1 – OVERVIEW, RATIONALE AND SPECIFIC AIMS

In the mid 1970s, Danish physicians made the seminal discovery that high levels of the marine omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the diet of Greenlandic Inuit was associated with reduced mortality from cardiovascular disease (CVD) [1]. This finding has fuelled over 50 years of intense investigation to better understand the health benefits associated with EPA and DHA, as well as their underlying mechanisms of action. Epidemiological and clinical studies now show that higher levels of EPA+DHA in the body have beneficial effects on numerous CVD risk factors, including triglyceride (TG) concentrations, blood pressure (BP), inflammation, endothelial function and platelet coagulability [2]. Many, but not all, studies have concluded that omega-3s can reduce the risk of congestive heart failure, coronary heart disease, ischemic stroke, and sudden cardiac death. Despite a lack of consensus, science advisories of the American Heart Association support the intake of omega-3s for the prevention of cardiovascular events [3, 4]; a message also endorsed by the Heart & Stroke Foundation of Canada (HSFC).

Unfortunately, recent data from the Canadian Health Measures Survey revealed that most of the Canadian adult population have alarmingly low levels of EPA+DHA [5]. **As such, ensuring that individuals have optimal levels of EPA+DHA is important for cardiovascular health.** Two methods are currently used to determine an individual's omega-3 status, but both have significant limitations. The first, self-reported assessments of food intake such as food records and food frequency questionnaires (FFQs) are inaccurate and subject to reporting biases [6-9], and are unable to account for individual differences in omega-3 bioavailability. The second method is to measure EPA and DHA in red blood cells (RBCs) obtained from a blood sample. The combined amount of EPA+DHA in RBCs, expressed as a percentage of total fatty acid content, is known as the Omega-3 Index (O3I) [10]. The O3I has recently emerged as a validated diet-sensitive biomarker of cardiovascular health [11]. While the O3I is considered the "gold standard" for determining an individual's omega-3 status, measuring it requires specialized equipment that is not amenable for routine screening. Further, the required blood sample often poses a significant barrier for children and adults who have anxiety and a fear of needles [12-17]. To overcome the challenges associated with dietary assessment and blood analyses, researchers are exploring the use of urinary biomarkers to non-invasively assess an individual's nutrient status [18-20]. We recently identified a panel of 17 urinary biomarkers that were significantly correlated with the O3I following high-dose EPA-only or DHA-only supplementation relative to a placebo control ([21], see attached paper). We therefore propose that urinary biomarkers that reflect an individual's omega-3 status could provide an unbiased and non-invasive approach for risk assessment of cardiovascular health.

The **overall objective** of our proposed research is to determine if urinary biomarkers that reflect an individual's omega-3 status can provide a non-invasive method to monitor changes in CVD risk factors. We will achieve our objective through **two complementary aims** that will be addressed with a prospective study in which individuals are randomly assigned to groups consuming different doses of omega-3s for a 12-week intervention period followed by a 12-week washout period. **Aim 1** will determine if a dose- and time-response relationship exists between changes in urinary metabolites and the O3I. **Aim 2** will assess whether the reductions in CVD risk factors (e.g., TG concentration, inflammatory markers, and resting BP) associated with increased omega-3 intake correlate with changes in urinary biomarkers. The proposed research is both novel and important in the search for non-invasive biomarkers of cardiovascular disease risk, which has enormous potential to advance precision nutrition strategies that aim to improve cardiovascular health. Importantly, our proposed research aligns strongly with the HSFC mandate to promote healthy living through prevention and healthy lifestyles. We anticipate that the outcomes of this project will: a) demonstrate the accuracy of urinary biomarkers to reflect changes in the O3I that will negate the need for blood sampling, and b) strengthen the evidence regarding the use of omega-3s for the primary prevention of CVD.

2 – BACKGROUND

2.1 Omega-3 Fats in the Diet. The three predominant omega-3 fatty acids consumed in the diet are alpha-linolenic acid (αLNA), EPA, and DHA [22]. The most highly consumed omega-3 is αLNA, which is rich in green leafy vegetables and vegetable-based cooking oils (e.g. flaxseed, rapeseed, and soybean oils) [23]. Typical consumption of αLNA in Western societies ranges from 0.5-1.7 g/d [23]. The benefit of increasing αLNA for cardiovascular health outcomes is not well established [24]. In contrast, EPA+DHA, which are high in fatty fish (e.g., salmon) and fish oils, are consumed to a much lower extent. Estimates suggest that less than 20% of the world's population consumes ≥0.25 g/d of EPA+DHA (equivalent to two servings of salmon per week) [25]. The low levels of EPA+DHA consumption is particularly alarming given that low levels of these fatty acids are associated with increased risk for numerous cardiometabolic diseases [26].

2.2 Methods for Determining Omega-3 Status in the Body. Most of the EPA+DHA in the body is derived from the diet, with a small amount coming from the endogenous conversion of αLNA [27]. The most widely accessible method to estimate omega-3 status is through the use of food records and FFQs; however, their accuracy is limited for several reasons. First, the levels of EPA+DHA in the body are influenced by several unmodifiable (sex, genetics, age) [28-30], modifiable (physical activity, alcohol, smoking, diet) [28-31], and bioavailability (chemical form of supplements) [32] factors. Second, changes in fish farming practices due to increased global demand has led to a ~50% reduction in EPA+DHA content in commonly consumed fatty fish, such as Atlantic salmon [33], thus making it difficult to know exactly how much of these fatty acids an individual is truly consuming. Third, it is well known that dietary assessment tools (food records, FFQs) are subject to substantial error arising from intake-related and person-specific biases [6-9]. Due to the array of factors that limit the accuracy of assessing EPA+DHA status with food records and FFQs, more accurate and objective methods to determine the actual levels of these important fatty acids in the body are necessary.

The most accurate way to determine an individual's EPA+DHA status is through the collection of blood or tissue samples. For example, serum, plasma, RBCs, and adipose tissue can all be used to measure EPA+DHA levels in the body. Serum and plasma provide information about acute and short-term changes in dietary fatty acid intake, RBCs provide information about moderate-term intake due to the slow turn-over of these cells (120-d lifespan), and adipose tissue provides information about long-term intake [29, 34]. Katan *et al.* [35] reported the different rates of incorporation of EPA into serum, RBCs and adipose tissue in men consuming fish oil for one year. Specifically, they showed that EPA reached half-maximal levels after ~5 days in serum and 28 days in RBCs, while adipose tissue levels did not plateau during the one-year study. More recent studies showed that increases in plasma EPA+DHA are detected within hours of consumption [36-38]. This demonstrates that serum and plasma fatty acids change rapidly in response to dietary intakes and are therefore not suitable to determine an individual's long-term EPA+DHA status. In contrast, adipose tissue has a slower rate of fatty acid turn-over and is considered the best choice to assess long-term fatty acid intake; however, it is costly and not feasible to routinely collect adipose tissue samples due to the invasiveness of the procedure [29, 34]. **RBCs are therefore considered optimal to assess EPA+DHA levels in an individual because their levels in RBCs are more stable than in serum and plasma, while blood sampling is far less invasive than adipose tissue sampling.** Further, EPA+DHA levels in RBCs are strongly correlated with membrane EPA+DHA content in tissues, such as heart, liver, muscle, and kidney [22, 39].

2.3 The O3I: A Validated Diet-Sensitive Biomarker for Cardiovascular Health. The amount of EPA+DHA in RBCs, expressed as a percentage of total fatty acids, is known as the O3I [40]. The O3I was first proposed by Harris and von Schacky in 2004 as a novel biomarker to assess coronary heart disease risk [40]. Through several prospective and retrospective analyses, threshold values were established for the O3I in relation to cardiovascular risk. Specifically, **an O3I of >8% was associated**

with low CVD risk, while an O3I of <4% was associated with high CVD risk [40]. These threshold values were corroborated in a meta-analysis of 10 cohort studies, which showed that the relative risk for fatal coronary heart disease (CHD) in individuals with an O3I of >8% was reduced by 30% compared to those with an O3I of <4% [41]. **Data collected in the Canadian Health Measures Survey revealed that more than 40% of Canadian adults have an O3I of <4% (i.e., levels associated with high risk) and fewer than 5% of Canadian adults have an O3I of >8% (i.e., levels associated with low risk)** [5]. However, we [42-44] and others [45-48] have shown that the O3I is modifiable with changes in omega-3 intake. For example, the O3I was shown to increase from 4.3% at baseline to 7.7% in participants who consumed ~2 g/d EPA+DHA from fish oil for 8 weeks [45]. More recently, it was shown that tightly controlled intakes of 0.25, 0.5 and 1.0 g/d EPA+DHA resulted in linear increases in the O3I in both males and females [49]. While diet is the main predictor of changes in the O3I, unmodifiable factors such as sex, age, and genetics also influence the O3I [28-30]. This reinforces the importance of directly measuring EPA+DHA levels rather than estimating their intake by dietary assessment. However, the fear and anxiety associated with needle procedures (venipunctures) is a significant obstacle for many individuals who are considering participation in clinical research. Indeed, the majority of children and up to 60% of adults have reported experiencing pain and/or fear related to needle procedures, which can be associated with increased risk of vasovagal syncope and seizures [12-17]. **Thus, non-invasive biomarkers of the O3I have great potential to overcome this barrier and broaden the scope of clinical research focused on omega-3s and cardiovascular health.**

2.4 Urinary Metabolites as Biomarkers of Omega-3 Intake. Urine has served as the primary diagnostic tool to assess an individual's health for centuries [50]. For example, bubbles on the surface of urine may indicate proteinuria, sweet smelling urine may indicate diabetes, and urine sediment may indicate kidney disease. Although conventional urinalysis quantifies specific metabolites or metabolite classes of clinical significance (e.g., organic acidurias), analytical developments now enable the simultaneous measurement of several hundred small molecules in an untargeted manner for biomarker discovery [51, 52]. The human urinary metabolome comprises a diverse array of metabolites of both endogenous and exogenous origin [53], including a large fraction of unknown compounds. Studying urine has several advantages over other biofluids, such as blood. For example, urine can be obtained non-invasively in large quantities, repeat sampling is more feasible, and sample pre-treatment prior to analysis can be less complex [53]. Over the past few years, urine metabolomic studies have garnered considerable interest to objectively assess an individual's dietary intake and nutrient status [18, 54, 55]. To date, the application of metabolomics has identified specific dietary biomarkers of food intake such as protein [56, 57], citrus fruits [58], dairy and soy [59], and tea [60], as well as complex eating patterns associated with healthy (Prudent) and unhealthy (Western) diets [61].

Yet, there are sparse studies demonstrating that urinary metabolites are associated with omega-3 intake [62, 63]. For example, urinary trimethylamine-*N*-oxide (TMAO) levels were found to distinguish high from low fish intake [62]. However, another study found that urinary TMAO differentiated individuals consuming a high from a low-protein diet, suggesting that this metabolite may not be specific to fish intake *per se* but rather total animal protein intake [57]. Another urinary metabolite, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), was reported to be higher in individuals after consuming a fish oil supplement [63]; however, urinary CMPF is also associated with gestational diabetes and kidney function. It is also notable that these metabolites have not been independently validated and their specificity as biomarkers of omega-3 intake remains equivocal. *The approach proposed in the current project is fundamentally distinct from these past studies in that we are focusing on urinary biomarkers related to changes in the O3I rather than dietary intakes, thus allowing us to account for any unmodifiable, modifiable and bioavailability factors known to influence EPA+DHA levels in RBCs.* As such, we posit that studying the relationship between the urinary metabolome and the O3I can provide an accurate, sensitive, and non-invasive assessment of an individual's omega-3 status

compared to existing methods; thus, paving the way to develop an alternate approach to monitor an individual's cardiovascular health.

2.5 Omega-3s and Cardiovascular Health: Addressing the Controversy

While there is broad consensus regarding the anti-inflammatory and triglyceride lowering properties of EPA+DHA, considerable debate about their beneficial role for both primary and secondary prevention of CVD exists. This controversy stems primarily from large RCTs that have failed to reproduce results from observational studies. Briefly, trials such as the *Japan EPA Lipid Intervention Study* (JELIS, [64]) and *The Reduction in Cardiovascular Events with Icosapent Ethyl-Intervention Trial* (REDUCE-IT, [65]) provided evidence that EPA can reduce CVD risk. In contrast, more recent trials used mixed EPA+DHA formulations, such as the *A Study of Cardiovascular Events in Diabetes* (ASCEND, [66]), the *Vitamin D and Omega-3 Trial* (VITAL, [67]) and the *Statin Residual Risk Reduction with Epanova in High Cardiovascular Risk Patients with Hypertriglyceridemia* (STRENGTH, [68]) trial found little evidence for an effect, although ASCEND reported a significant reduction in vascular death and VITAL reported lower rates of myocardial infarction (MI) in those receiving EPA+DHA. Despite these conflicting results, the inclusion of studies with null findings in a recent meta-analysis still demonstrated significant reductions in MI and CHD events with omega-3s [69]. These contradicting results could be explained by differences in doses, study populations, and the placebo control formulation [2, 70, 71]. First, dose-response effects have been consistently reported for omega-3s. The various RCTs used EPA+DHA dosages ranging between <1 g/d up to 4 g/d. Unsurprisingly, null effects tended to be observed in low dose supplementation studies. Second, the background dietary habits of study populations were not considered in these RCTs. A secondary analysis of the VITAL study showed that omega-3 supplementation had significant benefits on MI and CHD in participants who had low levels of fish consumption, while no benefits were observed in participants with high fish consumption [72]. Finally, the placebo control was inconsistent between studies, e.g., REDUCE-IT used a mineral oil and STRENGTH used a corn oil; thereby making direct comparisons difficult and potentially obscuring treatment outcomes. While future research considering these factors is imperative, it is notable that a substantial body of evidence supports the benefits of EPA and DHA on cardiovascular risk factors.

3 – PRELIMINARY DATA

Mutch (Co-PI) previously conducted a double-blind, placebo-controlled parallel-arm RCT to examine the independent effects of EPA and DHA supplementation on the O3I [42]. Briefly, young males and females (18-30 y) were randomized into one of three groups: 1) EPA, 2) DHA, and 3) olive oil (placebo control). Participants consumed ~3 g/d of these supplements for 3-mo, with fasting blood and morning first-void urine samples collected before and after supplementation. EPA supplements increased the O3I from ~3.5% at baseline to ~6.5%, while DHA supplements increased the O3I from ~3.5% at baseline to ~8.4%. Olive oil had no effect on the O3I, as expected. With Britz-McKibbin (Co-PI), we recently published an exploratory secondary analysis of this RCT in which we identified 17 candidate urinary metabolites, using a combination of variable selection by discriminant analysis and linear mixed model regression analyses, whose abundance in urine changed following an increase in the O3I [21]. The most significant urine metabolite was identified as S-carboxypropylcysteamine (CPCA). Creatinine-normalized (to account for hydration status) CPCA was consistently increased in participants following either EPA or DHA supplementation compared to the olive oil control, which was unchanged from baseline ($p>0.05$). Similar increases in CPCA following EPA or DHA supplementation were seen when males and females were analyzed separately, suggesting that CPCA is not a sex-specific urinary biomarker. Based on receiver operating characteristic (ROC) curves, we found urinary CPCA is capable of differentiating individuals consuming EPA and DHA from those who do not. Moreover, urinary CPCA was significantly correlated ($r=0.3$) with the O3I, and was also able to distinguish between individuals with a low O3I (<4%) and a high O3I (>8%). Other abundant RBC fatty acids such as palmitic acid, oleic

acid, linoleic acid and αLNA were *not* correlated with urinary CPCA. Although the biochemical link between increased EPA+DHA and increased urinary excretion of CPCA remains unclear, CPCA was reported to be a by-product of impaired valine catabolism mediated by enoyl-CoA hydratase (ECHS1) [73]. ECHS1 is also involved in mitochondrial β-oxidation of fatty acids [74], a process activated by omega-3s. The increase in urinary CPCA associated with increased EPA and DHA may therefore reflect a shift away from branched-chain amino acid catabolism towards fatty acid β-oxidation; thus, pointing to a new biochemical mechanism regulated by omega-3s. Altogether, these analyses reinforce the potential of urinary metabolites to serve as non-invasive biomarkers of the O3I and clearly support the rationale to further pursue this exciting line of investigation. While this discovery could be highly impactful to the cardiovascular field, these results stem from an exploratory secondary analysis of a previous RCT and thus need to be examined in an optimally designed and adequately powered study.

4 – THE NEED FOR A TRIAL Although numerous reports in the literature describe the identification of potential candidate biomarkers of omega-3 intake, none have been validated to support their clinical relevance in terms of reflecting changes in the O3I. Dragsted *et al.* [75] described a validation pipeline for dietary biomarkers of food intake that establishes both their biological validity and analytical performance to ensure their clinical utility (**Table 1**). While our future goal is to investigate urinary biomarkers in free-living populations, the requisite starting point is a human intervention trial that carefully controls for known confounding variables that allow for the study of the effects of specific nutrients in complex biological samples. The study we propose will enable us to evaluate urinary biomarkers to assess omega-3 status and CVD risk factors by addressing the criteria outlined by Dragsted *et al.* [75]. This rigorous approach to biomarker research will minimize the risk of pursuing "false leads".

TABLE 1: Criterion #	**How will this be assessed in the proposed research?**
1. Plausibility: A biomarker should be specific to the nutrient of interest.	Comparing urinary metabolites in omega-3 supplemented groups with the placebo control.
2. Dose-Response: A biomarker must be sensitive to changes in nutrient intake.	Evaluating changes in urinary metabolites in conjunction with different doses of EPA+DHA.
3. Time-Response: Repeated measures over time will uncover its day-to-day variability and establish optimal timing of measurement.	Examining changes in urinary metabolites in relation to the O3I at multiple time points during the intervention and follow up periods.
4. Robustness: A biomarker should not be affected by other foods and/or specific nutrients.	Analyzing changes in urinary metabolites in relation to dietary assessment data collected at multiple time points during the trial.
5. Reliability: A novel biomarker should reflect changes observed with a gold standard method.	Evaluating changes in urinary metabolites in relation to changes in the gold standard O3I (i.e., EPA+DHA content in RBCs).
6. Analytical Performance. The precision and accuracy of biomarker measurement must be determined using standards.	If commercially available, candidate urinary metabolites will be analyzed side-by-side to confirm precision and accuracy, as well as establish reference intervals.

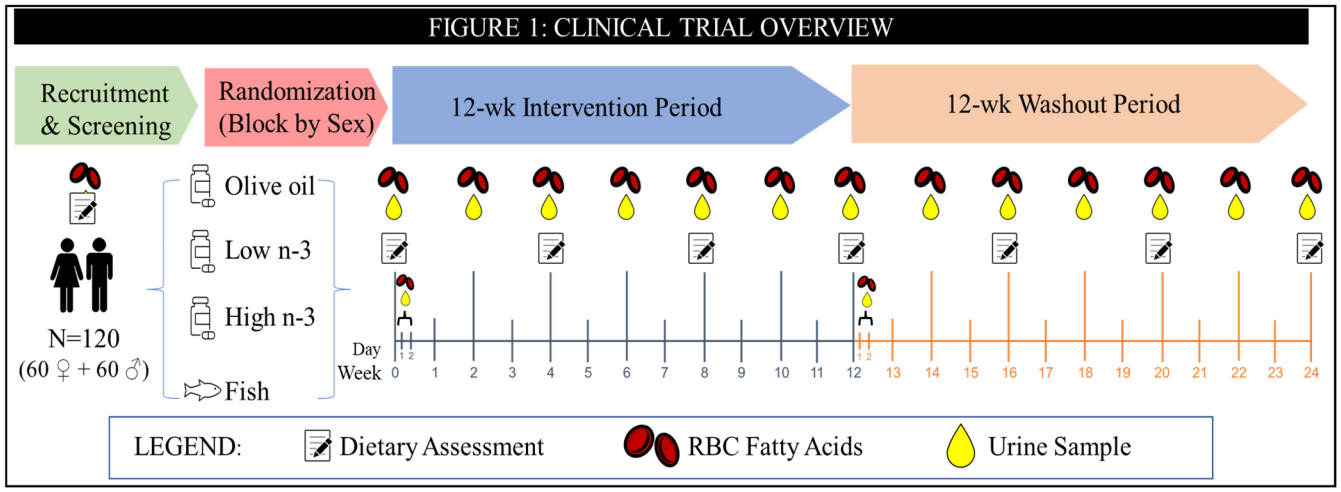
5 – METHODS AND EXPERIMENTAL APPROACH

Dose-response relationships between the O3I and triglycerides [76], inflammation [77], and blood pressure [78] have been previously reported. However, we are interested to determine whether urinary metabolites that are strongly correlated with the O3I are similarly able to capture changes in these CVD risk factors, thus providing a new and non-invasive method to assess an individual's cardiovascular health. The trial will be led by a newly recruited PDF and supported by MSc students at the University of Guelph together with a PDF involved in the urine metabolomic analyses at McMaster University. Our team has a longstanding commitment to Equity, Diversity, and Inclusion in our groups, as well as in the participants we recruit for clinical studies. All protocols will receive Research Ethics Board approval, and all participants will provide written informed consent.

Aim 1: To determine if a dose- and time-response relationship exists between changes in specific urinary metabolites and the O3I.
Hypothesis: *A subset of the previously identified urinary metabolites, including CPCA, will show dose- and time-response increases that are significantly correlated with increases in the O3I.*

Aim 2: To assess whether reductions in CVD risk factors (e.g., TG concentration, inflammatory markers, and resting BP) associated with an increased O3I are also observed with urinary biomarkers.
Hypothesis: *Changes in urinary biomarkers associated with increased omega-3 status will be significantly correlated with changes in TG concentration, inflammatory markers, and resting BP.*

5.1 Study Design: See **Figure 1** for an overview of the proposed trial and timing of sample collection. We will conduct a placebo-controlled, parallel-arm RCT in which male and pre-menopausal female participants (18-45 y) will be randomly allocated to an intervention group for 12 weeks followed by a subsequent 12-week washout period. The trial will be registered at ClinicalTrials.gov.



5.2 Participant Inclusion/Exclusion Criteria: We will recruit adults between the ages of 18-45y who will self-report their sex and gender, and have an O3I ≤ 4% at screening. Individuals will be excluded if they have an allergy to fish, are current smokers, chronically use medications known to influence CVD risk (e.g., statins, anti-hypertensives, *etc*), have a history of chronic disease, consume >1 serving of fish or shellfish (or other EPA/DHA fortified foods) per week, have used a fish oil supplement in the previous 3 months, and have needle phobia/reluctance to give blood. During screening, we will use an omega-3 diet survey to ensure that participants have not consumed fish oil supplements in the past 12 weeks and/or are not frequent consumers of foods rich in or fortified with omega-3 fatty acids. We will also inquire about the use of gender affirming hormone therapy due to links with increased CVD risk [79, 80]. Older adults will be excluded from this trial because it was reported that their O3I is generally >5% [5]. Indeed, past reports examining dietary habits and supplement usage have reported that older adults consume more fish and use omega-3 supplements more than younger adults due to increased awareness of their cardiovascular and cognitive benefits [81, 82]. This higher intake of fish and fish oil supplements would result in a higher baseline O3I. Moreover, older adults are often using medications to control blood lipids (statins) and blood pressure (antihypertensives), amongst others. We will avoid these confounders by recruiting only healthy adults between 18-45y with an O3I<4% and not using medications.

5.3 Study Endpoints: The overall objective of our proposed research is to determine if urinary biomarkers that reflect an individual's omega-3 status can provide a non-invasive method to monitor changes in CVD risk factors. To accomplish this, we will first confirm that the 17 candidate urinary

metabolites previously identified by the Co-PIs change in relation to the O3I in a dose- and time-responsive manner. We will then investigate if changes in the abundance of these urinary biomarkers are correlated with changes in various CVD risk markers (e.g., TG concentration, inflammatory markers and resting BP). Exploratory analyses will include comparisons between sexes to determine if changes in urinary metabolites reflect changes in CVD risk markers differently between males and females.

5.4 Sample Size: Since our primary objective is to determine if urinary metabolites that reflect the O3I can accurately capture changes in CVD risk markers, we performed a sample size calculation with our collaborator (Dr. Darlington) using results from our recently published exploratory analysis [21] showing that CPCA was positively correlated with the O3I with an estimate of r=0.30. A sample size of 113 participants is required for sufficient power to detect a correlation coefficient of 0.3 between CPCA and the O3I as significant (α, two tailed: 0.05; β=0.10) [83]. To account for an anticipated drop-out rate of up to 10%, we will recruit 120 participants (n=60 males; n=60 females). Based on the low variability in O3I in the general Canadian population (as reported in [5]) we divided this sample size evenly across the 4 intervention groups (placebo control, low dose, high dose, and fish) to ensure our cohort shows a wide range in O3I values after the 12-week intervention period. Further, we propose to recruit an equivalent number of males and females to enable an exploratory sex-based analysis. While we acknowledge that we may be underpowered for this exploratory analysis, these results will help inform future precision nutrition studies using urinary metabolomics. In total, 120 participants will be recruited: n=30 per intervention group (15 males, 15 females) × 4 intervention groups.

5.5. Overview of Study Protocol: As illustrated in **Figure 1**, participants will complete one screening visit (~30 min) and 17 testing visits (~15 min each). Consenting participants will be asked to complete a short omega-3 dietary fats and screening questionnaire to determine their habitual intake of fatty fish, omega-3 fortified foods and dietary supplements. The screening questionnaire will be completed in person with the PDF at our dedicated Human Nutraceutical Research Unit (HNRU). A fasted blood sample will be collected by venipuncture by trained phlebotomists for the isolation of RBCs and subsequent determination of the O3I. Individuals who consume little-to-no omega-3s in their diets and have an O3I of <4% will be invited to continue in the study. Participants will then be block randomized by sex to ensure an equivalent number of males and females in the following 4 intervention groups:

a) ***Placebo Control Group:*** Participants will consume 3 softgel capsules containing olive oil per day for the 12-wk intervention period. We have shown that olive oil does not change the O3I [42].
b) ***Low n3-LCPUFA Group:*** Participants will consume 2 softgel capsules containing olive oil and 1 softgel Omegor Vitality 1000 capsule per day containing 0.9g of EPA+DHA (2:1 ratio) in a TG form for the 12-wk intervention period.
c) ***High n3-LCPUFA Group:*** Participants will consume 3 softgel Omegor Vitality 1000 capsules per day providing 2.7g/d of EPA+DHA (2:1 ratio) in a TG form for the 12-wk intervention period.
d) ***Fish Group:*** Participants will consume 2 servings of Kirkland farmed Atlantic salmon (~6oz per serving, each providing ~1.8g EPA+DHA), 2 times per week for the 12-wk intervention period.

Participants in the placebo control, low, and high supplement groups will be blinded to their group through the consumption of an equivalent number of capsules per day. See Letter of Support from UGA Nutraceuticals for a product and safety report on Omegor Vitality 1000 capsules. Support for the low and high n3-LCPUFA doses comes from literature showing a dose-response relationship between fish oil intake and the O3I [30, 84], as well as fish oil intake and reductions in TG concentration [42, 85, 86] in both healthy participants and individuals with hypertriglyceridemia. The inclusion of a fish group will enable us to verify urinary metabolites that change in relation with the O3I versus fatty fish intake. Rationale for the number of fish servings comes from the Dietitians of Canada and American Dietetic Association [87], and is further supported by a report on the Canadian Health Measures Survey [5].

5.6 Study Visits: The intervention and washout periods will each last 12 weeks. The rationale for having a 12-week intervention period and a 12-week washout period comes from past studies. We and others have reported that EPA+DHA supplementation increases the O3I significantly within this time frame [44, 88, 89]. Furthermore, studies in which a washout period was included provide support that the O3I returns to baseline levels within 12 weeks after cessation of EPA+DHA supplementation [44, 88]. Our prospective study design will allow us to determine whether the 17 previously identified urinary metabolites change both *acutely* and *chronically* in response to a change in omega-3 intake.

- Daily Study Visits: In the two days immediately following the Baseline Study Visit at Week 0, participants will return to the HNRU for fasted blood and urine sample collection. Similarly, two daily study visits will also follow the start of the washout period (Week 12 of the trial) where fasted blood and morning first-void urine samples will be collected. The purpose of these back-to-back daily study visits at Weeks 0 and 12 of the trial is to assess whether urinary metabolites change *acutely* in response to a change in omega-3 intake in individuals whose circulating EPA and DHA levels are low and high.
- Weekly Study Visits: Participants will return to the HNRU for fasted blood and urine sample collection once every 14 days at Weeks 2, 4, 6, 8, 10, and 12 during the intervention period of the trial, as well as Weeks 14, 16, 18, 20, 22, and 24 of the washout period. The purpose of these bi-weekly study visits during the supplementation and wash-out periods of the trial is to assess whether urinary metabolites change *chronically* in response to a change in omega-3 intake.

Participants will be provided instructions for the collection, storage, and transport of morning first-void urine samples. Immediately upon arrival at the HNRU on a study visit day, participants will hand in their urine sample. Next, participants will have their resting blood pressure measured with our collaborator (Dr. Millar). Finally, fasted blood samples will be collected by venipuncture from supine participants in the morning after a minimum 12-h overnight fast. Matching urine and RBC samples will be stored at -80°C to ensure maximum stability of metabolite profiles, as previously reported [90].

5.7 Recruitment, Compliance, and Loss to Follow Up: Participants will be recruited by advertising on the U of Guelph campus (posters, emails), on the HNRU website, through local businesses and community centres (posters), on social media and in the local papers. Based on a previous trial conducted by Dr. Mutch, it took 6 months to recruit 90 participants into an EPA and DHA supplementation study [42]. This equates a recruitment rate of 15 participants per month. The current study requires 120 participants. Since the proposed trial is more intensive, we estimate it will take 12 months to complete the recruitment. Compliance will be assessed by several complementary methods: 1) capsule counting every 4 weeks, 2) routine dietary assessments, and 3) measuring EPA+DHA content in RBCs to ensure participants consumed supplements or fish. Participants will be instructed to maintain their regular exercise and dietary habits throughout the study, which also favours compliance. Based on our previous less intensive EPA+DHA supplementation study, the rate of loss to follow up was 2 out of 90 participants (~2% rate of loss). Since the proposed trial is more intensive, a 10% rate of loss was deemed prudent.

5.8 Study Methodologies and Analysis:

Dietary Assessment: Co-Investigator Dr. Duncan will ensure that participants complete 24-h dietary recalls at Screening, Baseline and Weeks 4, 8, 12, 16, 20, and 24 during the trial. The rationale for having participants complete dietary assessments is to ensure no significant changes in dietary patterns occur during the study (e.g., significant change in caloric intake, adopting a new dietary pattern, unintentional consumption of omega-3s). Participants will complete these assessments using the free, web-based Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool developed by the U.S. National Cancer Institute and adapted for Canada (ASA24-Canada-2018) to reflect the Canadian food supply, portion sizes and nutrient composition. The ASA24-Canada-2018 Dietary Assessment Tool guides participants through a 24-h dietary recall and includes prompts about their dietary supplement

use, thereby capturing any omega-3 and/or fish oil supplement intake. The dietary supplement database in the ASA24-Canada-2018 is based on Health Canada's Licensed Natural Health Product Database and supplemented with the NHANES Dietary Supplement Database [91]. Participants will be fully oriented to the ASA24-Canada-2018 and will complete it the day before their visit to the HNRU with instructions to ensure it is a typical day for them. The ASA24-Canada-2018 automatically codes data entered by participants enabling researchers to access nutrient intakes including omega-3s.

CVD Risk Factor Analysis: Serum will be isolated from fasted blood samples and shipped to LifeLabs Inc. for the analysis of a blood lipid panel consisting of TG, total cholesterol, HDL-C, and LDL-C, as well as high-sensitivity C-reactive protein (hs-CRP), and fasting glucose. *(see attached quote)*

Fatty Acid Analysis: In conjunction with our collaborator (Dr. Ma), RBCs will be isolated from fasted blood samples, and fatty acids analyzed by gas chromatography-flame ionization detector, as previously described [42-44, 92-95]. Briefly, lipids will be extracted from RBCs using chloroform:methanol (2:1 v:v) containing butylated hydroxytoluene (0.01%) as an antioxidant and heptadecanoic acid (17:0) as an internal standard [96] according to the Folch method. After lipid extraction and transmethylation with BF_3/methanol, the lipid phase containing fatty acid methyl esters will be analyzed using an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA) with a Supelco SP 2560 fused-silica capillary column (DB-FFAP; 15m×0.1mm) and flame-ionization detector. Individual fatty acids are detected based on the retention times of standards and will be reported as a % of total fatty acids (relative abundance). %EPA and %DHA will be summed to calculate the O3I. *(see attached quote)*

Urinary Metabolomic Analysis: Multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS) will be used for urinary metabolite analyses. MSI-CE-MS offers a high throughput platform for untargeted metabolomics with stringent quality control that is ideally suited for the analysis of ionic/polar metabolites in small volumes (<10 μL) of highly saline urine specimens without complicated sample workup procedures [61, 97, 98]. Urine metabolomic analyses will be performed using an Agilent G7100A CE system equipped with a coaxial sheath liquid Jetstream electrospray ion source with heated nitrogen gas to an Agilent 6550 iFunnel Q-TOF-MS system. Diluted urine samples will be analyzed in duplicate by MSI-CE-MS under two conditions based on a background electrolyte (BGE) comprised of 1.0 M formic acid with 15 % *vol* acetonitrile (pH = 1.80) and 50 mM ammonium bicarbonate (pH = 8.50) when using positive (polar basic metabolites) and negative (polar acidic metabolites) ion mode detection [99]. A dilution trend filter in MSI-CE-MS will be used for molecular feature selection and metabolite authentication in a pooled urine sample that also serves as a quality control (QC) while rejecting spurious ions, degenerate signals, and background compounds. Mean responses for all urine metabolites will be normalized to internal standards and annotated based on their accurate mass (*m/z*), relative migration time (RMT) and detection mode (+/-). Molecular feature detection and metabolite identification will be done with Mass Hunter Molecular Feature Extractor, Molecular Formula Generator tools and an in-house compound database [97].

Statistical Analysis: The distribution of urinary metabolites, as well as RBC fatty acids, will be tested for normality. Mixed effect regression models accounting for age, BMI, sex, and gender will be performed to assess the relationships between urinary metabolites and the O3I, as well as urinary metabolites and CVD risk factors. Multivariate data analysis of urinary metabolites will be performed using Metaboanalyst 5.0, including principal component analysis and partial least-squares-discriminant analysis. Normalized metabolomic data sets will be log transformed and autoscaled for analysis. Sensitivity and specificity of individual urinary metabolites with the O3I will be assessed with ROC curves. Exploratory analyses of sex will be conducted using mixed effect models. Bonferroni *post hoc* procedures will be used to account for multiple testing. Machine learning algorithms will be used to test

the sensitivity and specificity of models using single or multiple panels of creatinine-normalized urinary metabolites. All statistical analyses will be overseen by our collaborators (Drs. Darlington and Feng).

5.9 Timeline: Obtaining research ethics approval and recruiting participants will occur in Year 1. The RCT will take place during Years 1 and 2, with urinary metabolite and RBC fatty acid analyses occurring in Years 3 and 4. Years 3 and 4 will also be used for knowledge dissemination.

5.10 Anticipated Pitfalls and Challenges: It is possible that the urinary metabolites, including CPCA, identified in our previous study are not found to associate with changes in the O3I in the present study. Our previous study supplemented participants with either EPA or DHA, while the proposed study will use a combined EPA+DHA supplement. Although the total amount of supplemented n3-LCPUFA is equivalent between these studies (~3 g/d), the formulations are different. However, we don't anticipate this to be an issue since CPCA, for example, was significantly correlated with the O3I in both EPA and DHA supplemented individuals in our previous study. Nevertheless, reproducibility of specific urinary metabolites identified in our past study remains the greatest challenge. We consider the proposed research to be considerably more robust due to the dose-response aspect of the intervention study. As with many clinical trials, drop-outs can pose a challenge. However, the proposed trial will be our seventh omega-3 intervention study and we have an excellent track record of participant compliance and completion rates.

5.11 Expertise of the Team: Our team has expertise conducting human dietary interventions and metabolomics investigations. The Co-PIs are investigators whose programs are supported by a variety of funding agencies. Co-PI Mutch (*h*-index 44) is an expert in fatty acid metabolism and human clinical nutrition research, while Co-PI Britz-McKibbin (*h*-index 44) has extensive expertise in bio-analytical chemistry, metabolomics and bioinformatics. The Co-PIs have an established track record of collaboration. Our team comprises collaborators with expertise in dietary assessment (Duncan), cardiovascular physiology (Millar), nutritional biochemistry (Ma), and statistics (Darlington, Feng).

6 – IMPACT AND ANTICIPATED FINDINGS

Specific urinary metabolites will accurately reflect a person's omega-3 status and be strongly correlated with common CVD risk markers. We anticipate these associations will be independent of biological sex and gender, thus rendering these biomarkers generalizable to the population. We also believe that urinary CPCA, as well as some (if not all) of the other candidate urinary metabolites, will be replicated as biomarkers of the O3I in the proposed dose- and time- response trial and found to associate with CVD risk markers. Establishing an accurate and non-invasive method to assess omega-3 status would present a major breakthrough in precision nutrition. Our ***long-term vision*** is to develop an accessible, easy-to-use, and affordable over-the-counter dried urine spot test for a specific metabolite (i.e., colorimetric assay) that will empower individuals to self-monitor their CVD risk and tailor their omega-3 intake accordingly (either independently or with assistance from a healthcare provider). Achieving this long-term vision is only possible through the foundational knowledge that will be generated through the current proposal. We anticipate that the outcomes of this research will strengthen the evidence regarding the use of omega-3s for the primary prevention of CVD, broaden the scope of future clinical research, and inform precision nutrition strategies aimed at preventing and mitigating CVD risk in Canadians.

7 – KNOWLEDGE TRANSLATION

Our proposed research aligns with the HSFC mandate to promote healthy living through prevention and healthy lifestyles. Our KT plans will therefore emphasize how omega3s can be used to support cardiovascular health. Outcomes will be disseminated in public lectures, podcasts, scientific journals, national/international conferences and press releases generated with assistance from institutional Public Relations offices in conjunction with the HSFC. Once completed, we will apply for a CIHR Planning and Dissemination grant to bring together experts in academia, industry, and healthcare to expand the investigation of urinary biomarkers in free-living populations, both national and international.

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