDL-c levels when compared to their minor allele carrier counterparts (CA+CC), while the opposite relationship was seen for TC/HDL-c. However, these associations failed to replicate in GOLDN (p=0.29 and p=0.97, respectfully). In *FASN,* rs2229422 was significantly associated with TAG levels in the GONE FISHIN' cohort, with major allele carriers (AA) having lower TAG compared to minor allele GA/GG carriers (p=0.04). However, we were unable to confirm this finding, as this SNP was not genotyped in the GOLDN cohort. None of the other candidate SNPs were associated with any of the examined lipid traits.

3.4.6 Genotype × Sex Interactions Related to Blood Lipids

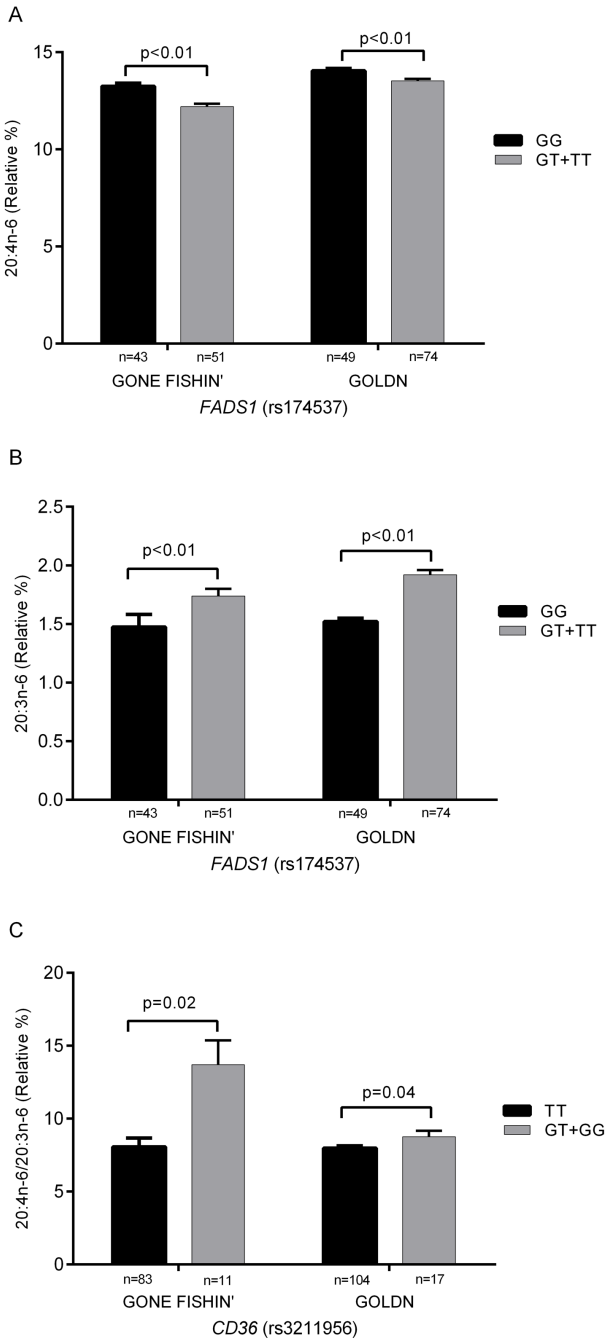
Overall, no consistent genotype-sex interactions were detected with blood lipids in the present study. As mentioned above, an association was identified between rs1800775 in *CETP* and the TC/HDL-c ratio in the GONE FISHIN' cohort. We also found a significant genotype-sex interaction for this association in the same cohort (p_{int}=0.03). Specifically, male homozygotes for the major allele (AA) had a significantly lower TC/HDL-c ratio compared to male carriers of the minor allele (CA/CC). No difference between genotypes was observed between women. However, this genotype-sex interaction was not replicated in the GOLDN cohort.

3.4.7 Gene-Fatty Acid Associations

We next investigated associations between SNPs and RBC FAs in the combined male and female population of both cohorts. In *FADS1,* rs174537 was significantly associated with AA levels in both cohorts ($p<0.01$), with major allele carriers (GG) having higher levels compared to minor allele carriers (GT+TT) (**Figure 3-1, A**). We also observed that this same SNP was significantly associated with dihomo-γ-linoleic acid (DGLA, 20:3n-6) in both cohorts ($p<0.01$), with major allele carriers having significantly lower levels compared to minor allele carriers (**Figure 3-1, B**).

Finally, major allele carriers (TT) for the rs3211956 SNP in *CD36* had a higher estimated FADS1 ratio (i.e., AA/DGLA) compared to minor allele carriers (GT+GG) in both cohorts (**Figure 3-1, C**).

Figure 3-1: Significant associations between fatty acids and SNPs in *FADS1* and *CD36*. Relative percentage for AA (20:4n-6) (A) and DGLA (20:3n-6) (B) in participants stratified as major (GG) or minor (GT+TT) carriers of rs174537 in *FADS1*. (C) FADS1 activity estimate (i.e., 20:4n-6/20:3n-6) in participants stratified as major (TT) or minor (GT+GG) for rs3211956 in *CD36.* Data is reported as mean% ± SEM. P-values correspond to post-hoc analyses following a 2-way ANOVA.



3.4.8 Genotype × Sex Interactions related to Fatty Acids

No genotype-sex interactions were detected between any of the candidate SNPs investigated in the current study and RBC FA levels.

3.5 Discussion

The present study investigated if sex differences in blood lipids and FA were associated with common genetic variants previously reported in the literature. The primary findings from the current study were: 1) HDL-c was higher and the TC/HDL-c ratio was lower in women compared to men, 2) palmitoleic acid and the SCD desaturation index were higher in RBCs in women compared to men, and 3) despite finding weak associations in the total population between SNPs in *CETP, FASN,* and *FADS1* with HDL-c, TAG, and specific FAs levels, respectively, we found no evidence that these traits were modulated by the candidate SNPs in a sex-specific manner. Overall, our findings suggest that the SNPs investigated in the current study do not contribute significantly to sex differences in blood lipids and FA in young adults.

3.5.1 Sex Differences in Blood Lipids and Fatty Acids

Risk of cardiovascular disease (CVD) mortality is two-to-five times greater in men versus women [185]. In general, risk factors for CVD, which include HDL-c and TC, are more favorable in women compared to men. In agreement with previous reports [185; 186; 187], we found that women had higher HDL-c levels and a lower TC/HDL-c ratio compared to men. These differences align with previous findings in the Framingham Offspring Study [188]. Interestingly, a past study by Freedman et al. found that not only was HDL-c higher in women than men, but so was the proportion of larger anti-atherosclerotic HDL particles [188]. Together, this would confer

a reduced risk of CVD in women. Further, these aforementioned sex differences were most apparent in younger adults compared to older adults. In contrast to Freedman et al. [188], we did not observe sex differences in LDL-c and TAG levels. It is possible that these discrepant findings stem from differences in sample size and demographics (i.e., Freedman et al. had a larger sample and investigated associations in older adults compared to the current study).

We also observed sex differences in several RBC FA and estimates of enzyme activity. Most notably, we found that women had higher levels of RBC palmitoleic acid. While palmitoleic acid can be obtained in low quantities through the consumption of plant oils and animal fats, it is primarily produced *de novo* from the delta-9-desaturation of palmitic acid [8]. In agreement with increased palmitoleic acid levels, we also found that women had a higher SCD-16 desaturation index. Together, this suggests that females have increased SCD activity compared to men. Stark et al. previously reported that post-menopausal women had lower relative levels of serum palmitoleic acid compared to pre-menopausal and post-menopausal women receiving hormone therapy [189]. This suggests that the higher levels of palmitoleic acid and the SCD-16 desaturation index in young women may be related to hormone differences between the two sexes. Additionally, blood levels of palmitoleic acid have been proposed to serve as a marker of AT lipolysis and hepatic *de novo* synthesis [190]. Consequently, RBC palmitoleic acid levels may serve as a potential blood indicator for a person's overall metabolic health.

We also found sex differences in EPA and DHA levels, with women having less EPA and more DHA in RBCs. Interestingly, when estimating the activity of the FADS pathway using the product-to-precursor ratio of EPA / ALA, we observed that men had a higher FADS pathway

activity. While seemingly contradictory, these findings can be reconciled when considering past studies that investigated ALA conversion into DHA using tracers. Specifically, it was shown that women have a higher capacity to convert ALA into DHA [191]. Consequently, this means that women would be expected to have higher levels of DHA in relation to EPA. In contrast, men have a lower ability to synthesize DHA, which would lead to an accumulation of EPA. This differential pathway activity is particularly intriguing given emerging evidence showing that EPA and DHA have distinct effects on blood lipids, glycemic control, and inflammation [192; 193].

Finally, we also found that women had higher levels of RBC myristic acid compared to men. This is noteworthy given that previous studies have reported positive correlations between circulating myristic acid levels and CVD risk [172; 194; 195]; however, these studies did not stratify their cohorts by sex when investigating these associations. As such, caution is warranted when interpreting our finding that women had higher RBC myristic acid levels. Indeed, the relative amounts of myristic acid compared to other SFA (e.g., palmitic and stearic acids) are considerably lower in RBCs – and no sex differences were observed with these more abundant SFA. Moreover, when considered collectively, young females in our study had a more favourable CVD risk profile compared to men. Nevertheless, future studies examining sex differences in FA profiles should consider monitoring diet intake and *de novo* lipogenesis, both of which could explain these minor sex differences in myristic acid.

3.5.2 The Relationship between Blood Lipids, Genotype and Sex Differences

We examined a panel of SNPs located in 8 genes known to influence blood lipids, and found that only rs1800775 in *CETP* was significantly associated with HDL-c levels and the TC/HDL-c ratio. While rs1800775 was genotyped in both the GONE FISHIN' and GOLDN, the

identified associations were not consistent between the two cohorts. The lack of reproducibility could stem from a multitude of reasons that include differences in lifestyle and environmental factors, small sample sizes, and demographics between the two cohorts. Nevertheless, our finding in the GONE FISHIN' cohort aligns with previous reports by other research groups and thus warrants further discussion.

The *CETP* gene encodes the cholesterol ester transfer protein, which plays a critical role in reverse cholesterol transport [159]. Past research has shown that homozygote carriers of the major allele (AA) for the rs1800775 SNP in *CETP* have significantly higher HDL-c levels compared to their minor allele counterparts (CA+CC) [196; 197]. An inverse relationship was also seen between this SNP and the TC/HDL-c ratio [198]. In our study, we found similar associations in GONE FISHIN', but not GOLDN. While rs1800775 in *CETP* might be valuable for CVD risk prediction in the general population, we did not find evidence that this SNP had a differential effect on HDL-c levels in men and women. Nevertheless, past evidence suggests a relationship between SNPs in the *CETP* gene and sex differences in blood lipids may exist. For example, Papp et al. revealed that a haplotype consisting of the rs5883 and rs9930761 SNPs in *CETP* was associated with HDL-c levels and CVD risk in men, but not women [199]. Moreover, Anagnostopoulou et al. also reported an interaction between sex and two other SNPs in *CETP*, rs708272 (also known as TaqIB) and rs5882 (also known as I405V), on postprandial TAG response following an oral fat tolerance test [200]. Collectively, these previous reports highlight that further investigation is necessary to clarify the sex-specific effects of genetic variation in *CETP* on both fasting and postprandial lipids.

3.5.3 The Relationship between RBC Fatty Acids, Genotype and Sex Differences

There is considerable evidence in the literature that SNPs in the *FADS1* gene are associated with serum, plasma, RBC, and tissue FA profiles [201]. The present study replicated previous findings between the rs174537 SNP in *FADS1* and specific FA, but found no evidence that this association varied in a sex-specific manner. *FADS1* encodes the delta-5-desaturase (D5D), which regulates the conversion of DGLA into AA [201]. Previous reports have shown that individuals carrying minor alleles in *FADS1* SNPs have lower desaturase activity compared to major allele carriers [201]. As expected, minor allele carriers (GT+TT) for the rs174537 SNP in *FADS1* had lower RBC AA levels compared to major allele carriers (GG), and correspondingly more DGLA. This observation may have implications for CVD risk, because this same SNP was previously shown to not only influence AA synthesis, but also pro-inflammatory oxylipin production in whole blood [202]. However, we did not find any evidence of a sex-genotype interaction between *FADS1* and omega-6 polyunsaturated fatty acids (n-6 PUFA) in RBCs.

Interestingly, we also observed a higher FADS1 activity estimate (AA/DGLA) in minor allele carriers (TT) of the rs3211956 SNP in *CD36* compared to major allele carriers (GT+GG). The *CD36* gene encodes an integral membrane protein, which binds and facilitates the uptake of FA from blood into tissues around the body [203]. Although not possible to definitively conclude from our data, this association may suggest a degree of FA specificity by CD36. In other words, CD36 protein in minor allele carriers for the rs3211956 SNP may have a preferential affinity for DGLA uptake into tissues compared to AA, which would consequently lead to an increase in the FADS1 activity estimate in RBCs. Although we did not detect a sex-genotype interaction with the rs3211956 SNP (nor has one been reported previously in the literature), sex differences in

hepatic CD36 gene expression have been demonstrated by Stahlberg et al [204]. It may be valuable to continue investigating the role of CD36, both at the level of SNPs as well as gene/protein expression, as this may uncover differences in FA transport mechanisms between men and women that could alter CVD risk.

3.5.4 Strengths and Limitations

The present study has several strengths and limitations that should be considered. A major strength of this study was the use of two independent age-matched cohorts to investigate sex differences in lipids and FA in young adults. However, we acknowledge that the sample sizes of the age-matched cohorts were small, and that the sex distribution was not equivalent. Future studies with a comparable number of men and women, as well as increased sample size, would improve the power to identify sex-genotype interactions. From a technical perspective, the two studies used different approaches for genotyping. As such, not all of the candidate SNPs investigated in the GONE FISHIN' cohort were available in the GOLDN cohort. Finally, we acknowledge that investigating the influence of these common SNPs in diseased or at-risk populations may reveal sex differences that are not apparent in healthy young adults.

3.5.5 Conclusion

The findings from current study have both confirmed and identified sex differences in specific blood lipids / FA in RBCs between young, healthy male and female adults. Our results do not support the hypothesis that these differences are related to SNPs in key candidate genes associated with lipid and FA metabolism. However, previous studies in the literature suggest that such sex-genotype interactions might exist. Further investigations examining factors that

contribute to sex differences in blood lipids and FA will continue to advance our understanding of differences in CVD risk between men and women.

4 FADS1 genotype is distinguished by human subcutaneous adipose tissue fatty acids, but not inflammatory gene expression

Presented as published:

Klingel SL, Valsesia A, Astrup A, Kunesova M, Saris WHM, Langin D, Viguerie N, Mutch DM. (2018) FADS1 genotype is distinguished by human subcutaneous adipose tissue fatty acids, but not inflammatory gene expression. *Int J Obes (Lond).* doi:10.1038/s41366-018-0169-z.

4.1 Abstract

Background: Single nucleotide polymorphisms (SNPs) in *FADS1/FADS2* genes are associated with changes in serum and tissue polyunsaturated fatty acids (PUFA) content. PUFA regulate inflammatory signalling pathways in adipose tissue; however, the effect of SNPs in *FADS1/FADS2* on adipose tissue inflammation is equivocal. The present study examined if SNPs in *FADS1/FADS2* modify human subcutaneous adipose tissue (SAT) fatty acid (FA) profiles and the expression of genes associated with inflammation/immune function, lipid metabolism and cellular differentiation.

Methods: SAT fatty acids and the expression of 117 genes were measured in 174 men and women from the DiOGenes Study using gas chromatography (GC) and qRT-PCR, respectively. Associations between FA, gene expression, and SNPs in *FADS1/FADS2* were investigated by linear regression and multivariate analysis.

Results: Four SNPs (rs174537, rs174546, rs174556, rs174601) in *FADS1/FADS2* were significantly associated with SAT FA. All SNPs were in high linkage disequilibrium (LD) with the commonly reported rs174537 SNP in *FADS1*. Minor allele carriers for rs174537 (GT+TT) had reduced 20:4n-6 (p=1.74E-5), lower delta-5 desaturase enzyme activity (p=2.09E-9), and lower *FADS1* gene expression (p=0.03) compared to major GG carriers. Multivariate analysis revealed that 20:4n-6 and 20:3n-6 explained ~19% of the variance between rs174537 genotypes, while gene expression explained <7%. Receiver operating characteristic (ROC) curves indicated that rs174537 genotype can be distinguished with SAT FA (AUC=0.842), but not gene expression (AUC=0.627). No differences in SAT inflammatory gene expression were observed

between rs174537 genotypes. SAT 20:3n-6 levels were positively correlated with the expression of several inflammatory genes, and inversely correlated with *FADS1* expression.

Conclusion: This study showed that *FADS1* genotype is distinguished by SAT FA profiles, but not inflammatory gene expression.

4.2 Introduction

Obesity is a low-grade inflammatory state associated with increased risk of type-2 diabetes and cardiovascular disease [205]. The pro-inflammatory state underlying obesity stems, in large part, from cellular and metabolic adaptations within adipose tissue (AT) [206]. Considerable evidence shows that high levels of saturated fats (SFA) trigger numerous signaling pathways that promote AT inflammation, while high monounsaturated fats (MUFA) do the opposite [207; 208]. However, the role of polyunsaturated fats (PUFA) is more complex, with n-3 PUFA generally thought to have anti-inflammatory and inflammation-resolving properties, and n-6 PUFA to have pro-inflammatory effects. Both n-3 and n-6 PUFA are particularly relevant when it comes to the regulation of AT inflammation due to their pleiotropic effects on gene expression, cytokine secretion and oxylipin production [209].

Endogenous PUFA metabolism is controlled through sequential desaturation and elongation steps in the fatty acid desaturase pathway [135]. This pathway is functional in AT, with adipocytes expressing the fatty acid desaturase 1 (*FADS1*) and 2 (*FADS2*) genes that code for the delta-5 and delta-6-desaturase enzymes (D5D and D6D), respectively [210]. Briefly, linoleic acid (LA, 18:2n-6) is first converted by D6D into γ-linolenic acid (GLA, 18:3n-6), then elongated to di-homo-γ-linolenic acid (DGLA, 20:3n-6), and then converted by D5D into arachidonic acid (AA, 20:4n-6). Similarly, α-linolenic acid (ALA, 18:3n-3) is desaturated and elongated into eicosapentaenoic acid (EPA, 20:5n-3), which can then undergo further conversion into docosahexaenoic acid (DHA, 22:6n-3). Previous studies have reported relationships between the aforementioned FA, as well as estimates for D5D and D6D activities, and markers of inflammation in plasma [211; 212; 213] and AT [214].

Polymorphisms in *FADS1/FADS2* genes influence FA composition in plasma, serum, erythrocytes, and AT [155; 201], and have also been associated with various cardiometabolic risk factors [155; 215]. Additionally, there is some evidence to suggest that single nucleotide polymorphisms (SNPs) in the *FADS1/FADS2* gene region may be associated with inflammation. Indeed, variants in *FADS1/FADS2* genes have been associated with plasma high-sensitivity C-reactive protein [212], oxylipin synthesis [202], and subcutaneous AT (SAT) inflammatory gene expression [214]. This latter study is of particular relevance to the present investigation due to the reported association between *FADS1/FADS2* genotype and the *NFκB* pathway in SAT, suggesting that variants in this gene cluster may influence the inflammatory status of this depot. However, this past study was performed in a small sample size of morbidly obese individuals, and no associations were identified between *FADS1/FADS2* SNPs and the expression of individual inflammation-related genes. Thus further investigation regarding the impact of SNPs in *FADS1/FADS2* genes on SAT inflammation is of importance.

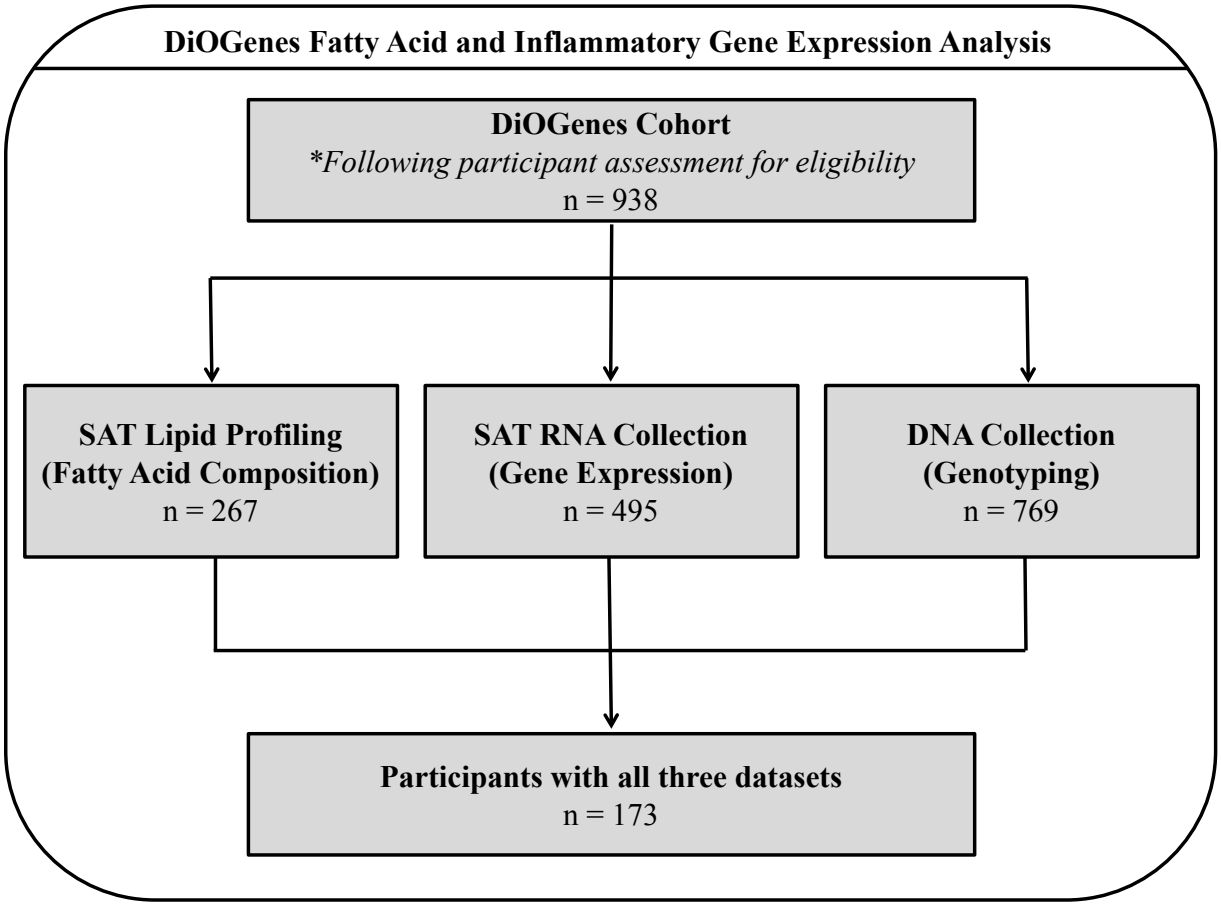
The present study examined if *FADS1/FADS2* genotypes could be distinguished by SAT FA composition and the expression of obesity-related transcripts involved in inflammation/immune function, lipid metabolism, and cellular differentiation in participants from the DiOGenes (Diet, Obesity and Genes) study. We hypothesized that individuals carrying major alleles in the *FADS1/FADS2* gene cluster would show increased fatty acid desaturase pathway activity and increased inflammatory gene expression in SAT.

4.3 Material and Methods

4.3.1 Cohort Characteristics

This study is part of the European Framework project entitled Diet, Obesity and Genes (DiOGenes), registered in ClinicalTrials.gov (NCT00390637). For a thorough description of the overall objective and goals of this multicenter, randomized, controlled dietary intervention study, see Larsen et al [216]. Briefly, this study examined the effects of dietary macronutrients in particular protein and glycemic load on weight regain and cardiovascular risk factors. Inclusion and exclusion criteria for study participation were previously outlined [216]. The DiOGenes study consisted of 938 eligible participants from 8 European countries; however, the present study used only a subgroup of 173 men and women aged 27-63 y who had SAT FA and gene expression data available, as well as a DNA sample for genotyping (**Figure 4-1**). Only baseline biological samples and clinical data were used in the present investigation.

Figure 4-1: Summary diagram depicting the subjects from the DiOGenes Cohort used for the current subcutaneous adipose tissue fatty acid and inflammatory gene expression analysis.



4.3.2 Anthropometric and Clinical Measurements

Anthropometric measures (age, height, body weight, and BMI) were collected from each participant using standard protocols previously described in Larsen et al [216]. Fasted blood samples were collected via a venflon for the analysis of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides (TAG) and high-sensitivity C-reactive protein (hsCRP). Low-density lipoprotein cholesterol (LDL-C) was calculated according to Friedewald's equation.

4.3.3 Subcutaneous Adipose Tissue (SAT) Collection

SAT samples were collected from the periumbilical area by needle aspiration under local anesthesia following an overnight fast, as previously described [216]. Tissue samples were stored at -80 °C until required for analyses.

4.3.4 Subcutaneous Adipose Tissue Fatty Acid Analysis

Gas chromatography (GC) was used to determine the FA composition of SAT, as previously reported [217]. Briefly, total lipid was transmethylated to FAME with 1 M sodium methoxide in dry methanol under nitrogen atmosphere in darkness. The reaction mixture was neutralized with 1 M acetic acid, FAME were extracted twice into hexane and passed through a column (5 × 20 mm) of anhydrous sodium sulphate. The combined extracts were dried under nitrogen, dissolved in an appropriate volume of isooctane and stored at −20 °C until analyzed. GC was performed with a Trace GC gas chromatograph equipped with a capillary split/splitless injector and flame ionization detector (FID), combined with AS 2000 autosampler (Thermo Finnigan USA). Analyses of FAME were performed on fused-silica capillary columns coated

with chemically bonded stationary phases Select FAME (100 m × 0.32 mm I.D). Individual FA are indicated as a relative percentage (%) of total detected FA. FA contributing <0.25% of the total FA profile were excluded for the present analysis. FA classes were calculated by summing values for individual FA, i.e., SFA (12:0, 14:0, 16:0, 18:0, 20:0), MUFA (14:1n-5, 16:1n-7, 18:1n-9, 20:1n-9), *trans* fatty acids (TFA, 18:1c11*t*), n-6 PUFA (18:2n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6), and n-3 PUFA (18:3n-3, 22:5n-3, 22:6n-3). As previously reported [179], product-to-precursor ratios were used to estimate the following activities: delta-6 desaturase (18:3n-6: 18:2n-6), delta-5 desaturase (20:4n-6: 20:3n-6), as well as overall fatty acid desaturase pathway activity (20:4n-6: 18:2n-6).

4.3.5 Subcutaneous Adipose Tissue Gene Expression Analysis

Details regarding the extraction of total RNA (ribonucleic acid) and quantitative RT-PCR (qRT-PCR) from SAT samples have been previously described [218]. Briefly, SAT samples were homogenized in QIAzol (Qiagen) prior to extraction using the RNeasy total RNA Mini Kit (Qiagen). Total RNA quality was checked using ethidium bromide-stained agarose gel and concentration was determined using the Experion analyzer (BioRad, Marnes-la-Coquette, France). A total of 271 unique transcripts were quantified using 96.96 Dynamic Arrays (Fluidigm), as previously described [218]. Raw data was obtained from the system's software using the default global threshold setting (BioMark Real-time PCR Analysis V2.1.1, Fluidigm). Genes with very high or missing Ct values were excluded. The present analysis investigated 117 obesity-related transcripts associated with inflammation/immune function, lipid metabolism, and cell differentiation, according to Gene Ontology Biological Process classification.

4.3.6 DNA Extraction and Genotyping

DNA was extracted from EDTA-blood buffy coats stored at -80 °C by KBioscience. As previously described [219], a quality check of genomic and amplified DNA samples was conducted prior to genotyping. Samples were then quantified and normalized to approximately 100 ng/ml. Genotyping of DiOGenes participants was performed using an Illumina 660W-Quad SNP chip on the Illumina iScan Genotyping System (Illumina, San Diego, CA, USA) in accordance with manufacturer's protocols. SNPs that met the following criteria were excluded for the present analysis: i) low call rate (<95%), ii) violated Hardy-Weinberg equilibrium (HWE, FDR <20%), and iii) extreme minor allele frequency (MAF). Given the focused nature of the present study, we examined all SNPs within +/- 10 kb for *FADS1* (n=10 SNPs) and *FADS2* (n=21 SNPs). Pairwise linkage disequilibrium (LD) between SNPs was assessed with the SNP Annotation and Proxy Search (SNAP) [220].

4.3.7 Statistical Analyses

All data were assessed for normality using the Shapiro Wilk's test, and $\log_{10}$ transformed if necessary to achieve normality.

<u>*Genetic analyses:*</u> We initially conducted genetic analyses using both additive and dominant models. In general, additive models revealed no statistical differences between heterozygous and minor homozygous individuals (data not shown). Given the small number of individuals in the minor homozygous groups, we opted to use a dominant model (i.e., MM vs. Mm + mm) for all SNP analyses to increase our statistical power. Thus the term "minor allele carrier" refers to heterozygous and minor homozygous individuals combined together. Associations between SNPs and SAT FA were examined with linear regression, and accounted

for age, sex, BMI, and recruitment site as covariates. Multiple testing was accounted using a Bonferroni correction. In a second round of analyses, we included the rs174537 SNP in *FADS1* as an additional covariate to perform conditional analyses. This SNP has been previously reported, and reproduced by numerous independent research groups, to influence PUFA levels in blood [139; 221; 222].

Sparse partial least squares-discriminant analyses (sPLS-DA) analyses: These analyses aimed to assess whether SAT FA or SAT mRNA levels could predict population sub-groups as defined by the rs174537 genotype (i.e., major vs. minor allele carriers). Data were split randomly into a training set (corresponding to 80% of the data) and a test set (corresponding to the remaining 20% of the data). Model training and evaluation was performed using the *mixOmics* R package [223]. The sPLS-DA models were constructed on the training set, using internal 5-fold cross-validation (as implemented with the *tune.splsda* function, with misclassification measured as Balanced Error Rate, the distance for classification error rate set as 'max.dist', and the maximal number of components limited to six). This led to the identification of the optimal parameters (i.e., optimal number of components and optimal number of variables per component) and to build the model (with the *splsda* function). The performance of the resulting model was assessed using the testing dataset (using the perf function, with 5 M-fold validations repeated 10 times). Receiver operating characteristic (ROC) curves were derived using the *auroc* function and enabled to visualize the performance on testing dataset.

Fatty acid and mRNA associations: Associations between specific FA and the expression of individual genes were analyzed by linear regression adjusting for age, sex, BMI, and recruitment site as covariates (JMP 12 Statistical Software, SAS Institute, Cary, NC, USA).

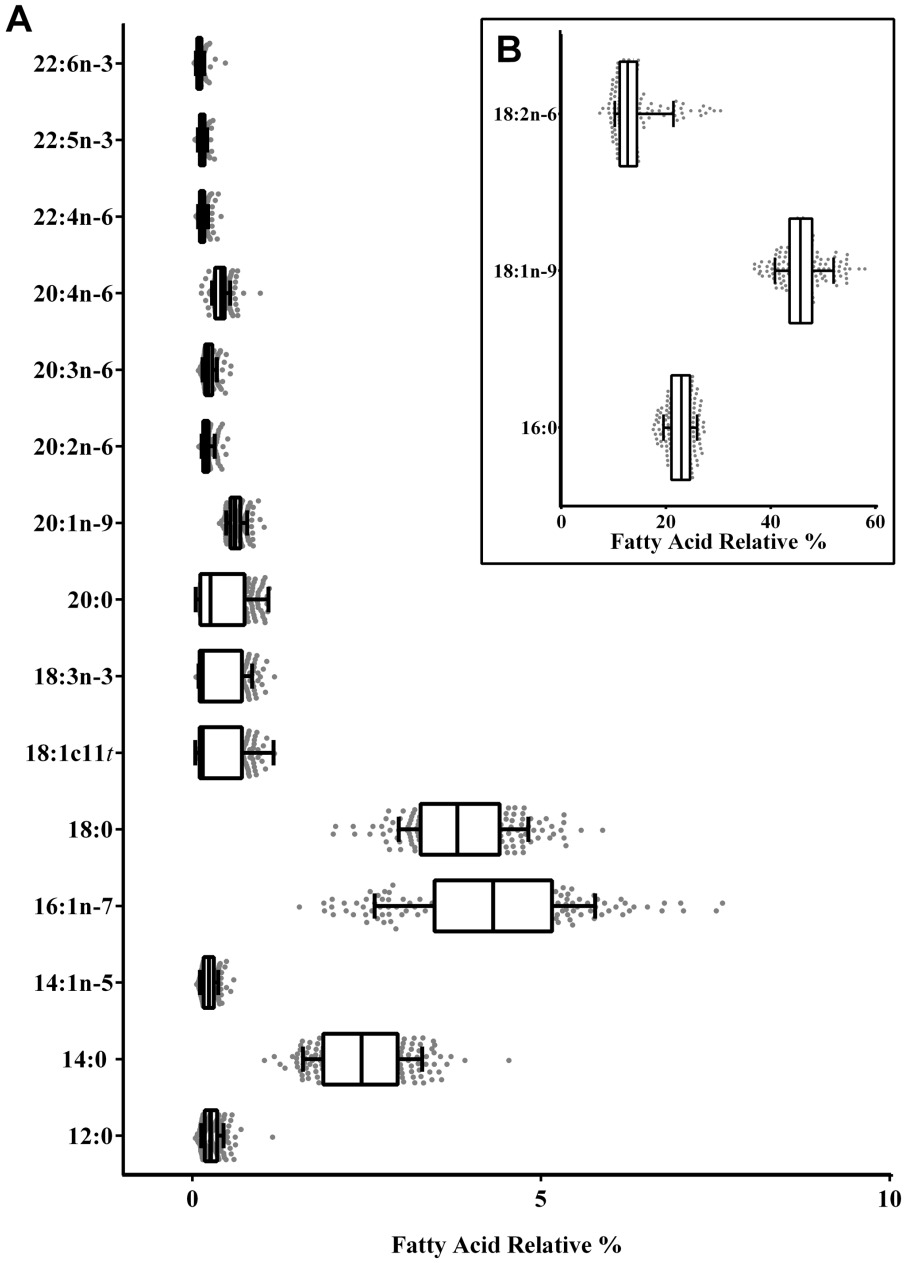
Adjustment for multiple testing was performed using Bonferroni correction (117 genes × 2 FA = 234 associations; p=2.14E-04 (0.05/234)).

4.4 Results

4.4.1 SAT Fatty Acid Profile

The present analysis was conducted in 173 participants (111 females, 62 males) who had a mean age of 42.8 ± 0.4 y and a mean BMI of 34.4 ± 0.4 kg/m^2. The relative abundance of FA classes in SAT were, from highest to lowest, MUFA (53.48% ± 0.32), SFA (26.64% ± 0.24), n-6 PUFA (14.91% ± 0.34), TFA (0.99% ± 0.03), and n-3 PUFA (0.82% ± 0.03). The dominant individual FA in SAT were 18:1n-9, 16:0, and 18:2n-6 (**Figure 4-2**).

Figure 4-2: Relative fatty acid levels in subcutaneous adipose tissue. A) Low relative abundance FA (< 10%) and B) high relative abundance FA (>10%) are shown. Relative FA data is reported as mean % ± SEM (Standard error of the mean).



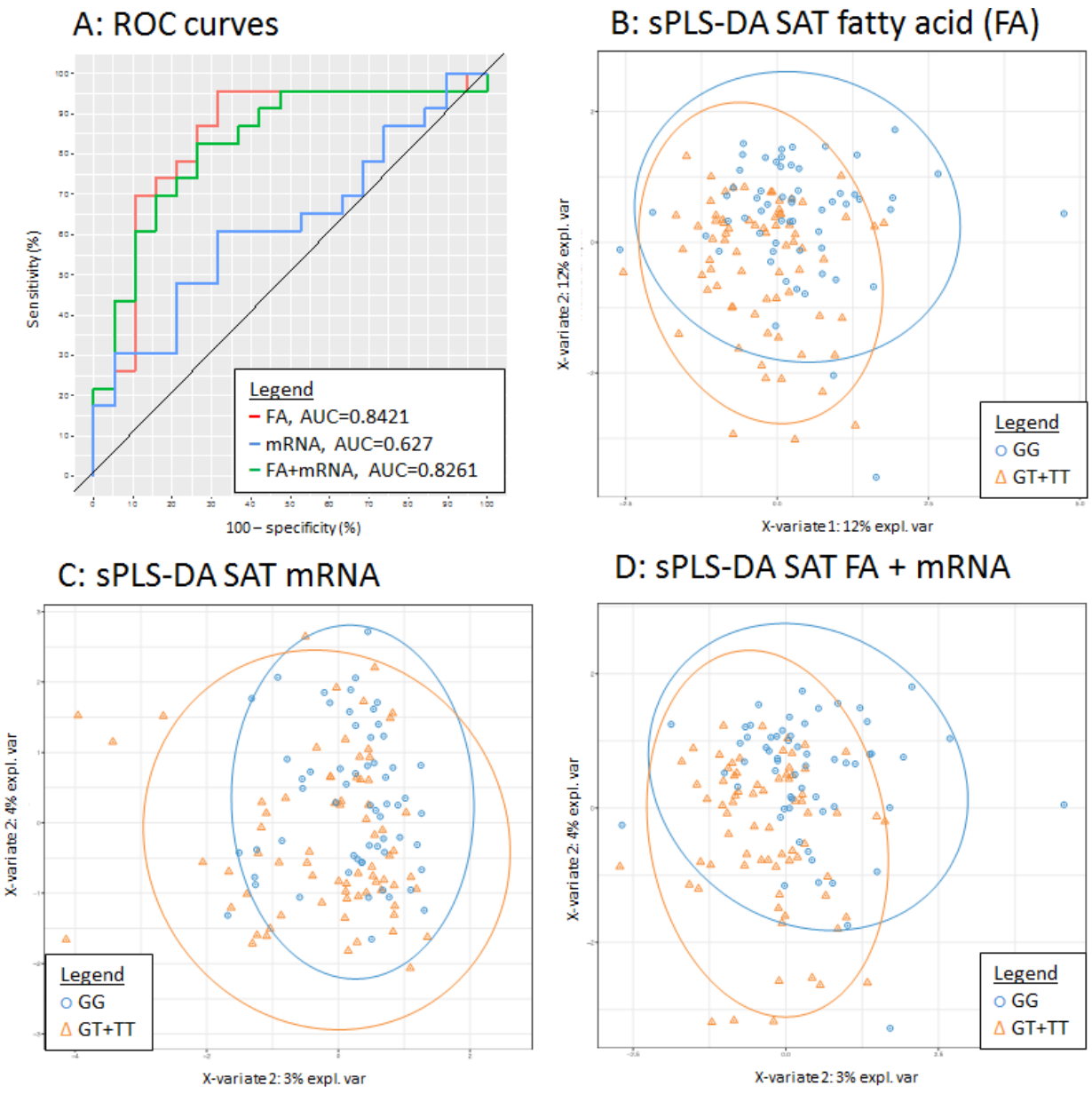
4.4.2 Variation in the FADS1/FADS2 gene cluster is associated with SAT fatty acids

Four of the 31 SNPs in the *FADS1/FADS2* gene cluster were significantly associated after multiple testing with the estimate for overall FADS pathway activity (i.e., 20:4n-6: 18:2n-6 ratio): rs174537 (p=4.12E-5), rs174546 (p=8.66E-6), rs174556 (p=4.00E-4), and rs174601 (p=6.44E-7). We then conducted a second round of analyses in which the commonly reported rs174537 was included as a covariate to assess LD between the 4 aforementioned SNPs. Two SNPs (rs174546 and rs174556) were no longer significantly associated with overall FADS pathway activity in this second analysis, while rs174601 was only borderline significant (p=0.02). This demonstrates that the four SNPs are in high LD with one another. Due to this high LD, all subsequent analyses were conducted with only rs174537.

We next examined associations between rs174537 and individual FA and relevant estimates of desaturase activity in SAT. This SNP was significantly associated with SAT levels of AA (p=1.74E-5) and the estimate for D5D activity (p=2.09E-9), with major allele carriers (GG) having higher values in both cases compared to minor allele carriers (GT+TT). Furthermore, GG carriers tended to have lower amounts of DGLA compared to GT+TT carriers (p=0.09). The association between rs174537 genotype and the estimate for D6D activity was not statistically significant. Predictive models using sPLS-DA (see Methods) aimed to study the contribution of SAT FA in obese individuals, according to their rs174537 genotype. As depicted in **Figure 4-3, A**, the sPLS-model achieved strong performance at predicting genotype classes (AUC=0.842). We then investigated our sPLS-DA model to identify which FA in particular were the best discriminators of the two genotype groups. This analysis indicated that SAT FA explained ~24% of the variance between GG and GT+TT genotypes (**Figure 4-3, B**). Further

examination of the variables contributing to the first two components revealed that AA was the only FA to contribute to component 1 (explaining ~12% of the data variance) and DGLA was the top FA contributing to component 2 (explaining ~7% of the data variance).

Figure 4-3: Multivariate analysis of fatty acids (FA) and gene expression according to rs174537 genotype. A) ROC curves (FA - red line; mRNA - blue line; FA +mRNA - green line). SPLS-DA score plots for FA (B), mRNA (C), and the combination of FA+mRNA (D) are shown according to rs174537 major (GG) and minor allele carrier (GT+TT) genotypes.



4.4.3 Relationship between discriminating fatty acids and SAT gene expression

FA are known to influence SAT gene expression. Given that AA and DGLA were identified by sPLS-DA to be the primary discriminating variables for rs174537 genotype, we next examined if these two FA were associated with the expression of SAT genes related to inflammation/immune function, lipid metabolism, and cellular differentiation. As indicated in **Table 4-1**, statistically significant associations after multiple testing were identified for 14 of the 117 genes examined. Thirteen (out of 14) of the significant correlations were detected with DGLA. Higher DGLA levels in SAT were inversely associated with *FADS1* and *FASN* gene expression, which are genes that encode enzymes involved in lipogenesis. An inverse association was also seen with *FADS2* gene expression (p=4.0E-4), but did not meet our threshold for statistical significance when accounting for multiple testing. In contrast, the majority of statistically significant positive associations detected with DGLA were with genes associated with inflammation/immune response (**Table 4-1**). Four significant positive associations were detected between AA and genes linked to inflammation/immune response. No associations were detected between these FA and any genes related to cellular differentiation.

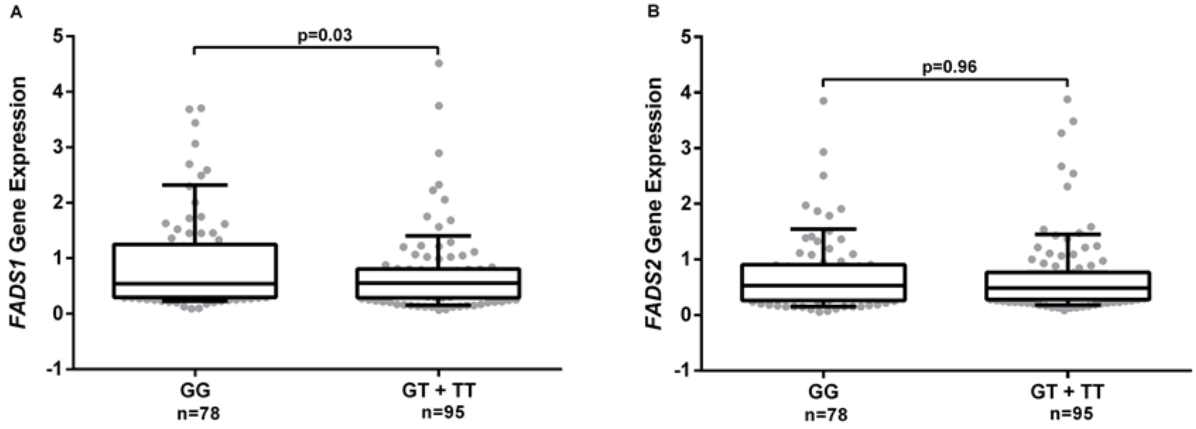
Table 4-1: Correlations between subcutaneous adipose tissue DGLA and AA levels, and gene expression. P-values in bold correspond to linear regression models that met our criteria for statistical significance after accounting for Bonferroni multiple testing. Models with a p < 2.1E-04 were considered statistically significant. Models accounted for BMI, age, sex, and recruitment site as co-variates.

				DGLA (20:3n-6)		AA (20:4n-6)	
Gene Symbol	Entrez GeneID	Gene Name	GO Biological Process	R	P Value	R	P Value
NCEH1	57552	Neutral cholesterol ester hydrolase 1	cholesterol metabolism	0.63	2.43E-05	0.51	8.19E-04
C1QB	713	Complement C1q B chain	complement system	0.62	6.43E-05	0.52	5.10E-04
C1QC	714	Complement C1q C chain	complement system	0.62	4.23E-05	0.50	2.21E-03
C2	717	Complement C2	complement system	0.62	2.00E-04	0.49	7.00E-03
CCL18	6362	C-C motif chemokine ligand 18	lymphocyte chemotaxis	0.66	3.16E-07	0.49	1.40E-02
CCL3	6348	C-C motif chemokine ligand 3	chemotaxis	0.63	2.00E-05	0.53	1.08E-04
CD163	9332	CD163 molecule	acute phase response	0.65	7.20E-07	0.53	1.09E-04
CD68	968	CD68 molecule	scavenger receptor activity	0.62	9.37E-05	0.51	1.56E-03
FADS1	3992	Fatty acid desaturase 1	lipogenesis	-0.64	3.33E-06	0.47	8.59E-02
FASN	2194	Fatty acid synthase	lipogenesis	-0.63	1.29E-05	-0.48	5.46E-02
FCGBP	8857	Fc fragment of IgG binding protein	immunoglobulin mediated immune response	0.61	6.00E-04	0.52	2.00E-04
GPD1L	23171	Glycerol-3-phosphate dehydrogenase 1 like	glycerophospholipid metabolism	-0.65	2.02E-06	-0.52	3.05E-04
IL1RN	3557	Interleukin 1 receptor antagonist	pro-inflammatory cytokine	0.62	4.37E-05	0.53	1.73E-04
MRC1L1	4360	Mannose receptor C-type 1	endocytosis signaling	0.62	7.26E-05	0.52	3.77E-04

4.4.4 SAT gene expression does not distinguish FADS1 genotype

There is some suggestion that SNPs in *FADS1/FADS2* may be associated with differences in SAT gene expression [214; 224]. We therefore examined the relationship between rs174537 and SAT gene expression. A sPLS-DA analysis revealed that SAT gene expression did not predict rs174537 genotype. Indeed, <7% of the variance between GG and GT+TT genotypes is explained by the 117 genes examined in the present study (**Figure 4-3, C**). The inability to distinguish genotype groups with gene expression was further evidenced by the low area under the curve (AUC=0.627) in the ROC analysis (**Figure 4-3, A**). It is also notable that combining gene expression with FA data had no discernable impact on the ability to classify the two genotype groups compared to FA data alone (**Figure 4-3A, 4-3D**). Although rs174537 genotype was not associated with overall gene expression, we did identify a specific association between this SNP and *FADS1* ($p<0.03$), but not *FADS2*, gene expression (**Figure 4-4A, 4-4B**).

Figure 4-4: Relationship between the rs174537 and *FADS1/FADS2* gene expression. Individuals were grouped according to rs174537 genotype (GG vs. GT+TT) prior to analysis of A) *FADS1* and B) *FADS2* gene expression in subcutaneous adipose tissue. Values are expressed as mean gene expression ± SE.



4.5 Discussion

The present study investigated whether SAT FA composition and inflammatory gene expression could be used to distinguish *FADS1* genotype in an obese adult cohort. The primary findings of this research were: 1) variation in rs174537 in *FADS1* is associated with both SAT FA (DGLA and AA) and estimated D5D activity; 2) multivariate analysis revealed that DGLA and AA explained ~19% of the variance between rs174537 genotypes; 3) levels of these two FA were associated with the expression of genes linked to inflammatory/immune function and lipid metabolism; and 4) that inflammatory gene expression does not distinguish rs174537 genotypes to any appreciable extent. Collectively, our data shows that *FADS1/FADS2* genotype can be classified with SAT FA composition, but not inflammatory gene expression.

The relationship between *FADS1/FADS2* SNPs and SAT FA profiles has not been extensively studied; however, our findings agree with the few previous reports in the literature that used both smaller (N=89) and larger sample sizes (N>1000). Aslibekyan et al. were the first to report that individuals carrying minor alleles in the *FADS1/FADS2* gene cluster had lower AA and higher DGLA in SAT compared to major allele homozygotes [225]. These same authors also showed that minor allele carriers had lower SAT EPA levels, demonstrating that the modifying effect of *FADS1/FADS2* SNPs was similar for both n-3 and n-6 PUFA. Although the relative abundance for SAT EPA were similar between this past study and our study, we did not detect a statistical association between rs174537 and EPA levels. This is most likely due to a combination of our smaller sample size and the low abundance of EPA in SAT. More recently, Vaittinen et al. demonstrated that the minor allele for a variant in *FADS2* (rs174616) was associated with a reduction in the estimated D6D enzyme activity in the triglyceride fraction of

SAT as well as visceral adipose tissue [214]. Pairwise analysis using the Broad Institute SNAP database indicated a low LD between rs174537 and rs174616 ($R^2 = 0.43$); thus it is not surprising that we did not identify an association between rs174537 in *FADS1* and estimated D6D activity. We also identified a highly significant association between rs174537 and estimated D5D enzyme activity, which further reinforces the modifying effect of this SNP on both DGLA and AA levels. Interestingly, our findings suggest that this SNP-FA relationship may result from differences in *FADS1* gene expression. Specifically, we found that individuals carrying the minor T allele for rs174537 had lower *FADS1* gene expression compared to major allele carriers. Although the molecular mechanism linking this SNP with *FADS1* gene expression requires further clarification, Rahbar et al. showed that the minor T allele for rs174537 is associated with increased methylation of a putative enhancer region in *FADS1* [226]. Therefore investigating the methylation status of the *FADS1/FADS2* gene region in SAT in future studies is warranted.

Alterations in D5D and D6D activities have been associated with a myriad of metabolic and cardiovascular risk factors, including insulin resistance, obesity and hepatic steatosis [215]. Inflammation represents a common underlying feature of the aforementioned risk factors. While the relationship between PUFA and inflammation is complex, these FA regulate inflammatory status by influencing gene expression, signaling pathways, and oxylipin profiles [209]. Thus, it stands to reason that polymorphisms in *FADS1/FADS2* genes could modify the relationship between FA and inflammatory status by altering PUFA biosynthesis. However, the evidence to date is equivocal. For example, Aslibekyan et al. did not find that polymorphisms in the *FADS1/FADS2* gene cluster modified circulating hsCRP or vascular cellular adhesion molecule-1 [225]. In contrast, Roke et al. reported a weak association between a SNP in *FADS2* and hsCRP

levels in young healthy adults, with minor allele carriers having 13% lower hsCRP compared to major allele carriers [212]. The present study did not detect any associations between SNPs in *FADS1/FADS2* genes with hsCRP levels (data not shown). Further, variation in rs174537 was reported to influence oxylipin biosynthesis in zymosan-stimulated whole blood [202]. Specifically, Hester et al. demonstrated that carriers of the minor T-allele had a reduced capacity to produce 5-lipoxygenase metabolites compared to homozygotes of the major allele. Finally, Vaittinen et al. recently advanced this area of investigation by studying the modifying effect of SNPs in both *FADS1* (rs174547) and *FADS2* (rs174616) on SAT inflammatory gene expression in morbidly obese individuals before and after gastric bypass surgery [214]. These authors showed that both SNPs were moderately associated with the expression of an NFκB pathway gene panel in SAT after surgery, but not before. The authors postulated that the high level of inflammation in subjects prior to surgery may have masked any effect related to *FADS* genotype. Our study adds to this area, and indicates that polymorphisms in *FADS1/FADS2* genes are not distinguished by SAT gene expression. Indeed, multivariate analysis revealed that <7% of the variance between rs174537 major and minor allele genotypes could be explained by gene expression, as further evidenced with a ROC analysis. The panel of 117 genes examined in the current study, which are all linked with obesity and weight change, corresponded to transcripts classified by Gene Ontology as being associated with inflammation/immune function, lipid metabolism and cell differentiation [218]. Although our results seemingly conflict with those of Vattinen et al. [214], it is important to note that this past study was unable to identify statistically significant associations between SNPs and individual gene expression when accounting for multiple testing (similar to the current study). This suggests that the association between SNPs in *FADS1/FADS2* genes and SAT gene expression is minimal.

Interestingly, multivariate analyses between DGLA and AA with SAT gene expression exposed some intriguing findings. DGLA was inversely associated with *FADS1* gene expression, with a similar trend seen with *FADS2*. An accumulation of DGLA in the context of lower *FADS1* gene expression is logical from a biochemical perspective. While the association (P=4.0E-4) between DGLA and *FADS2* expression did not meet our criteria for statistical significance when accounting for multiple testing, the inverse relationship with *FADS2* gene expression is counter-intuitive as one would expect lower DGLA with reduced *FADS2* gene expression. Although feedback inhibition of *FADS2* gene expression has been previously reported in adipocytes treated with AA and EPA [(210)], it is not known if DGLA is similarly capable of reducing *FADS2* expression.

The subset of genes whose expression correlated positively with SAT DGLA levels consisted primarily of pro-inflammatory genes (*CCL3*, *CCL18*, *CD68*, *CD163*, *IL1RN*) and genes related to the complement system (*C1QB*, *C1QC*, *C2*). A previous study in which diet-induced obese mice were treated with a selective D5D-inhibitor reported that several pro-inflammatory genes (including *CD68*) were reduced compared to obese controls, which would suggest an improved inflammatory status in epididymal fat in the context of higher DGLA [(227)]. In contrast, our study found that higher DGLA levels in SAT were associated with increased pro-inflammatory gene expression. We acknowledge that establishing cause-and-effect regarding this positive association between DGLA and pro-inflammatory gene expression is not possible. DGLA and associated oxylipins are widely recognized to have anti-inflammatory effects [(228)]. Indeed, DGLA serves as a precursor for the synthesis of anti-inflammatory oxylipins, while simultaneously reducing the production of AA-derived inflammatory oxylipins through enzyme competition. Thus it is entirely plausible that higher DGLA levels may reflect a potential tissue

response to help mitigate SAT inflammation in obese individuals. Irrespective, while examining correlations between individual FA and gene expression can provide useful insights, it is important to reiterate that rs174537 did not modify SAT inflammatory gene expression.

In conclusion, the current study demonstrated that *FADS1* genotype is associated with different SAT FA profiles in an obese population, but not inflammatory gene expression. Future studies should investigate these relationships in additional subsets of the general population to ensure they are generalizable. SAT levels of DGLA and AA explain upwards of 19% of the variance between *FADS1* major and minor alleles, while gene expression explains less than 7% of the variance. Collectively, this suggests that obese SAT inflammatory gene expression does not distinguish *FADS1* genotype to any appreciable extent. However, given the known association between individual FA and inflammatory signaling pathways, knowledge of *FADS1/FADS2* genotype can provide important insights to better understand the roles of FA as regulators of metabolic and cardiovascular risk.

5 Treatment with High-Dose DHA, but not High-Dose EPA, is Associated with Reductions in Serum TAG levels in Young Adults

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5.1 Abstract

Background: Increased circulating triglyceride (TAG) levels are known to be a strong indicator of cardiometabolic risk. EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) are effective at lowering TAG levels in populations with increased cardiometabolic risk. It is not known, however, whether all individuals respond similarly to EPA and DHA treatment, and whether possible important factors such as baseline health status, biological sex and *FADS1* (fatty acid desaturase 1) genotype have an impact on the change in TAG. Furthermore, investigation of these factors within healthy individuals is pertinent to understand the preventative effects (i.e. before disease onset) of EPA and DHA.

Methods: In a randomized, double blind, placebo-controlled study, 45 men and 45 women who were young (21.60 ± 0.23 y) and healthy, were randomized into one of three treatment groups: 3 g/day olive oil, 3 g/day EPA, or 3 g/day DHA. At baseline, and following 12 weeks of treatment, red blood cell (RBC) fatty acids (FA) were quantified for confirmation of compliance, serum TAG was analyzed for change in levels and *FADS1* genotype for the rs174537 single nucleotide polymorphism (SNP) was determined.

Results: While neither EPA nor olive oil significantly reduced serum TAG concentrations (-0.03 ± 0.06 vs. 0.01 ± 0.06 mmol/L), DHA supplementation significantly reduced ($p<0.05$) serum TAG concentrations compared to olive oil (-0.22 ± 0.06 vs. 0.01 ± 0.06 mmol/L, $p=0.03$). In the DHA group, the decrease in TAG levels was strongly correlated with the baseline TAG status ($R^2= 0.65$, $p<0.01$); however, significant correlations were also found in the EPA and olive oil group ($R^2= 0.20$, $p=0.01$ and $R^2= 0.31$, $p<0.01$, respectively). The change in TAG was similar between sexes, and between major and minor allele carries of the *FADS1* genotype. In contrast to

TAG, no changes in other cardiometabolic risk factors were observed in either the EPA or DHA group.

Conclusion: The physiological benefits of DHA treatment are greater than that of EPA for reducing serum TAG levels in healthy individuals, implicating DHA supplementation as a viable approach for disease preventation. While the beneficial effect of DHA supplementation to lower TAG levels does not depend on biological sex or *FADS1* genotype, individuals with higher baseline levels may have a greater risk-reduction in response to DHA supplementation.

5.2 Introduction

The incidence and prevalence of chronic health conditions such as obesity, type-2 diabetes (T2D), and cardiovascular disease are increasing at alarming rates worldwide [159]. These conditions have detrimental implications on the health and well-being of those affected and are a significant economic burden. Increased circulating triglyceride (TAG) [229], as well as increased low-density lipoprotein-cholesterol (LDL-c) and high sensitivity C-reactive protein (hsCRP) [230], are well-known cardiometabolic risk factors. Although pharmaceuticals can help to treat or slow the development of cardiometabolic risk, they are not always effective, and often have unwanted side effects [159]. Modifying dietary habits is now recognized as a safe and effective way to help reduce cardiometabolic risk [231], however there remains a paucity of information pertaining to the ideal dietary intervention for disease prevention.

Omega-3 polyunsaturated fatty-acids (n-3 PUFA) have numerous beneficial health effects, such as the production of anti-inflammatory and inflammation-resolving bioactive lipid species, as well as improving the circulating lipid profile (i.e. TAG, LDL-c, etc.) [72; 232]. Two critical n-3 PUFA are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which can be obtained through the diet from fatty fish, algal sources, or produced endogenously to a limited extent [153]. Increases in EPA and DHA intake can be quantitatively measured in distinct blood fractions such as serum, plasma, and red blood cells (RBC). As the n-6:n-3 PUFA ratio is a strong indicator of cardiometabolic risk [233; 234; 235], concomitant increases in n-3 PUFA and decreases in n-6 PUFA levels in serum, plasma and RBC membranes are markers of risk reduction [234; 236; 237].

In populations with increased cardiometabolic risk, increasing n-3 PUFA intake is an effective therapeutic method to lower TAG levels [72]. However, whether DHA and EPA differ in their impact on cardiometabolic risk is unclear. A recent systematic review by Innes and Calder concluded that both EPA and DHA lower TAG levels, but suggested that the effects on other cardiometabolic risk factors are not as consistent [75]. They provided evidence to suggest that DHA may lower TAG levels to a greater extent than EPA. Of note, Grimsgaard and colleagues enrolled healthy middle-aged (36-56 y) men to receive 7-week treatment with EPA, DHA, or a corn oil (rich in n-6 PUFA) control [238]. They found that DHA decreased TAG by 26% whereas EPA only decreased TAG by 21%, which trended toward a significant difference ($p=0.14$) [238]. More recently, Allaire and Lamarche completed a crossover trial in which healthy men and women with abdominal obesity and low-grade inflammation received 10 weeks of treatment with EPA, DHA, or corn oil [70]. They found that DHA led to a more substantial decrease in serum TAG than EPA when compared to control (-0.25 ± 0.04 vs. -0.16 ± 0.03 mmol/L, $p=0.005$) [70]. While these studies have both individually demonstrated different effects of EPA and DHA on TAG, which favor DHA, further investigations are required to address the preventative use of these FA, and specifically in populations of young healthy men and women. Furthermore, TAG responses to n-3 PUFA supplementation remains heterogeneous, and responses are not always as predicted [239; 240; 241; 242]. Therefore, within these specific FA, with different claimed effects on blood lipid levels [243] and TAG responses [70; 238], factors that affect TAG response should be explored to determine if variability is present.

Although the variability in serum TAG response to n-3 PUFA has been researched, it is still unclear in the literature whether all individuals respond similarly to EPA and DHA treatment, and possible important factors may be biological sex and genetic makeup. As biological sex is

known to influence long chain PUFA levels [244] and desaturase activity [147; 149; 150], it is possible that men and women may respond differentially to n-3 PUFA. It is known, for example, that there are significant sex differences in incorporation of EPA and DHA into plasma fractions and cells after 12 months of supplementation with different doses of a combined EPA and DHA supplement [191]. Further, some studies have suggested differences in the response of men and women to EPA and DHA treatment for cardiometabolic risk factors such as blood lipids [11; 70], but more research is required on these differential effects. In addition to biological sex, EPA and DHA concentration is influenced by genetic variants that effect PUFA metabolism. The delta-5-desaturase (D5D) and delta-6-desaturase (D6D) are encoded by the fatty acid desaturase 1 (*FADS1*) and 2 (*FADS2*) genes, which are membrane-bound enzymes that catalyze the rate-limiting formation of long-chain PUFA. Polymorphisms in these genes have been shown to influence PUFA composition in erythrocytes [139; 213; 245; 246], as well as be associated with cardiometabolic risk factors [201; 247]; however, it is not completely known if these genetic variations might play a role in the variability of response to EPA or DHA supplementation.

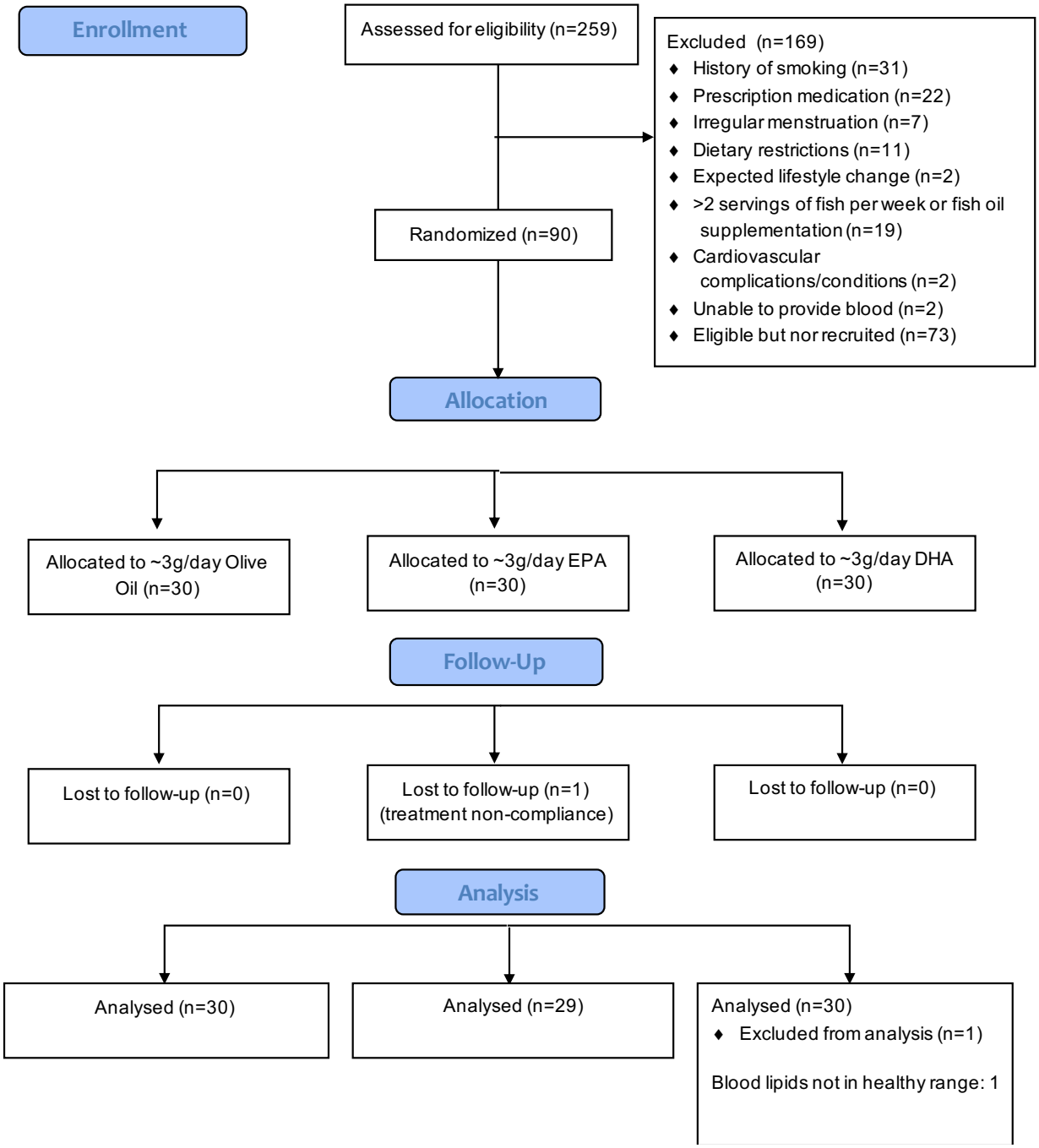
Therefore, the purpose of this study was to recruit young, healthy men and women to examine the independent effects of EPA and DHA on serum TAG levels and other lipid and FA associated with cardiometabolic risk. As well, we aimed to investigate if the variability in response to treatment within these young, healthy adults, can be explained by factors such as baseline lipid values, biological sex, or *FADS1/2* genotype. We hypothesized that both EPA and DHA treatment would affect FA levels by promoting a shift to increased n-3 PUFA incorporation into erythrocytes, as well as decreased TAG levels and improved cardiometabolic risk factors.

5.3 Methods

5.3.1 Participants and Recruitment

90 young adults (45 male, 45 female) between the ages of 18–30 years were recruited through emails and poster advertisements displayed around the University of Guelph campus. Individuals completed an online screening questionnaire prior to enrolment into the study, and were deemed ineligible to participate if they regularly consumed n-3 PUFA supplements and/or fish more than two times per week, currently smoke any substances (or have smoked regularly in the past), have baseline lipid levels that are outside of healthy ranges (as set by the Canadian Cardiovascular Society), are taking any medication which may interfere with blood lipid levels, or have any serious medical conditions. Female participants must have reported having a regular menstrual cycle in the online screening questionnaire and would expect to menstruate (approximately) every 28 days to be eligible for the study **(Figure 5-1)**.

Figure 5-1: Consolidated Standards of Reporting Trials flow chart of participant recruitment



5.3.2 Study Design

We used a double-blind, randomized, placebo-controlled study design. Participants were randomized into one of three treatment groups (Olive oil, EPA, and DHA), with 15 men and 15 women per group, using a randomization generator algorithm in Microsoft Excel. Purified EPA (KD-PÜR EPA700TG), DHA (KD-PÜR DHA700TG), and olive oil were obtained from KD Pharma. EPA and DHA were in triglyceride form. Encapsulation of the 3 oils was performed by InnovaGel so that all softgel capsules would be visually identical. Capsules were placed into de-identified bottles by colleagues not involved in the study and provided to participants to ensure double blinding. Participants were instructed to consume 4 capsules daily (2 in the morning and 2 in the evening) with food. Each capsule contained 800mg of the treatment, for a total of 3 g/day. The formulations had approximately 99% purity, and were supplied by KD Pharma (Bexbach, Germany).

Participants were instructed to maintain their regular exercise and dietary habits throughout the entire study. For women, baseline and final (week 12) study visits were scheduled during the follicular phase of their menstrual cycle to minimize variability in lipid levels within an individual, as recommended by Mumford and colleagues [248]. Compliance was assessed by analyzing EPA, DHA, and olive oil levels in the RBC fraction of serum, and participants were deemed compliant if there was an increase in the specific FA being supplemented after the 12-week treatment period. Ethical approval for the study was granted by the University of Guelph Human Research Ethics Board (REB #:15OC009). The trial was registered at clinicaltrials.gov (NCT03378232).

5.3.3 Bioclinical Measures

Blood collection occurred at the Human Nutraceutical Research Unit at the University of Guelph (Guelph, Ontario). Blood samples were collected at baseline and at the final visit (12 weeks) from participants by a certified phlebotomist via venous puncture, following an overnight (10-12 h) fast. Serum blood samples were collected from each participant in yellow top vacutainers (BD Vacutainer, 5.0 mL, SST (serum separation tubes)) coated with silicone and micronized silica particles to accelerate clotting, and then sent to Lifelabs Medical Laboratory Services (Kitchener, Ontario, Canada) for the analysis of TAG, total cholesterol (TC), LDL-c, HDL-c (high-density lipoprotein-cholesterol), hsCRP and glucose. Anthropometric measures (age, height, body weight, and calculated BMI) were also collected on the day of the visit using standard protocols.

5.3.4 Gas Chromatography

Blood samples were collected at baseline and at the final visit (12 weeks) and were centrifuged (e.g. 4000 g at 4°C for 10 min) to separate serum and RBCs, and then aliquoted and stored at −80 °C until analysis. RBCs were then sent to Poland for Gas Chromatography (GC) analysis by Chabowski and colleagues, where lipids were extracted according to the Folch method [177]. Briefly, lipids from isolated RBCs were extracted in chloroform-methanol (2:1 v/v) containing butylated hydroxytoluene (0.01%) as an antioxidant and heptadecanoic acid (C17:0) as an internal standard, as previously described [249]. After lipid extraction and transmethylation with BF_3/methanol, the lipid phase containing FAME was dissolved in hexane and analyzed using a Hewlett-Packard 5890 Series II gas chromatograph with Varian CP-SIL capillary column (100 m, internal diameter of 0.25 mm) and flame-ionization detector. Individual FA were

detected in accordance with the retention times of standards. FAME or FA standards were purchased from Larodan (Solna, Sweden). FA data are reported as relative abundance (% composition).

Individual FAs are reported as a relative percentage (%) of total FAs. FA contributing to <0.25% of the total FA profile were excluded for the present analysis. As previously reported [179], product-to-precursor ratios were used to estimate the following activities: delta-5 desaturase (20:4n-6: 20:3n-6), and overall n-6 *FADS* pathway activity (20:4n-6: 18:2n-6). The Omega-3 Index was calculated by adding EPA and DHA[250].

5.3.5 DNA Extraction and Genotyping

Blood samples were collected at baseline and at the final visit (12 weeks), and DNA was extracted from the whole blood using the Qiagen Paxgene Blood DNA kit (Qiagen, Toronto, Canada), according to the manufacturer's instructions. Using the NanoDrop 2.0 (Fisher Scientific, Waltham, MA), a concentration of 50 ng/ul, and a 260/280 ratio of 2.0 was required. Genotyping for *FADS1* and *FADS2* was performed using the Sequenom MassArray platform at the Sick Kids Genetic Analysis Facility (Toronto, Canada). A total of 90 samples were analyzed, of which no Mendelian errors were detected. Two DNA samples were randomly selected for replication and 100% concordance was achieved.

5.3.6 Statistics

A one-way ANOVA was used to compare the change in anthropometric, bioclinical, and RBC FA data between the groups following 12-weeks of treatment, and a repeated measures 2-way ANOVA was then completed comparing TAG levels at baseline and final time points

among the three treatment groups. This was followed by a Tukeys HSD (honest significant difference) to account for multiple comparisons. A student's t test was used to compare within group differences for sex and genotype at baseline and for change (final-baseline) for RBC FA measures. A $p<0.05$ was considered statistically significant. Linear regression analyses were completed to investigate the relationship between change in TAGs and baseline TAGs, as well as change in EPA and DHA levels.

Deviations from Hardy Weinberg Equilibrium (HWE) were tested for *FADS1* within the total study population, as well as within the male and female subgroups, using a χ^2 test. This conservative approach ensured that both HWE and minor allele frequency (MAF) were comparable in the total population, as well as within the three treatment groups. Because of low numbers for homozygote minor alleles for most single nucleotide polymorphism (SNP)s, we combined heterozygous and minor homozygous subjects into a single group termed "minor allele carriers". To determine a single, dominant SNP to be used in this investigation, linear regression was used to examine the associations between SNPs, lipids, and/or FA with models adjusted for age, sex and BMI as well as corrected for multiple testing. In a second round of analyses, we included the rs174537 SNP in *FADS1* as an additional covariate, due to the high LD within this gene region. GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) and JMP 13 Statistical Software (SAS Institute, Cary, NC, USA) were used for these analyses.

5.4 Results

5.4.1 Study Population Characteristics

This randomized double-blind placebo-controlled study recruited 90 (45 men, 45 women) young-healthy participants into three treatment groups, whereby each participant received a dietary supplement containing either olive oil (placebo control), EPA, or DHA. Participants visited the laboratory at baseline and after 12 weeks of consuming their respective treatment. A total of 89 participants completed the study. Upon analysis, one participant's data was excluded for having the majority of their lipid levels within a "high risk" range at baseline, as established by the Canadian Cardiovascular Society [251]. These cut-offs were a TAG level <2.2 mmol/L, HDL-c >0.9 mmol/L, LDL-c <4.1 mmol/L, and TC <6.2 mmol/L [251]. Each treatment group had close to equal numbers of men and women, and there were no differences in mean age or BMI among the treatment groups **(Table 5-1)**.

At baseline, RBC FA levels were measured with gas GC **(Table 5-2).** The dominant (>10% of total FA) RBC FA species were 16:0, 18:0, 18:1n-9, 18:2n-6 and 20:4n-6, and there were no differences among treatment groups for any FA (**Table 5-2**). Likewise, calculated D5D enzyme activity estimate, n-6 FADS Pathway Activity, and Omega-3 Index did not differ among treatment groups **(Table 5-2)**. At baseline, serum was also collected from participants to assess bioclinical measures. There were no differences found among treatment groups for TC, LDL cholesterol (LDL-c), HDL-c, TC/HDL-c ratio, TAG, or hsCRP **(Table 5-1)**. Blood glucose, however, was increased (~0.2 mmol/L) in the EPA group in comparison to the olive oil group **(p=0.02, Table 5-1)**. Thus, together, these data indicate that the baseline characteristics of our study population were largely similar among treatment groups.

Table 5-1: Study population characteristics and baseline serum bioclinical measures in all treatment groups. Data is reported as mean ± SEM (Standard error of the mean). BMI, body mass index; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol. A one-way ANOVA was used to analyze data, followed by a Tukey's HSD.

Parameter	Olive Oil	EPA	DHA	P Value
Number of Participants	n=30 (15F, 15M)	n=29 (14F, 15M)	n=29 (15F, 14M)	-
Age (y)	21.13 ± 0.39	21.44 ± 0.40	22.24 ± 0.40	0.13
BMI (kg/m^2)	24.05 ± 0.59	23.02 ± 0.61	23.72 ± 0.62	0.47
Total Cholesterol (TC;mmol/L)	4.45 ± 0.15	4.52 ± 0.15	4.26 ± 0.15	0.43
LDL-c (mmol/L)	2.38 ± 0.12	2.45 ± 0.12	2.24 ± 0.12	0.45
HDL-c (mmol/L)	1.64 ± 0.07	1.70 ± 0.07	1.63 ± 0.07	0.79
TC/HDL-c ratio	2.81 ± 0.11	2.74 ± 0.11	2.70 ± 0.11	0.78
TAG (mmol/L)	0.93 ± 0.06	0.84 ± 0.06	0.86 ± 0.06	0.47
Glucose (mmol/L)	4.58 ± 0.06^{a}	4.81 ± 0.06^{b}	4.73 ± 0.06^{ab}	0.02
hsCRP (mg/L)	2.10 ± 0.36	1.40 ± 0.37	1.68 ± 0.37	0.40

Table 5-2: Baseline RBC fatty acid levels in all treatment groups. Data is reported as mean ± SEM. A one-way ANOVA was used to analyze data, followed by a Tukey's HSD.

Parameter (%)	Olive Oil (n=30)	EPA (n=29)	DHA (n=29)	P Value
14:0	0.45 ± 0.02	0.42 ± 0.02	0.43 ± 0.02	0.51
16:0	22.75 ± 0.21	22.70 ± 0.21	22.55 ± 0.21	0.78
16:1n-7	0.55 ± 0.04	0.51 ± 0.04	0.46 ± 0.04	0.24
18:0	15.71 ± 0.13	16.02 ± 0.13	16.06 ± 0.13	0.13
18:1n-9	13.60 ± 0.17	13.70 ± 0.18	13.87 ± 0.18	0.54
18:2n-6	15.24 ± 0.28	15.17 ± 0.28	15.12 ± 0.29	0.96
18:3n-3	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.76
20:0	0.39 ± 0.01	0.38 ± 0.01	0.39 ± 0.01	0.61
20:3n-6	1.52 ± 0.06	1.55 ± 0.06	1.45 ± 0.06	0.50
20:4n-6	16.18 ± 0.21	16.14 ± 0.21	16.04 ± 0.21	0.90
20:5n-3	0.50 ± 0.03	0.51 ± 0.03	0.51 ± 0.03	0.92
22:6n-3	3.19 ± 0.11	2.98 ± 0.12	2.96 ± 0.12	0.31
22:0	1.57 ± 0.04	1.52 ± 0.04	1.58 ± 0.04	0.42
24:0	3.89 ± 0.10	3.91 ± 0.10	4.09 ± 0.10	0.29
24:1	4.27 ± 0.11	4.38 ± 0.10	4.33 ± 0.11	0.76
D5D (20:4n-6/20:3n-6)	10.93 ± 0.48	11.15 ± 0.49	11.55 ± 0.49	0.66
n-6 FADS Pathway Activity (20:4n-6/18:2n-6)	1.08 ± 0.03	1.07 ± 0.03	1.08 ± 0.03	0.98
Omega-3 Index (20:5n-3+22:6n-3)	3.68 ± 0.13	3.50 ± 0.13	3.47 ± 0.13	0.47

5.4.2 RBC Fatty Acid Composition and Estimates of Enzyme Activity

After 12 weeks of treatment with each dietary supplement, we observed significant changes in RBC FA composition **(Table 5-3).** Importantly, the RBC content of 18:1n-9 was increased in the olive oil treatment group (+0.42% ± 0.16), 20:5n-3 was increased in the EPA treatment group (+3.44% ± 0.13), and 22:6n-3 increased in the DHA treatment group (+4.16% ± 0.14). As these are the predominant FA species within each respective dietary supplement, this data indicates that participants were compliant with the assigned supplementation regime.

The saturated fatty acids (SFA) 14:0 and 16:0 were decreased after DHA treatment when compared to EPA ($p<0.01$) but not when compared to olive oil **(Table 5-3)**. Likewise, the monounsaturated fatty acid (MUFA) 16:1n-7 was decreased after DHA treatment when compared to EPA ($p=0.04$), while the FA 18:1n-9 decreased after the DHA treatment as compared to olive oil (**Table 5-3)**. There were also substantial changes in PUFA levels (**Table 5-3)**. For the n-6 PUFA, the EPA treatment significantly reduced 18:2n-6, as compared to the olive oil treatment ($p<0.01$, **Table 5-3).** DHA and EPA treatments decreased 20:4n-6 levels when compared to olive oil ($p<0.01$), with the reduction after DHA treatment greater than that after EPA ($p<0.01$) (**Table 5-3)**. As well, both DHA and EPA treatment decreased 20:3n-6 levels when compared to olive oil ($p<0.01$); however, the reduction after EPA treatment was greater than that after DHA ($p<0.01$) (**Table 5-3)**. For the n-3 PUFA, both EPA and DHA treatment led to an increase in 20:5n-3 levels when compared to olive oil ($p<0.01$), but the increase after EPA was greater than that after DHA ($p<0.01$) (**Table 5-3)**. DHA treatment increased 22:6n-3 levels compared to both EPA and olive oil, while EPA treatment led to a non-significant decrease in 22:6n-3 in comparison to olive oil (**Table 5-3)**.

When examining calculated estimates of enzyme activity (**Table 5-3)**, D5D was increased after EPA treatment when compared to DHA and olive oil ($p<0.01$). In contrast, the n-6 FADS pathway activity estimate was decreased only after DHA treatment ($p<0.01$). The omega-3 index estimate increased in both DHA and EPA groups when compared to olive oil, with DHA supplementation resulting in a larger increase as compared to EPA ($p<0.01$). Together, these data clearly demonstrate that 12 weeks of supplementation with either EPA or DHA leads to changes in many RBC FA levels, and that each may have unique effects.

Table 5-3: Change in RBC fatty acid levels in all treatment groups. Data is reported as mean ± SEM. A one-way ANOVA was used to analyze data, followed by a Tukey's HSD.

Parameter (%)	Olive Oil (n=30)	EPA (n=29)	DHA (n=29)	P Value
14:0	0.01 ± 0.02^{ab}	0.06 ± 0.02^{a}	-0.04 ± 0.02^{b}	1.66 E-3
16:0	0.27 ± 0.14^{ab}	0.69 ± 0.14^{a}	-0.10 ± 0.14^{b}	8.82 E-4
16:1	-0.02 ± 0.03^{ab}	0.02 ± 0.04^{a}	-0.10 ± 0.03^{b}	0.04
18:0	0.02 ± 0.17	0.18 ± 0.17	-0.13 ± 0.17	0.44
18:1n-9	0.42 ± 0.16^{a}	0.04 ± 0.16^{ab}	-0.39 ± 0.13^{b}	2.39 E-3
18:2n-6	0.07 ± 0.32^{a}	-1.42 ± 0.33^{b}	-0.62 ± 0.33^{ab}	7.73 E-3
18:3n-3	0.01 ± 0.01	0.02 ± 0.01	-0.02 ± 0.01	0.14
20:0	-0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.12
20:3n-6	0.02 ± 0.03^{a}	-0.39 ± 0.03^{b}	-0.27 ± 0.03^{c}	1.13 E-13
20:4n-6	-0.67 ± 0.20^{a}	-2.22 ± 0.21^{b}	-3.20 ± 0.21^{c}	7.86 E-13
20:5n-3	0.04 ± 0.13^{a}	3.44 ± 0.13^{b}	0.70 ± 0.13^{c}	2.02 E-32
22:6n-3	-0.18 ± 0.14^{a}	-0.46 ± 0.14^{a}	4.16 ± 0.14^{b}	6.20 E-42
22:0	-0.03 ± 0.04	-0.01 ± 0.04	-0.04 ± 0.04	0.86
24:0	-0.04 ± 0.11	0.08 ± 0.11	0.02 ± 0.11	0.78
24:1	0.08 ± 0.12	-0.06 ± 0.12	0.01 ± 0.12	0.71
D5D (20:4n-6/20:3n-6)	-0.57 ± 0.26^{a}	1.81 ± 0.27^{b}	-0.32 ± 0.27^{a}	4.43 E-9
n-6 FADS Pathway Activity (20:4n-6/18:2n-6)	-0.05 ± 0.03^{a}	-0.04 ± 0.03^{a}	-0.18 ± 0.03^{b}	1.86 E-3
Omega-3 Index (20:5n-3+22:6n-3)	-0.14 ± 0.19^{a}	2.98 ± 0.20^{b}	4.85 ± 0.20^{c}	1.99 E-30

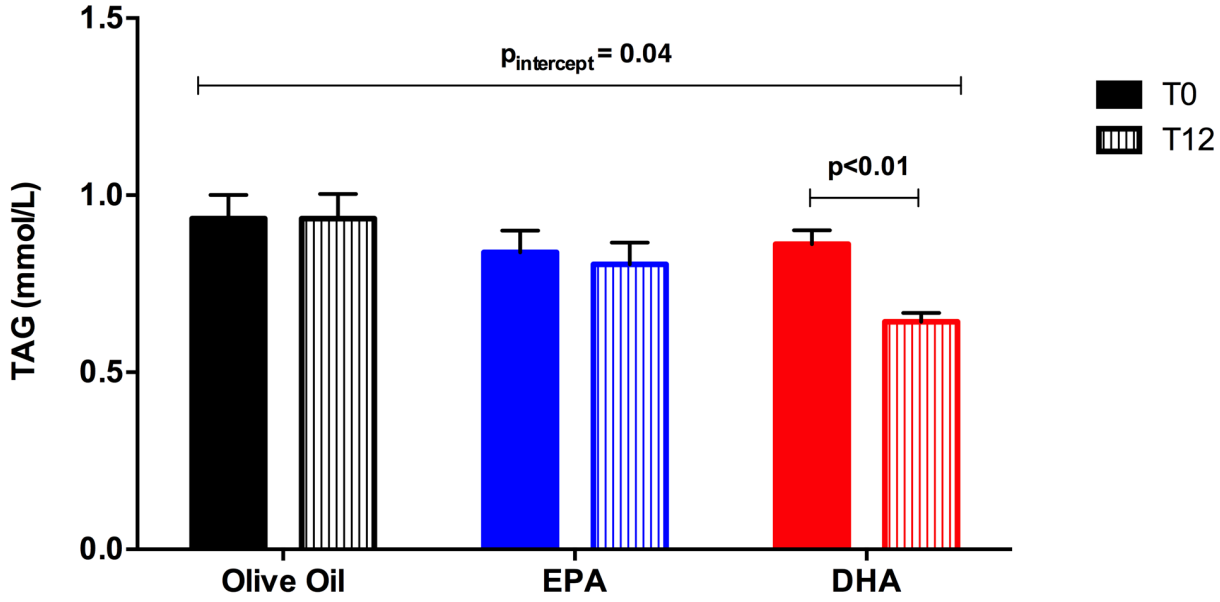
5.4.3 Serum Bioclinical Measurements

The primary outcome measure of our study was to evaluate whether EPA and DHA influenced serum bioclinical measurements. After 12 weeks of supplementation, there were no significant differences among treatment groups for TC, LDL-c, HDL-c, TC/HDL-c ratio, glucose, or hsCRP **(Table 5-4)**. Notably, after 12 weeks of supplementation the DHA treatment group had a significant reduction in TAG levels when compared to olive oil (-0.22 ± 0.06 vs. +0.01 ± 0.06 mmol/L, p=0.03), whereas the change after EPA treatment was not different than that of olive oil (-0.03 ± 0.06 vs. +0.01 ± 0.06 mmol/L) (**Table 5-4**). To verify these results in TAG change, a repeated measures 2-way ANOVA was then completed comparing TAG levels at baseline and final time points among the three treatment groups. Similar to the results for the change in TAG levels, significant reductions were only found in the DHA treatment group (**Figure 5-2**). Thus, these data suggest that DHA supplementation lowered circulating TAG levels whereas EPA supplementation did not, in this young healthy cohort.

Table 5-4: Δ-Change in bioclinical measures by treatment group following 12-week intervention. Data is reported as mean ± SEM. BMI, body mass index; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol. A one-way ANOVA was used to analyze data, followed by a Tukey's HSD.

Parameter (%)	Control (n=30)	EPA (n=29)	DHA (n=29)	P Value
Total Cholesterol (TC;mmol/L)	0.19 ± 0.11	-0.01 ± 0.11	0.16 ± 0.11	0.38
LDL-c (mmol/L)	0.12 ± 0.08	-0.06 ± 0.08	0.10 ± 0.08	0.25
HDL-c (mmol/L)	0.07 ± 0.05	0.06 ± 0.05	0.14 ± 0.05	0.50
TC/HDL-c ratio	0.01 ± 0.07	-0.07 ± 0.07	-0.08 ± 0.08	0.68
TAG (mmol/L)	0.01 ± 0.06[a]	-0.03 ± 0.06[ab]	-0.22 ± 0.06[b]	0.03
Glucose (mmol/L)	0.11 ± 0.06	0.13 ± 0.06	0.14 ± 0.06	0.93
hsCRP (mg/L)	-0.21 ± 0.31	-0.01 ± 0.31	-0.15 ± 0.31	0.90

Figure 5-2: Serum TAG levels at baseline and final time points for each treatment group. Data is reported as mean ± SEM. A repeated measures two-way ANOVA was used to analyze data, followed by a Tukey's HSD.



5.4.4 Individual Effects of Treatment on Serum TAG Levels

To better investigate the pattern and individual variability of TAG levels following olive oil, EPA and DHA supplementation we performed linear regression analysis. These data highlight that the magnitude change in either EPA or DHA did not relate to changes in serum TAG levels (**Figure 5-3, A-F),** suggesting an alternative explanation for the observed individual variability. Since EPA and DHA have previously been shown to reduce serum TAG in unhealthy individuals, we next considered the possibility of participant's baseline TAG concentration as a determinant of how they responded to supplementation within this cohort. For the olive oil control group, 13 individuals increased TAG levels whereas 17 individuals decreased TAG levels after 12 weeks of supplementation. Although this is an approximately equal distribution, the change in TAG levels was moderately negatively correlated with baseline TAG levels (R^2= 0.31, $p<0.01$) **(Figure 5-4, A-C).** For the EPA treatment group, 13 individuals increased TAG levels whereas 16 individuals decreased TAG levels after 12 weeks of supplementation. EPA treatment also led to an approximately equal number of individuals increasing and decreasing TAG levels, and the change in TAG levels was moderately negatively correlated with baseline TAG levels (R^2= 0.20, $p=0.01$) **(Figure 5-4, D-F).** For the DHA treatment group, only 4 individuals increased TAG levels whereas 23 individuals decreased TAG levels, with 2 participants seeing no change after 12 weeks of supplementation. This indicates that the majority of participants had a decrease in TAG levels after DHA treatment. Further, the change in TAG levels was strongly negatively correlated with baseline TAG levels (R^2= 0.65, $p<0.01$) **(Figure 5-4, G-I).** Although all individuals saw a negative association between baseline TAG levels and the change in TAG, it can be noted that the strongest association was seen within the DHA treatment group.

Figure 5-3: Correlations between serum TAG levels and absolute change in EPA or DHA in treatment groups. Figures A and B show the correlation between change in TAG and change in EPA and DHA within the Olive Oil treatment group. Figures C and D show the correlation between change in TAG and change in EPA and DHA within the EPA treatment group. Figures A and B show the correlation between change in TAG and change in EPA and DHA within the DHA treatment group.

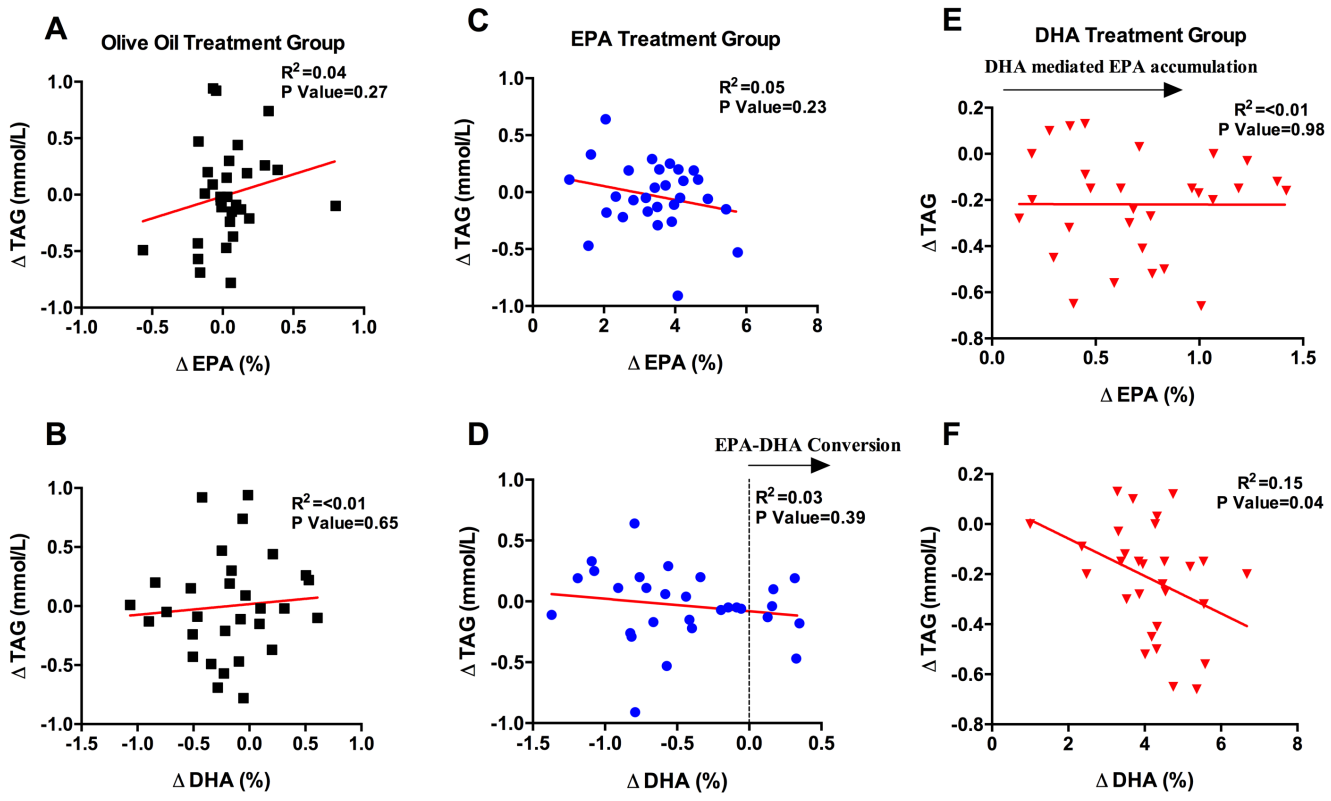
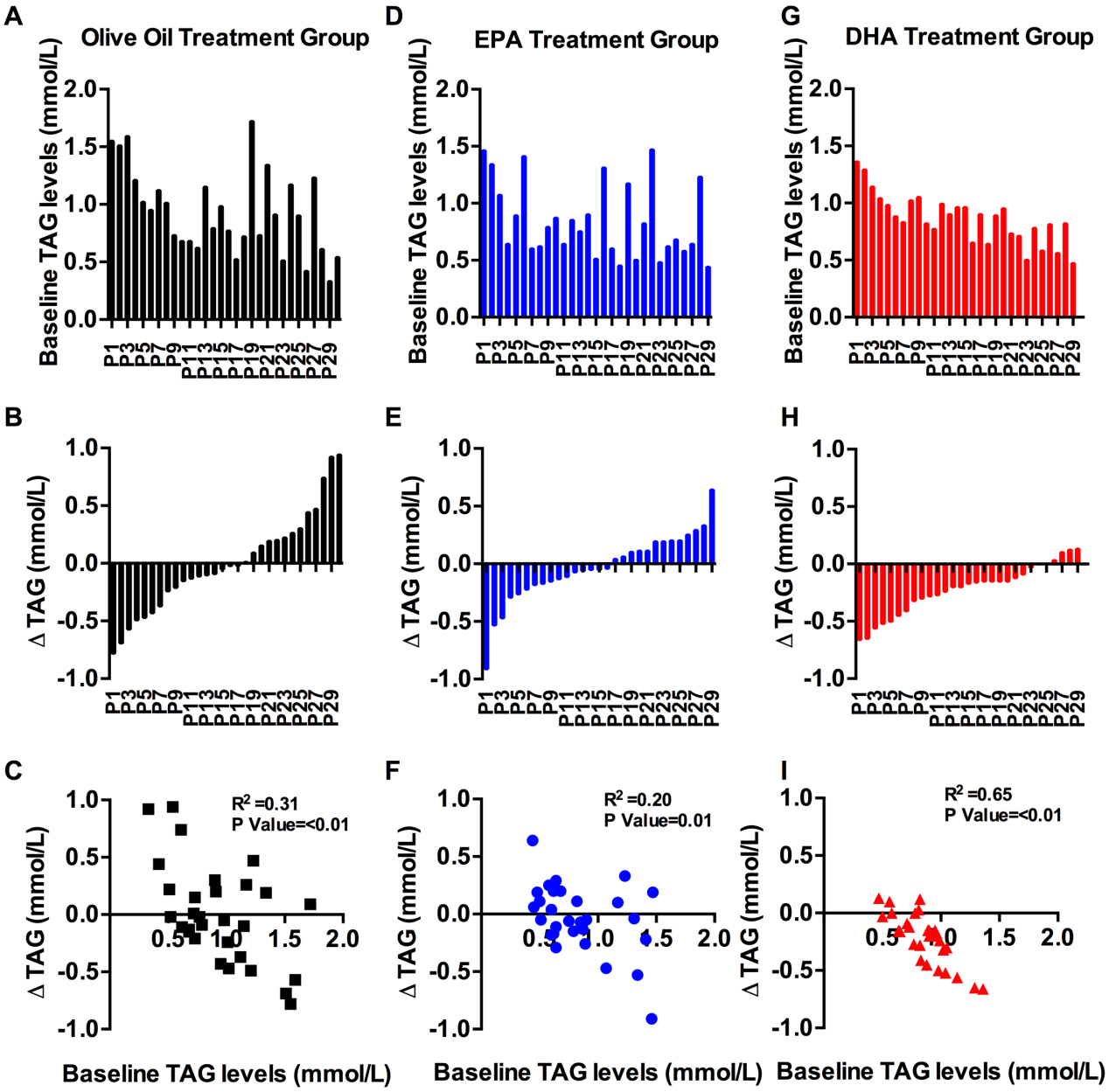


Figure 5-4: Individualized depiction of TAG levels at baseline and change over time. Figures A-C shows within the Olive Oil treatment group the A) Individual baseline TAG levels, B) individual change in TAG levels, and C) the correlation between baseline TAG levels and change in TAG levels. Figures D-F shows within the EPA treatment group the D) Individual baseline TAG levels, E) individual change in TAG levels, and F) the correlation between baseline TAG levels and change in TAG levels. Figures G-I shows within the DHA treatment group the G) Individual baseline TAG levels, H) individual change in TAG levels, and I) the correlation between baseline TAG levels and change in TAG levels.

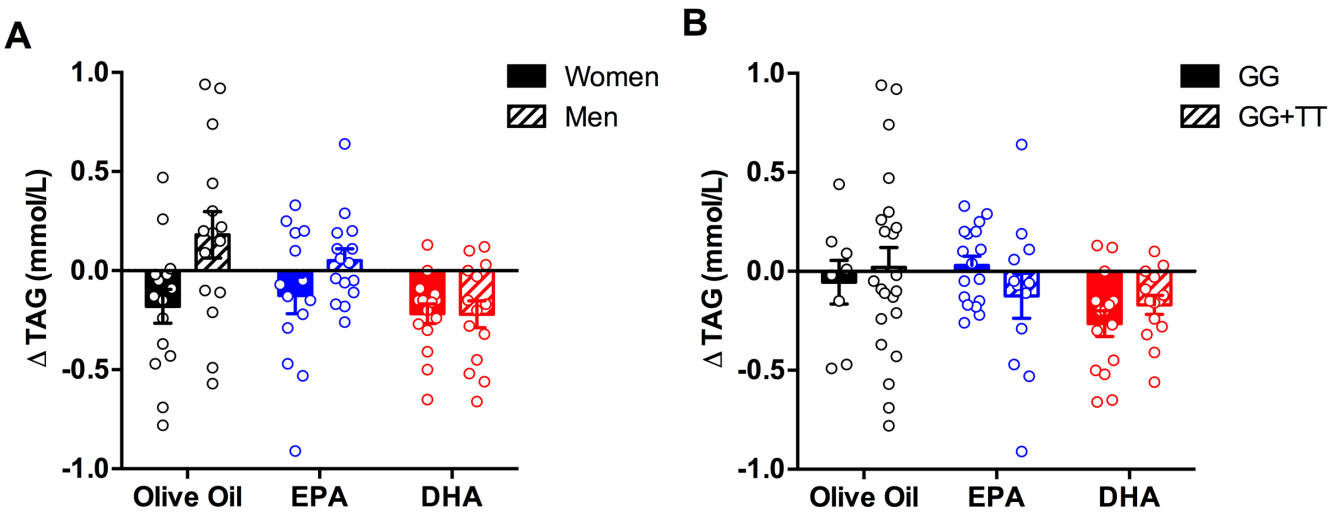


5.4.5 The Influence of Sex and *FADS1* Genotype on Serum TAG Levels

Given the large individual variability within each treatment group for TAG levels, we next wanted to identify whether biological sex or *FADS1* genotype were influencing the response, as these two factors are known to affect TAG levels [252; 253]. For biological sex, baseline TAG levels trended to be slightly higher in women compared to men (+0.94 ± 0.05 vs. +0.81 ± 0.04 mmol/L, p=0.05). However, the change in TAG levels after 12 weeks of supplementation was similar **(Figure 5-5, A)**. For the olive oil group, there was a significant difference in the treatment response between sexes (p=0.02), as women had a decrease and men had an increase in serum TAG. For EPA, there was no significant difference in the treatment response between women and men. In the DHA group, women and men both responded with a similar decrease in TAG levels of ~0.2 mmol/L.

For analysis of the *FADS1* genotype, we compared the response of individuals who were major allele carriers of the rs174537 SNP (GG) to those who were minor allele carriers (GT+TT) **(Figure 5-5, B)**. At baseline, TAG levels were almost identical between major and minor allele carriers (+0.89 ± 0.05 vs. +0.85 ± 0.04 mmol/L, p=0.68). The change in TAG levels after supplementation of olive oil and EPA was similar between major and minor allele carriers. As well, genotype did not affect the observed reduction in TAG level after DHA treatment as both major and minor allele carriers had a similar decrease of ~0.2 mmol/L. Together, these data indicate that within the DHA group, the beneficial treatment effect to lower TAG levels does not appear to depend on biological sex or *FADS1* genotype.

Figure 5-5: Treatment group differences in change in TAG levels according to: A) Biological sex, and B) *FADS1* Genotype.



5.5 Discussion

Our randomized double-blind placebo-controlled study investigated the preventative, as well as differential, effects of EPA and DHA treatment compared to that of olive oil in a young healthy population of men and women on cardiovascular risk factors. The primary findings were that 1) DHA treatment decreased circulating TAG levels while EPA did not, and 2) the beneficial effect of DHA treatment to lower TAG levels does not depend on biological sex or *FADS1* genotype. It is of note that within the DHA group the change in TAG was strongly negatively correlated with baseline TAG levels; however, significant correlations were also found in the EPA and olive oil group. We observed no significant changes in TC, LDL-c, HDL-c, TC/HDL-c ratio, glucose, or hsCRP after treatment with EPA or DHA. Analysis of RBC FA levels revealed a number of differential effects of EPA and DHA; of particular relevance to cardiovascular risk [254; 255]. We found that DHA led to a greater increase in the omega-3 index than EPA; however, both EPA and DHA had beneficial effects on these outcomes. Overall, these findings suggest that DHA supplementation had physiological benefits on serum TAG levels while EPA did not. This could possibly be due to baseline TAG levels, however, our findings supported this in all three treatment groups.

A large body of evidence exists showing that consumption of EPA and DHA, either as part of diet or via dietary supplements, reduces risk of cardiovascular disease, as well as all-cause mortality [232]. A key biomarker in the risk of cardiovascular disease is TAG [159], and it has been well established that n-3 PUFA can aid in the reduction of serum TAG levels [256; 257; 258]. A meta-analysis that investigated the effects of dietary and non-dietary intake of n-3 PUFA on coronary heart disease incidence concluded that there were significant reductions in overall

mortality, as well as mortality due to myocardial infarction and sudden death in patients with coronary heart disease, with increased n-3 PUFA consumption [259]. However, the majority of this information is based on n-3 PUFA supplements or dietary n-3 PUFA sources containing both EPA and DHA and does not differentiate between the two FA. More recently, the divergent effects of EPA and DHA have been compared, specifically with TAG as an outcome, as in our current study. While some studies have demonstrated that DHA is more efficacious at lowering serum TAG [70; 238], others have shown no difference between EPA and DHA [260; 261; 262]. Ranges for baseline levels for these studies were generally higher (approximately 1.22-2.25 mmol/L). In contrast to these previous studies, our study is unique as we compared the differential effects of EPA and DHA for the first time in a young healthy population of both men and women. Moreover, our study population is within a fairly tight age range (18-30 years old); with most other studies having a larger range of 18-70 years [70; 238; 263], and two with a slightly more restricted age gap 40-69 year cutoff [264] and 40-75 [262]. Unlike other studies, our trial was 12 weeks in duration, which is longer than those previously reported to be 6 weeks [262; 263], 7 weeks [238; 264], and 10 weeks [70]. Thus, our work adds to previous research that have studied only men [238] , or men and women with mild abdominal obesity and low-grade inflammation [11; 70], to indicate that DHA has more potent TAG lowering effects than EPA in young, healthy men and women.

It is important to note that in our study, EPA did not lower TAG levels when compared to olive oil. Although this is surprising as many others have shown that EPA supplementation does lower TAG [70; 238; 265; 266], the discrepancy could be explained by age and health status of our participants, as we used a young healthy population and others have primarily used populations at a greater risk of CVD. Such is the case with the REDUCE-IT trial, which found after one year

of supplementation with icosapent ethyl in a cohort of patients with established cardiovascular disease or diabetes, there was an average decrease of 18.3% in TAG levels [267]. Thus if the majority of studies showing a beneficial effect of EPA alone are in populations with higher, or even at risk levels of TAG, then it may be possible that baseline TAG levels could be a predictor of TAG response to EPA treatment. Given our findings that higher serum TAG levels at baseline may lead to a greater treatment effect, we believe that this is likely the main reason for the discrepancy in the findings between trials.

To elucidate what factors aside from baseline TAG levels may indicate individual responsiveness to EPA and DHA, we assessed sex and *FADS1* genotype. Sex differences in lipid levels have been well established in the literature, and importantly in our investigation, TAG levels were shown to differ between males and females [252; 268]. Premenopausal women secrete more TAG rich very low-density lipoproteins (VLDL) particles from the liver but have increased rates of VLDL-TAG clearance as mediated by lipoprotein lipase (LPL). By this mechanism, premenopausal women have overall lower serum TAG levels than their male counterparts [252]. Within our cohort, however, males had slightly lower baseline levels than women (0.81 ± 0.05 vs. 0.94 ± 0.04). Although this is contrary to what was expected, these trends are normally identified in older, although still premenopausal, women [229] while we used a younger cohort of women in the current study. Within the age range of the women in our cohort, there was high use of oral contraceptives, which is known to increase TAG levels, thus this may explain at least some of the differences [269; 270; 271]. In this respect, our investigation aimed to explore if sex may moderate the impact of DHA and EPA treatment on TAG reduction. Our results showed that within the TAG changes resultant from the three treatment groups, there were no sex-specific differences. Furthermore, we were interested in seeing if TAG changes over the 12-week

supplementation period were moderated by *FADS1* genotype. Previous genome-wide association studies (GWAS) research has shown a relationship between *FADS1/2* genotype and plasma lipid concentrations [253; 272; 273; 274]. Although the majority of these studies did not investigate associations with our SNP of interest (rs174537), they did research the rs174547 and rs174576 SNPs, which are in strong linkage disequilibrium (LD) with our SNP. However, we did not find any genotypic differences in the change in TAG following treatment with olive oil, EPA or DHA. These findings allow us to conclude that sex and *FADS1* genotype did not play a role in the variation seen in TAG response.

In contrast to the beneficial effect of DHA on TAG in our study, we observed no change in most other cardiometabolic risk factors (LDL-c, TC, etc.). This result was somewhat unexpected as previous studies that also compared the effects of EPA and DHA treatment have shown that supplementation was able to lower these markers [70; 238; 260; 262]. As described previously, it is likely that the discrepancies in our study findings compared to previous research can be attributed to the young, healthy population we investigated in the current study.

In addition to more traditional cardiometabolic risk markers, RBC FA abundance is also a reliable marker of long term dietary fat intake and has been strongly connected to relative risk of cardiovascular disease as well as all cause mortality [275], which did improve after EPA and DHA treatment in our study. The omega-3 index can be a clinical marker for consumption of n-3 PUFA, and Allaire and colleagues found that DHA treatment had more profound effects on this index than EPA [11]. This data agrees with the findings from our study, with DHA supplementation increasing the omega-3 index ~5%, and EPA increasing the index just ~3% ($p<0.01$). Interestingly, we also found that EPA increased in the DHA group, which agrees with

a number of DHA supplementation studies [238; 276; 277]. In contrast, EPA supplementation did not lead to increases in DHA levels within RBC membranes, which was seen by some [70; 238; 263; 264], but not all [266] studies comparing the effects of EPA and DHA supplementation. Unfortunately, our analysis did not include the FA docosapentaenoic acid (DPA), and therefore we cannot conclude that EPA was elongated to DPA, but not DHA, which had been seen in a number of previous studies in both rats and humans [278; 279; 280; 281]. While our data have shown these specific FA changes, some studies have suggested that variability exists in the measurement and quantification of FA levels between laboratories, thus some of these differences between ours and prior studies could be due to methods of measurement [282].

5.5.1 Strengths and Limitations

This study has a number of strengths and limitations that require reflection. Currently, scientific literature in the combined effects of EPA+DHA greatly outnumbers the research on the individual effects of EPA and DHA. The current study utilizes both a high EPA and high DHA supplement to discriminate the cardiometabolic effects of each. While it might also be seen as a weakness that there was not a combined EPA + DHA group, we concluded this was unnecessary as there is a substantial amount of literature already using this treatment. With the goal of our research being to identify the individual effects of EPA versus DHA on markers of cardiometabolic risk such as TAG in a young, healthy population, we understand that the more subtle differences might be missed in a cohort with limited cardiometabolic risk. However, we wanted to explore if supplementation with EPA or DHA can be used as an intervention before the onset of cardiovascular disease. Our results suggest the use of DHA may be an effective preventive intervention for cardiometabolic risk. In addition, although the sample size might be

limited, the study groups all had equal distribution of males and females to allow for sex specific comparisons in each. Finally, there was variability in serum TAG levels in all groups following the 12-week study duration. Although we are able to hypothesize that baseline serum TAG levels play a role in this variability, we were unable to determine if there were subsequent changes in *de novo* lipogenesis that might cause inter-individual variability within our cohort.

5.6 Conclusion

In conclusion, data from this randomized, double blind, placebo-controlled study shows that following 12 weeks of treatment with either 3 g/day of olive oil, EPA or DHA, the physiological benefits of DHA supplementation are greater than that of EPA, and notably, that EPA supplementation had no effect on serum TAG levels. Thus far, there is insufficient evidence to strongly advise on the specific use of EPA or DHA alone for therapy or prevention of cardiovascular disease. To apply our study findings, for the use of n-3 PUFA supplements (DHA+EPA) on TAGs, baseline health should be an important consideration when beginning treatment, as those with healthy lipid levels may not confer any benefits on lipid parameters. Additional studies are needed to compare the differences of treatment with EPA and DHA directly, and to determine those individuals who might differentially benefit from DHA, EPA or DHA+EPA supplementation

6 Integrative Discussion

6.1 Summary of Thesis Aims and Main Findings

The primary objective of this thesis was to investigate the role of sex and genetics on cardiometabolic risk factors, with an overarching goal of advancing our knowledge as to how these non-modifiable factors may interact to influence cardiometabolic health. Specifically, it was found that:

1) Sex differences in cardiometabolic risk factors were not due to SNPs in common lipid metabolism genes.
2) SAT FA levels, but not inflammatory gene expression profiles, were distinguished by FADS1 genotype.
3) Supplementation with DHA was more effective at lowering circulating TAG than EPA, which may be explained by baseline TAG levels, but not by sex or *FADS1* genotype.

This thesis was comprised of three distinct studies, each with a unique aim and hypothesis. In Study 1, we aimed to determine interactions of sex and genotype between a panel of common candidate SNPs that have been previously associated with blood lipids and FA, as well as the lipid and FA profile. Using participants from the GONE FISHIN' and GOLDN cohorts, we found that blood lipids and FA vary between healthy young men and women, but that these differences were not associated with candidate SNPs in lipid and FA metabolism genes. Women were found to have higher HDL-c and lower TC/HDL-c, while also having higher RBC palmitoleic acid levels and SCD desaturation index. Therefore, Study 1 showed that sex

specific differences in blood lipids and FA may not be explained by specific candidate SNPs selected for this investigation. As these assessments were done for circulating levels of lipids and FA, it is important to acknowledge that this was not completed within tissues, and thus candidate SNPs in lipid and FA metabolism genes may in fact influence tissue phenotype. As excessive storage of lipids and FA within AT is a risk factor for CVD, and there are known sex differences in the anatomy and physiology of AT, there is a possibility that candidate SNPs may explain, or be explained by, AT phenotype.

In Study 2, we therefore aimed to examine if SAT FA composition and the expression of obesity-related transcripts involved in inflammation, immune function, lipid metabolism, and cellular differentiation could be distinguished by *FADS1/FADS2* genotype. Using participants from the the DiOGenes cohort [216], we found that the *FADS1* genotype was associated with SAT FA levels, but not inflammatory gene expression. Specifically, variation in the rs174537 SNP in the *FADS* gene, (a SNP associated with DNA methylation in the putative enhancer region)[226], was associated with SAT FA levels. This included levels of DGLA and AA, which accounted for ~19% of the variance between the rs174537 genotypes, and were also associated with the expression of genes linked to inflammatory, immune function, and lipid metabolism. In contrast, inflammatory gene expression does not distinguish the rs174537 genotype, and accounts for less than 7% of the variance between the rs174537 genotypes. These data provide important insight into the relationship between the *FADS1/FADS2* SNP and SAT FA profiles. Although these findings, and that of Study 1, add to the literature and highlight the role that sex and genetic variation play in the regulation of circulating and AT lipid, FA, and inflammatory gene expression, both studies were cross-sectional in design. Thus, it is of importance to understand

whether sex and genetic variation are also factors that can explain individual variation in response to treatment.

In Study 3, we therefore aimed to examine how sex and genetic variation impact the individual response to EPA or DHA treatment on circulating lipid and FA levels in young healthy men and women. Using a 12-week randomized double-blind placebo-controlled study, we found that the physiological benefits of DHA were greater than that of EPA, as DHA lowered TAG levels, while EPA did not. Additionally, we found that individuals who had higher baseline levels had lower TAG levels after treatment, with the DHA group having the strongest relationship. This was not dependent on biological sex or *FADS1* genotype. Together, the results of the three studies of this thesis advance our knowledge as to the role that sex and genetics have on aspects of cardiometabolic health. Furthermore, there are a number of themes that transpire across the three studies of this thesis that enable the results to be integrated into the larger scientific framework, for example, that of biological sex and genetics with regard to cardiometabolic health.

6.2 Attention to Biological Sex Differences in CVD Research

Biological sex differences were a key concept of this thesis, specifically in Study 1 when investigating sex-specific differences in lipid and FA metabolism genes, and in Study 3 when examining if n-3 PUFA TAG reductions were sex-specific. This is important, given that CVD is responsible for a third of the fatalities of both sexes in North America, but women are grossly underrepresented in clinical trials [283]. A consequence of this is a sex bias toward males in our understanding of lipid and FA metabolism [284; 285]. Although we did not see that SNPs in our candidate genes were important for distinguishing sex differences in blood lipids and FA levels

in Study 1, or that serum TAG response to EPA or DHA was dependent on sex in Study 3, our findings are nonetheless important on many levels. We add important insight into the interaction between sex specific differences and genetics, and whether this is physiologically relevant for CVD risk. Although we found results primarily of a confirmatory nature (e.g. HDL-c is higher and TC/HDL-c is lower in young women) [185; 186; 187], this information can add to the growing body of evidence that helps to further sex-specific health care and nutritional guidance.

As discussed in previous reviews, there are noted sex-specific differences in both lipid and essential FA metabolism [167; 188; 286]; however, there are a lack of studies that have closely examined sex differences in TAG serum response to n-3 PUFA treatments. Thus, by examining if the TAG response to EPA or DHA treatment could be explained by biological sex in Study 3, we generated important evidence to guide the optimal use of n-3 PUFA products in men and women. Moreover, our study used a population of young healthy men and women, meaning that any differences observed were likely less attributable to the aging process [287] and associated differences that develop that may preclude CVD, thus allowing for a direct comparison of risk factors between the sexes. However, using females of reproductive age poses a challenge in research, as the hormones associated with the reproductive cycle can introduce variability in data [173]. To ensure the representation of both sexes, and to allow for inclusive collection of accurate data on EPA and DHA treatment efficacy from both males and females, Study 3 ensured female participants were brought in at the same time during the follicular phase of the menstrual cycle[173]. Altogether, the underlying role that sex plays in the development of CVD and how it may influence the response to n-3 PUFA treatment is underdeveloped, and we add important data to this growing field.

6.3 *FADS1/2* in CVD Research

Another central theme of this thesis was to examine the relationship between the *FADS* genotypes and risk factors for CVD [65]. This work is timely as the field of nutrigenomics is an important and growing area of investigation to further understand the etiological foundation of CVD. More specifically, *FADS1/2* have been linked to a number of biological risk factors of CVD, but due to the pleiotropic nature of CVD the impact of this on the progression of the disease is unknown. In humans, expression levels of *FADS1* and *FADS2* vary depending on the tissue [288], but is high in the liver [135; 142]. Links have been identified between SNPs in *FADS1/2* and gene expression [288], with evidence of an association between SNPs in serum and plasma with RBC levels of AA and DGLA being plentiful [139; 170; 212; 289; 290; 291; 292; 293; 294; 295; 296; 297; 298], specifically for the rs174537 SNP [222; 293; 294; 299]. Therefore, Study 2 in this thesis was novel as we examined this relationship between SNPs and gene expression in AT, as well as finding that AA and DGLA help to discriminate the genotype. To further advance this research, it would have been beneficial if we could have also investigated these relationships within the liver, as it is the tissue with highest *FADS1* and *FADS2* gene expression [135; 142], therefore allowing us to get a more diverse repertoire of tissue analysis.

To further build on our work linking *FADS1/2* and lipid profile, in Study 3 we found that the rs174537 SNP in *FADS1* did not influence the change in serum TAG after DHA treatment. In a 2019 publication from the GLACIER study, a significant interaction effect was found such that the *FADS* genotype and associated differences in TAG levels could be related to magnitude of PUFA intake [300]. The goal of that study was to find "haplotype blocks" that harbour a number of regulatory variants that are functionally increased [300]. From these functional annotations, the

authors were able to determine that it is not one single causal variant that would be causing these effects, but instead multiple functionally increased variants within a region [300]. In the context of our findings, this could indicate that no change in TAG levels following EPA and DHA treatment between the *FADS1* genotypes could be due to the use of a single causal variant within our analyses, instead of consideration that it could be multiple variants in combination that create this biological association. Thus, in future work, or even in re-analysis of the samples on hand, we could measure additional variants in *FADS1/2* and assess whether minor allele carriers of two or more variants have a differential response to n-3 PUFA treatment, which may further identify the biological importance of *FADS1*.

Study 1 and Study 2 demonstrated clear n-3 PUFA differences between major and minor allele carriers for the rs174537 SNP in *FADS1*, which showed dominant effects over other *FADS1* SNPs, as shown in the statistical analysis of Study 2. Reasons for this SNP's dominance are not entirely understood. Epigenetic changes to *FADS1/2* have also begun to be elucidated, and these may provide insight on subsequent effects in tissues. For example, DNA methylation is an epigenetic modification that could impact *FADS* gene expression levels [301]. Chilton et al. found a strong association with the rs174537 SNP in human liver and the cg27386326 methylation site, which occurs within a putative enhancer region 3.5kb and 7.8kb from the *FADS1* and *FADS2* transcription initiation sites, respectively [301]. Interestingly, the methylation within this region was found to be 0.44 in the GG genotype, and 0.84 for the TT genotype, and furthermore, found to have significantly associated with PUFA biosynthesis [301]. In a follow-up study it was found that 86% of the CpG sites were highly methylated, specifically within the promoter regions of the *FADS1* and *FADS2* genes [226]. Furthermore, 8 methylated CpG sites within the putative enhancer region were significantly associated with the rs174537 site [226].

Excitingly, these studies investigated the rs174537 SNP, which is the exact SNP of interest throughout this thesis, therefore generating questions as to how their findings play a role in the results presented in this thesis. Hypothetically, the ability of the SAT FA composition to distinguish the *FADS1/FADS2* genotype in Study 2 could have been due to highly methylated regions within transcription initiation sites/putative enhancer regions altering the activity of these genes. This could in turn have created more methylation on the TT, and less methylation on the GG genotype, creating a larger gap in the PUFA biosynthetic capacities of these genotypes. Together, these studies collectively show that we are only beginning to understand the genetic and epigenetic factors at play that affect regulation of PUFA biosynthesis, and how these might ultimately affect ability to produce FAs and effect CVD risk.

6.4 Omega-3 Recommendations and Mixed Conclusions in Research

In Study 3, a novel formulation was used to create the EPA and DHA supplements, which included ~3 grams of EPA or DHA per capsule at 99% purity. As there are a number of n-3 PUFA trials in the literature, it is of value to compare how our formulation fits, and whether differences in formulation may influence the consensus and consistency between studies [302]. This includes questions as to which formulation should be primarily used (EPA vs. DHA), chemical form (TAG, EE, PL, etc.), dose, and duration, thus making the pharmaceutical or nutraceutical indications a moving target [302]. Recently, Allaire and colleagues completed a 10-week clinical trial that compared the effects of 2.7 g/day of EPA and DHA on TAG levels as well as markers of inflammation, blood lipids and FA in men and women at risk of CVD [69; 70]. This clinical trial is the closest comparison to Study 3 of my thesis, and they observed significant decreases in TAG levels following DHA treatment, but also after EPA [70]. As we only observed

a decrease in TAG with DHA, there are a number of differences between our study designs that could explain the discrepancy in results. To begin, their cohort of participants had significant CVD risk (abdominal obesity, elevated levels of CRP), and were of a broad age range (18-70 y) [70]. Finally, and probably most importantly, their baseline TAG levels were significantly higher than our cohort of individuals (avg: 1.4-1.5 mmol/L vs. 0.84-0.93 mmol/L, respectively) [70], which we now suspect plays a substantial role in the variability in response. Because of these key differences, it is understandable how variability in response to EPA and DHA might have occurred between studies. Yet, these findings are important when reviewing the use of pharmaceutical grade n-3 PUFA formulations for the dietary management of lipids, including Epanova and Lovaza [303; 304; 305; 306]. Epanova, a FFA supplement, and Lovaza, an EE formulation, are used as a TAG lowering treatment in patients with severe hypertriglyceridemia, and as an adjunct to a low cholesterol, saturated fat, and sodium diet [305; 306]. These formulations are comprised of ~1g of PUFA (550 mg EPA, 200 mg DHA, and 465 mg EPA, 375 mg DHA) per capsule, respectively, of EPA and DHA [305; 306]. In the trials examining the efficacy of these formulations, the participants enrolled had 400-1000% higher baseline TAG levels then the participants in our trial [307], even higher than that of Allaire [70]. In individuals with very high TAG levels, EPA led to 80% decreases in non-HDL-c levels, whereas DHA only resulted in reductions of 40%, and LDL-c increases were observed in DHA but not EPA treated cohorts [308]. Thus, in this high risk hypertriglyceridemic population [307], the EPA in the formulations could elicit extra benefits beyond DHA, which may explain why approximately balanced formulations are used. Altogether, there is mixed evidence as to when n-3 PUFAs should be recommended for CVD risk, and if so, which formulation, and how much. Based upon the evidence within our clinical trial, it is clear that 3g of DHA has lipid lowering potential and can provide a positive

nutritional impact on even those individuals who are healthy. However, EPA lacked the ability to lower TAG levels in our study, and therefore, in individuals who are healthy, thus we cannot conclude that it should be recommended for use in this population group. Yet, taking into consideration the studies previously discussed in which EPA was effective at lowering TAG in persons with higher baseline levels [70; 307; 308], baseline health status should be considered before recommendation of supplementation with n-3 PUFA.

As baseline health status may have important implications in the efficacy of n-3 PUFA formulations, it is of critical importance to understand how individual variation may be involved. Unfortunately, there have only been three studies to date that have investigated this. In a recently published study from the ComparED cohort, Allaire and colleagues investigated the intra-individual variation in TAG reductions following 10 weeks of 2.7 g/day of high dose DHA or EPA [309] and found that more individuals had a TAG reduction greater than 0.25 mmol/L after DHA as compared to EPA (45% vs. 32%, $p<0.001$) [309]. Consequently, they concluded that 2.7 g/day of EPA or DHA did not have a meaningful impact on proportion of individuals who had triglyceride values within a normal range, but was effective in individuals with higher baseline levels [309]. Similarly, a study by Grimsgaard and colleagues supplementing participants with 3.6-3.8 g/day of EPA or DHA for 7 weeks, extrapolated participants by baseline TAG serum levels and found that DHA had a more pronounced TAG lowering effect across all baseline concentrations than EPA [238]. These results reflect the findings within Study 3 of my thesis, as we concluded that baseline TAG levels could possibly be determinants of the response to the DHA intervention and that not all individuals have reductions, but, unlike their study, we did not observe an effect of EPA. In addition to this work, Nording and colleagues investigated the individual variation in lipidomic profile (FA, lipid classes, lipoprotein distribution and oxylipins)

in 12 healthy subjects following 6 weeks of 6 grams/day of fish oil (1.9 g/d EPA + 1.5 g/d DHA) [10]. They found that both n-3 and n-6 PUFA metabolites had large magnitude of variation among the subjects, such as 12-hydroxyeicosapentaenoic acid (an EPA lipoxygenase metabolite) which ranged from an 82% increase to a 5,000% decrease [10]. Likewise, half of the subjects decreased levels of the AA metabolites PGE_2 and TXB_2, while the other half either had no change, or an increase in levels [10]. Although this study did not investigate TAG levels, it showed the enormous variability in response to an n-3 PUFA intervention, and consequently variable disease risk. Taken together, the results from these studies indicate the need for clinical trials investigating individual variability in n-3 PUFA response and reinforces the finding that variability is still present even in low-risk individuals.

Although there is individual variation in the response to n-3 PUFA, the mechanisms by which reduced TAG levels are widely believed to be due to decreases in hepatic VLDL TAG production, as well as from increases in VLDL clearance [72]. This is largely gathered from results of tracer studies [310; 311; 312; 313; 314; 315; 316; 317], of which three were randomized controlled trials that used an EPA+DHA group or analyzed the two groups together [311; 312; 316], therefore whether there are differential effects of DHA and EPA are not fully known. Furthermore, only Park et al. used normolipidemic individuals to investigate the effects of EPA and DHA on change in TAG, and only in men, so information on individuals in a non-pathological state is lacking [316]. In Study 3, we found that DHA treatment resulted in significant TAG reductions whereas EPA did not. In a manuscript currently submitted to the American Journal of Clinical Nutrition [318], and not presented within this thesis, we continued analysis of Study 3 to examine LPL activity and markers of lipogenesis via both the lipogenic index and compound specific isotope analysis. The lipogenic index is commonly used as a non-invasive measure of *de novo*

lipogenesis, and is the ratio between palmitate (C16:0) and linoleate (C18:2n-6) [319]. The compound specific isotope analysis involves the quantification of the carbon isotopic signature ($^{13}C/^{12}C$ or $\delta^{13}C$) of FA in the body, with the concept being that dietary sugars are more enriched in ^{13}C, and therefore would indicate increased *de novo* lipogenesis from sugars [320]. Our results showed that EPA treatment increased the lipogenic index by 11% ($p<0.01$) and $\delta^{13}C$-16:0 from -23.2±0.2 to -22.8±0.2 milliUrey ± SEM ($p=0.03$), whereas DHA did not change either [318]. In both the EPA and DHA treatment groups, however, there was an increase in LPL activity ($p=0.09$ and $p=0.05$, respectively) [318]. Collectively, we can conclude that the beneficial reductions in TAG following DHA treatment could be mediated via an increase in LPL-mediated TAG clearance, and as EPA did not lower TAGs within our cohort potentially this could be due to an increase in both lipogenesis and LPL-mediated TAG clearance [318].

6.5 Limitations and Future Directions

There are a number of limitations within the studies of this thesis that require consideration. Firstly, an important alternative study design for Study 3 would have been to include individuals at moderate-to high cardiometabolic risk (e.g. age between 19-65 y, CRP levels between 1-10 mg/L, and abdominal obesity of ≥80 cm for women and ≥94 cm for men, as per the International Diabetes Federation cut-offs [321]) rather than to focus solely on healthy participants. This alternative approach would have better carried on from the results in Study 2 and would have expanded our work to look at the individual variation in those who are at risk of CVD, as opposed to looking for the more subtle results that might be found in healthy individuals. This is an approach to be considered for future research. Given that our results showed that DHA is more efficacious at lowering TAG than EPA, and that these changes seem

to be more pronounced in individuals with higher baseline levels, a logical follow-up study is to complete the same design but in individuals at risk for CVD. This has, at least somewhat, been completed by the Lamarche group [70; 309]; however, additional replication of this work is needed to strengthen the rationale for the use of specific n-3 PUFA formulations to reduce cardiometabolic risk. Furthermore, within Study 3 we were unable to control for daily variation in food composition. All participants were asked to consume the same foods the day before their baseline and final visit; however, this does not allow us to ensure that all participants consume the same macro and micronutrient profiles throughout the 12-week duration. As an example, the role of the "matrix effect" suggests consumption of n-3 PUFA with high-fat food can stimulate fat-digesting enzymes and increase bioavailability [322]. Although not advisable in individuals at high CVD risk, if this was a routine for certain participants, this could affect bioavailability of the treatment [322].

In addition, the Study 2 analysis focused on SAT FA and investigated the relationships this has with the *FADS1* genotype, as well as inflammatory genes. A limitation of this work is the absence of visceral adipose tissue (VAT) sampling to also investigate these relationships. VAT contains a larger number of inflammatory and immune cells, as well as having an increased number of larger adipocytes (hypertrophy), whereas subcutaneous adipose tissue allows for more preadipocyte differentiation (hyperplasia) [323]. As participants in our study had a BMI classified as overweight, whether the results would be different if we measured VAT between studies would be interesting to identify. Moreover, we also enrolled both men and women in our study. Given that men and women accumulate AT in different locations [324], if we also could obtain VAT samples it would be interesting to explore sex differences in this tissue.

Finally, in all of the studies of this thesis we explored the effects of a single genetic variant. Due to the polygenic nature of CVD, it would be interesting to develop a scoring system in which multiple loci are examined in combination. Since it is unlikely that one variant alone could possess pleiotropic effects that significantly increase the risk of CVD, it would be interesting to have an overall genetic risk score based on the presence or absence of a number of different SNPs. Early attempts to create these risk scores were dampened by the prematurity of understanding how genetics plays into a complex disease such as CVD [325]. While we would require a much larger sample size of our studies to thoroughly address this hypothesis, it is an important area of future research that could help improve the accuracy and effectivity of CVD risk prevention efforts.

6.6 Concluding Remarks

This thesis provides important insight into the role of genetic variance and sex in the regulation of blood FA, blood lipids, and AT FA. Collectively, the data presented highlight three main findings: 1) Sex differences in cardiometabolic risk factors are not due to SNPs in common lipid metabolism genes such as *FADS1*, 2) the *FADS1* genotype is associated with SAT FA levels but not inflammatory gene expression, and 3) DHA treatment is more effective at lowering circulating TAG than EPA, which could be explained by baseline TAG levels but not sex or *FADS1* genotype. Taken together, the findings in this thesis have helped to improve and expand our understanding of the wide ranging impacts of the *FADS1/2* genotypes on overall human health and risk of CVD, and underscore the importance of individual biology when studying the prevention and treatment of disease.

7 References

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