The effects of monounsaturated and n-3 polyunsaturated fatty acids on resting blood pressure and muscle sympathetic nerve activity

by

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THE EFFECTS OF MONOUNSATURATED AND N-3 POLYUNSATURATED FATTY ACIDS ON RESTING BLOOD PRESSURE AND MUSCLE SYMPATHETIC NERVE ACTIVITY

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This thesis investigated whether the n-3 polyunsaturated fatty acids, EPA and DHA, as well as olive oil (OO), a source of monounsaturated fatty acids, exert differential effects on resting hemodynamics and muscle sympathetic outflow. Ninety young healthy men and women were randomized to 3g/day of either EPA (n=30), DHA (n=30), or OO (n=30) for 12 weeks. Continuous measurements of resting blood pressure and heart rate, and muscle sympathetic nerve activity (MSNA) in a subset of the participants, were recorded before and after supplementation. DHA and OO reduced systolic and diastolic blood pressure relative to EPA, whereas EPA increased heart rate. DHA increased MSNA compared to EPA and OO, whereas OO did not change MSNA relative to EPA. Although DHA and OO elicit hypotensive effects in healthy young individuals, DHA likely acts through peripheral vascular mechanisms resulting in baroreflex mediated compensatory sympathoexcitation, while OO could act through a central resetting mechanism.
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BP: blood pressure
DBP: diastolic blood pressure
DHA: docosahexaenoic acid
EPA: eicosapentaenoic acid
MSNA: muscle sympathetic nerve activity
MUFA: monounsaturated fatty acid
n-3 PUFA: omega-3 polyunsaturated fatty acid
NE: norepinephrine
SBP: systolic blood pressure
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Chapter 1. Literature Review

1.1 General Introduction

Compared to Western societies, Japan has significantly lower rates of cardiovascular disease and mortality (2). Epidemiological studies on Japanese individuals living in coastal regions of Japan as compared to those having immigrated to more mainland regions have implicated dietary factors, specifically high fish intake, in mediating this reduced risk of cardiovascular mortality (3,4). Similar associations between fish intake and reduced incidence of cardiovascular death have been observed in other populations regularly consuming fish, including the Greenland Inuit and Swedish people (5–7). The beneficial effects of high fish consumption on cardiovascular mortality have been ascribed to the long chain omega-3 polyunsaturated fatty acids (n-3 PUFA’s) found abundantly in fatty fish (8). The n-3 PUFA’s act on multiple physiological pathways (9,10), promoting increased nitric oxide (NO) bioavailability (11) and vascular smooth muscle relaxation (12); which reduce resting blood pressure (BP).

Similar to the effects of a high fish diet, the Mediterranean diet has also been shown to reduce risk of cardiovascular disease (13). The Mediterranean diet consists primarily of plants such as fruits and vegetables, as well as a high intake of nuts, olive oil, and whole grains; meat intake, especially red meat, is consumed in smaller quantities compared to a Western diet (13). It is noteworthy that although the Mediterranean diet promotes a moderate intake of fish, the beneficial effects of these dietary patterns are not generally attributed solely to n-3 PUFA or fish intake (13,14). In particular, olive oil and nuts contain abundant amounts of monounsaturated fatty acids (MUFA), specifically oleic acid, as well as polyphenols (14). These compounds have been shown to have beneficial effects on BP, inflammation, and thrombogenesis (14,15).
1.2 Effects of diet on clinical cardiovascular outcomes and blood pressure

1.2.1 n-3 PUFA rich diet

Several meta-analyses have shown that higher fish intake is associated with reduced risk of CHD mortality (16–19). Interestingly, fish consumption is not associated with a reduced risk of non-fatal CHD, except in cases of high fish intake (≥5 servings/week) (17,18,20). In contrast, large prospective randomized controlled trials of fish oil supplementation and cardiovascular endpoints in patients with existing cardiovascular conditions have produced inconsistent results. Three early trials in cardiovascular patients reported a reduction in the risk of cardiovascular fatalities and acute events (21–23), while more recent trials have shown no effect of fish oil supplementation on cardiovascular events and fatalities (24–28). However, it should be emphasized that these trials all demonstrated significant heterogeneity in terms of the background medications used by patients. In particular, there was a higher prevalence of statin and β-blocker therapy in later studies (~50% of participants receiving statins vs ~10-20% in earlier studies) (24–28). There was also variability regarding the dosage and formulation of the fish oil supplements utilized.

The effects of a high fish diet on BP are not entirely consistent. A 2016 meta-analysis of prospective cohort studies showed that circulating levels of n-3 PUFA’s, but not fish intake or n-3 PUFA consumption, was associated with a reduced risk of developing elevated BP (defined as SBP ≥130 mmHg and/or DBP ≥ 85 mmHg) or hypertension (SBP ≥ 140 mmHg and/or DBP ≥ 90) (29). Cross-sectional data show an inverse relationship between fish intake and resting BP in older adults (30,31). In hypertensives, circulating levels of docosahexaenoic acid (DHA) but not eicosapentaenoic acid (EPA) are inversely associated with ambulatory DBP and heart rate (32), whereas erythrocyte levels of EPA but not DHA have been reported to display a small inverse
correlation with ambulatory SBP (33). The reason(s) for this discrepancy between biomarkers and intake are unclear, but may reflect differences in the type of fish consumed, the relative amounts and type of n-3 PUFA ingested, and interconversion between short and longer chain PUFA’s.

1.2.2 Mediterranean diet

Data from the Seven Countries Study in 1986 highlighted a reduced risk of death from coronary heart disease in countries with high rates of olive oil consumption (34). Further, a large meta-analysis of the impact of the Mediterranean diet on cardiovascular outcomes showed an inverse association between adherence to the Mediterranean diet and incidence of coronary heart disease and myocardial infarction (35). The effects of the Mediterranean diet on BP have been suggested to be a significant factor in improving cardiovascular risk profiles (36). Cross-sectional data demonstrate lower resting BP in individuals adhering to the Mediterranean diet or those with a high intake of MUFA (37–39). A meta-analysis of studies lasting a year or longer in patients with cardiometabolic disease or known cardiometabolic risk factors found that chronic adherence to the Mediterranean diet elicited reductions in both systolic blood pressure (SBP) and diastolic blood pressure (DBP) (40). Others have shown that chronic implementation of single components of the Mediterranean diet, such as high MUFA or polyphenol intake, are also associated with lowered BP (41,42). Extending upon these data, individuals with greater adherence to the Mediterranean diet have lower incidence of hypertension (39,43), although this has not always been a consistent finding (44).
1.3 Cardiovascular physiology

1.3.1 Regulation of blood pressure

BP regulation involves a complex interplay between neural, humoral, and vascular interactions over both short (seconds to minutes) and long (days to weeks) time scales (Figure 1) (1,45–47). In general, beat-to-beat regulation of BP is maintained through a negative feedback loop involving the arterial baroreceptors. These mechanically (stretch) sensitive fibers are located in the carotid sinus and aortic arch, and relay afferent feedback to cardiovascular control centres located in the brainstem, chiefly the nucleus tractus solitarius (NTS) (45). An increase in pressure increases excitatory firing from the baroreceptors to the NTS (46). This firing either directly or indirectly inhibits the rostral ventrolateral medulla (RVLM) (46,48), the key generator of tonic sympathetic outflow directed towards the heart, vascular smooth muscle, and kidneys (46,49). Loading of the arterial baroreceptors also increases parasympathetic outflow to the heart (45). Collectively, the changes in sympathetic and parasympathetic activity lead to reductions in either cardiac output (mediated through heart rate and stroke volume) and/or peripheral vascular resistance to return BP to its homeostatic set point. Conversely, a reduction in BP unloads the baroreceptors and results in increased cardiac output and/or peripheral vascular resistance (50,51). Importantly, arterial baroreflex control of sympathetic and parasympathetic activity is thought to be regulated separately (52,53). For example, healthy aging is associated with a decline in cardiac baroreflex sensitivity, a marker of reflex cardiac vagal modulation, without a change in sympathetic baroreflex sensitivity (54). Additionally, the sensitivity of the baroreflex appears to be greater during unloading of the baroreceptors as compared to loading (55,56).

In addition to the arterial baroreceptors, cardiopulmonary mechanoreceptors located in the heart and pulmonary vessels can also contribute to hemodynamic regulation during exercise.
or postural changes (57). In general, the cardiopulmonary baroreflex acts similarly as a negative feedback reflex; sympathoinhibitory during loading (58) and sympathoexcitatory during unloading conditions (59,60).

Over longer time scales (hours to weeks), BP is thought to be regulated primarily by neurohumoral and renal mechanisms (47). Within minutes of a drop in blood pressure, sympathetic outflow to the kidneys activates the renin angiotensin-aldosterone system (RAAS), causing the release of renin from juxtaglomerular kidney cells; ultimately converted to the potent vasoconstrictor, angiotensin II (47,61). Angiotensin II also causes aldosterone synthesis via aldosterone synthase from the adrenal glomerulosa (62). The release of aldosterone similarly acts to increase BP by increasing sodium retention and fluid volume (47). Over longer time scales, adjustment of body fluid by the kidneys is the primary mechanism of pressure regulation. Specifically, in response to low pressure the kidney will retain greater amounts of salt and water than is being excreted in order to increase blood volume, whereas during high pressure the kidney will excrete more salt and water to reduce blood volume (47). The purpose of this mechanism is to balance fluid intake and output so that mean arterial pressure returns to an equilibrium point.

Although the involvement of renal mechanisms in long-term blood pressure regulation is undisputed, a chronic role of the arterial baroreflex remains controversial (47,63,64). Given the important role of the arterial baroreceptors in beat-to-beat blood pressure control, it was initially hypothesized in the 20th century that the arterial baroreflex could also play a role in regulating long-term pressure (65). However, baroreceptor denervation in dogs showed that despite a transient increase in pressure, mean arterial pressure returns to baseline levels over time, suggesting that other mechanisms (i.e. renal) were predominantly responsible for long-term
control (47,66). Additionally, although reductions in BP occur following surgical removal of sympathetic splanchnic nerves in hypertensive patients, BP eventually returns to high levels within a few months (65,67). From this data, it was concluded that in baroreceptor intact animals and humans, the arterial baroreflex likely resets to a higher set point in instances such as chronic hypertension, and so the consensus at the time was that the arterial baroreflex was not involved in long-term BP regulation. However, further investigations with baroreceptor denervated animals have revealed limitations of the model, and the validity of the renal-centric stance has since been questioned due to these findings (68,69). More specifically, in the baroreceptor denervation model, central processing adaptations occur in response to a lack of baroreceptor feedback, leading to eventual normalization of sympathetic outflow and blood pressure (68,69).

With the development of implantable devices allowing chronic monitoring of sympathetic outflow to various organs in vivo, it has been shown that the arterial baroreflex plays an important role in regulating renal sympathetic outflow, specifically in inhibiting outflow during angiotensin induced hypertension (70). Additionally, it is now thought that the arterial baroreflex does not completely reset during longer term changes in BP, as chronic baroreflex stimulation lowers BP and sympathetic outflow (71). Overall, although fluid balance strategies from the kidneys play a significant role in long-term BP control, the arterial baroreflex has remerged as a likely candidate participating in long-term regulation, although the precise role remains to be fully explored.
1.3.2 The role of the sympathetic nervous system in cardiovascular regulation

Despite being described as the “fight or flight” system, the sympathetic nervous system (SNS) plays a pivotal role in resting homeostatic regulation of the cardiovascular system (63). This is highlighted by the fact that under resting conditions the body maintains a state of tonic sympathetic outflow. At the heart, sympathetic activation induces both positive chronotropic and inotropic effects on heart rate and myocardial contractility, respectively, whereas parasympathetic activation causes primarily negative chronotropy (45,49). Sympathetic outflow to the peripheral vasculature primarily causes the release of norepinephrine (NE), which can bind to adrenergic receptors on vascular smooth muscle (72,73). ATP and neuropeptide Y can also be released from postganglionic sympathetic nerve terminals as cotransmitters (74,75). Here, NE
can bind to α-adrenergic or β2-adrenergic receptors on vascular smooth muscle, with diverging functional consequences on vascular tone (72). Following binding to α-adrenergic receptors, NE increases smooth muscle intracellular calcium levels, which generally causes vasoconstriction and increases in total peripheral resistance (72,76,77). In contrast, NE binding to β2-adrenergic receptors causes vasodilation through production of NO through the L-arginine-eNOS pathway (78,79). Circulating NE from spillover at other organ beds, such as the kidneys or heart (80,81), or released by the adrenal medulla can also bind with adrenergic receptors and modify vascular tone (72,82). In humans, the general effect of sympathetic outflow to muscle vasculature is to induce vasoconstriction (76,83).

Although sympathetic outflow has been quantified by discrete measurements of plasma NE and NE spillover (81,84), microneurography is considered the current gold standard for direct measurement of continuous central sympathetic outflow. Developed in Sweden in the 1960’s by Hagbarth and Vallbo (85,86), sympathetic outflow to muscle or cutaneous vasculature can be measured by percutaneously inserting an insulated tungsten microelectrode into peripheral motor nerves, typically the median, radial, or ulnar nerve for upper limb measurements or the tibial or fibular nerve for lower limb assessment (87). Researchers interested in cardiovascular regulation primarily examine muscle sympathetic nerve activity (MSNA), which occurs in pulse synchronous bursts related to its control by the arterial baroreflex (85). The obtained neural signal can be rectified and integrated to provide a multi-unit signal composed of many individual action potentials that produce so called “burst” patterns of sympathetic activity (63). These bursts can be quantified on a per minute basis as burst frequency, or as bursts per 100 heartbeats, termed burst incidence, to account for differences in resting heart rate (88,89). Importantly, MSNA bursts are linked to the cardiac cycle, and while
they generally do not occur for every heartbeat, the maximum number of bursts cannot exceed the prevailing heart rate (87). MSNA, although highly variable between individuals (90), is highly reproducible in an individual within and between days, much more so than circulating NE (91–93). This makes it an ideal research technique.

It also important to note that MSNA exhibits considerable inter-individual variability and can be impacted by sex, age, and differences in body weight. For example, in young men, but not women, MSNA is correlated with total peripheral resistance and inversely related to cardiac output (94). The absence of relationships in women are thought to be due to a greater contribution of parallel beta-adrenergic vasodilation (72,94). Females have increased β2-adrenergic receptor density and post-receptor adenylate cyclase activity, which could account for the increased beta-adrenergic vasodilation (95–97). With healthy aging, both men and women demonstrate resting BP dependence with MSNA (72,98,99). In addition, body mass and adipose distribution also appear to modify sympathetic control, with overweight and abdominally obese individuals displaying higher levels of MSNA compared to lean and subcutaneous obese individuals, possibly due to inflammatory mechanisms or leptin induced sympathoexcitation (100–102). Although elevations in MSNA have been linked with adverse prognosis and clinical events in diseased populations (103), the impact of such variability in healthy individuals remains unknown.

1.3.3 Role of NO in regulating sympathetic outflow and blood pressure

NO is an important pleiotropic signalling molecule acting in both the peripheral and central nervous system. NO can be generated by two known pathways. Conventionally, nitric oxide synthase (NOS) can convert the amino acid L-arginine into NO in tissues such as the
vascular endothelium (104). However, NO can also be generated by an alternative oxygen-independent pathway in which nitrate and nitrite can be converted to NO. This system is most notable following dietary inorganic nitrate supplementation (e.g. beetroot juice). Here, nitrate is reduced to nitrite in the mouth as well as the gastrointestinal tract by commensal bacteria (105,106), and then further reduced to NO by nitrite reductases (106). NO has important contributions to BP regulation as it potently induces vascular smooth muscle relaxation and subsequent vasodilation (104).

NO is also thought to have a role in regulating central sympathetic outflow (107,108). NOS inhibition results in a significant increase in resting BP (109,110), but early studies showed conflicting results as to whether MSNA remained unchanged (109) or decreased (111,112) in response to this increased pressure. As MSNA is controlled by the arterial baroreflex (50), these studies may have been confounded by the concurrent changes in pressure. Subsequent research showed that skin sympathetic activity, which is not controlled by the arterial baroreflex (113,114), is increased during NOS inhibition despite increased BP, and is unchanged during matched hypertensive conditions induced by phenylephrine (non-NO dependent control) (115). This provided solid evidence that NO does indeed play a role in tonic inhibition of sympathetic outflow.

1.4 Physiological effects of fatty acids on the cardiovascular system

1.4.1 Fatty acids in a high fish diet

Fatty fish, such as salmon, as well as various marine oils like fish oil and krill oil, are rich sources of EPA and DHA (116). Accumulation of n-3’s in fish occurs due to bioamplification of these fatty acids across the food chain, which originate from algal food sources in aquatic
ecosystems (117). The precursor fatty acid to EPA and DHA is alpha linolenic acid (ALA), which is obtained primarily from plant food sources. Humans do not synthesize ALA endogenously and so it is considered an essential fatty acid (118). A series of elongase and fatty acid desaturase enzymes are responsible for lengthening ALA and adding cis double bonds to the chain, thereby converting ALA to EPA (20:5(n-3)) and the longer chain DHA (22:6(n-3)) (119). In humans, conversion of ALA to longer chain PUFA’s, especially to DHA, is limited, with approximately 5-10% of dietary ALA converted to EPA, and approximately 1% is converted to DHA (120–122). Thus, the most effective way to increase circulating and erythrocyte levels is through direct consumption (116). Importantly, DHA can be retro-converted to EPA following consumption of fish oil (116). The current recommendation for n-3 PUFA consumption from the American Heart Association is at least 1-2 servings of fatty fish per week or supplementing with approximately 1 g/day of EPA+DHA (123).

1.4.1.1 n-3 PUFA supplementation on blood pressure

The most recent meta-analysis on the effects of n-3’s on BP included 70 randomized controlled trials, and showed a significant overall reduction in both SBP (-1.5 mmHg) and DBP (-1 mmHg) (124). The average dose of n-3’s across all studies was 3.8 g of EPA+DHA/day, with an average study length of approximately 10 weeks. The effect size of overall BP reductions was larger in hypertensive subjects, with an average reduction in SBP and DBP of 4 mmHg and 3 mmHg, respectively. However, the reductions in SBP were not consistently related to dosage (range 1-5g/day), with reductions in SBP only occurring with 1-2 g/day and 3-4 g/day dosages; reductions in DBP were only observed with doses >2g/day. Although differences in dosage could play a role in determining overall hypotensive effects of n-3’s, the inter-individual
variability in the tissue and erythrocyte uptake and incorporation of n-3’s might also play a significant role in determining BP responses. In hypertensive patients, consumption of 3 fish meals per week for 6 months decreased BP only in those with concurrent increases in erythrocyte n-3 PUFA levels (125).

Aside from inter-individual differences in n-3 conversion and utilization, the biochemical form of the PUFA may also play a role in determining the effects on BP. A number of studies have examined krill or algal oil’s effects on BP in humans, but have not shown a consistent benefit (126–129). Interestingly, krill oil increases circulating levels of EPA and DHA to the same degree as fish oil at equal dosages, but the change in BP with krill oil was not different from the control group, whereas fish oil did elicit a significant reduction (126). This disagreement may be due in part due to different bioavailability of n-3 PUFA’s from krill oil vs fish oil. Specifically, n-3’s in krill oil are in phospholipid form, whereas n-3’s from fish and algal oil are in triglyceride form (130).

There are multiple proposed mechanisms by which fish oil and n-3 PUFA’s could elicit antihypertensive effects. Animal studies implicate a peripheral vascular mechanism as n-3’s reduce smooth muscle constriction during NE stimulation, and increase vessel relaxation in response to acetylcholine, following fish oil feeding (131–134). Observations of altered vascular reactivity in response to pharmacological stimulation have also been shown in human studies with fish oil supplementation (135,136). In addition, research has investigated the capacity for fish oil to alter flow mediated dilation (FMD), a measure of primarily NO-mediated endothelium-dependent vasodilation (137). Among young-to-middle aged individuals, four studies have shown improved FMD (i.e. greater increases in dilation to reactive hyperemia) with acute and short-term supplementation (3-12 weeks) in healthy and diseased populations (138–
141), whereas in older individuals a similar number of trials have shown null results (142–144). Two published meta-analyses support increases in FMD with fish oil (145,146). However, secondary analyses showed that improvements in FMD were not significant when including only high quality trials (i.e. double blindered, randomized, placebo controlled) (145). In addition to changes in endothelial function, circulating n-3’s are correlated with arterial compliance (147,148), and supplementation with fish oil has been shown to decrease arterial stiffness (149–155), although not consistently (144,156,157).

In contrast to these direct vascular mechanisms, there have been fewer studies examining the interaction of fish oil and n-3’s on the autonomic nervous system. Cardiac baroreflex sensitivity, the gain of the reflex change in heart rate in response to a given change in SBP (158), is improved following fish oil supplementation in post-myocardial infarction patients (159). In healthy individuals, 6g of n-3’s per day for 6 weeks improved cardiac baroreflex sensitivity in normotensive men and women (160), whereas two other studies in healthy participants showed no changes in sensitivity (161,162). It is currently unknown whether the improvements in cardiac baroreflex sensitivity with n-3’s were due to an attenuation in vascular stiffness leading to improved signal transduction at the baroreceptors (152) or an alteration in central processing. Frequent fish consumers have both lower vascular stiffness and higher baroreflex sensitivity when compared to people with infrequent fish intake (163). However, there are no studies which have examined a causal relationship between arterial baroreflex sensitivity, n-3’s, and potential improvements in vascular stiffness.

In addition to arterial baroreflex control of heart rate, human and animal work show that n-3’s can reduce circulating levels of NE, suggesting that n-3’s may reduce sympathetic outflow (164–166). As circulating NE reflects both the rate of release of NE from sympathetic nerve
terminals as well as the rate of reuptake by the NE transporter (81), these data provide only indirect evidence of altered sympathetic outflow with n-3’s. Direct intraneural recordings do not support a reduction in central sympathetic outflow, as 4-8 weeks of fish oil supplementation does not alter MSNA in normotensive or prehypertensive individuals (167,168). However, it should be noted that both of these prior studies also failed to show a reduction in resting BP (167,168). Thus, while peripheral mechanisms are likely to play a role in reducing blood pressure, the current evidence for a reduction in sympathetic outflow remains controversial.

1.4.1.2 n-3 PUFA supplementation on heart rate and heart rate variability

The effects of n-3’s supplementation on heart rate was recently summarized in a meta-analysis of 51 trials (169). Across all studies, there as a small but statistically significant reduction of heart rate (~2 beats/min). Unlike the effects of n-3’s on BP, when the studies were separated based on the health status of the participants, the chronotropic effects of n-3’s were similar between healthy subjects and clinical patients (169). The proposed mechanisms responsible for these benefits include altered automaticity of the sino-atrial node (10), compensatory heart rate reductions due to increased stroke volume (170), and alterations in sympathetic and vagal tone to the heart (10).

Several studies have examined the effects of n-3 PUFA supplementation on heart rate variability (HRV), a non-invasive indirect measure of cardiac sympathetic and parasympathetic modulation (171). HRV can be quantified using time domain measurements, such as the standard deviation of normal RR intervals (SDNN), which is considered a marker of both sympathetic and vagal tone (172). As well, spectral analysis of the RR intervals yields the distribution in high and low frequency bands (171). High frequency power is considered an index of parasympathetic
tone under the control of respiratory modulation, whereas low frequency power is considered to be a combination of parasympathetic and sympathetic influences (173). n-3 intake is positively correlated with time domain measurements of heart rate variability, specifically the SDNN (174). Supplementation with fish oil in various cardiovascular patient populations increases high frequency power (175), and can decrease the low to high frequency ratio (176,177), although not all studies have shown this (178). It also appears that these benefits extend primarily to clinical populations, as n-3 supplements have had inconsistent benefits on HRV in healthy populations (161,179–181).

1.4.2 Fatty acids present in the Mediterranean diet

The primary source of lipids in the Mediterranean diet come from olive oil and nuts (182). Nuts and olive oil both contain large amounts of the MUFA, oleic acid, and moderate amounts of the n-6 PUFA, linoleic acid (183,184). Specific types of nuts in the Mediterranean diet, such as walnuts, also contain small amounts of the n-3 PUFA ALA (183). Oleic acid, which is an 18 carbon chain with a single double bond at the ninth carbon (185), has been shown in rats to be the principal compound in olive oil responsible for eliciting reductions in BP (184,186).

In addition to oleic acid, olive oil is also a direct source of nitrated unsaturated fatty acids, including nitro oleic acid and nitro linoleic acid, which are found in both the oil and the olive peel (187,188). Additionally, the unsaturated fatty acids in olive oil are precursors to nitro fatty acids, due to their endogenous nitration in acidic environments such as the stomach (188). Nitro fatty acids have been shown in vitro to limit signalling of inflammatory transcriptional factors such as NF-κB (189,190), and in animal models have been shown to lower BP and protect against the development of hypertension (191,192). Aside from direct signalling effects,
nitro fatty acids can also act as donors of NO, which independently has important antioxidant and cardioprotective effects (187,193).

It is important to consider that manufactured olive oils possess a highly heterogenous fatty acid composition, with oleic acid content varying by as much as thirty percent between different oils (between 50-80 percent of total fatty acids) (194). The phenolic compounds and antioxidants can also vary in quantity and types of compounds present in the oil (195,196). The type of cultivar, processing method, and the ripeness of the olive all have an impact on the fatty acid and polyphenol compositions in different olive oils (194,195,197), and unfortunately this variation and composition is scarcely reported in the literature. Interestingly, olive oil is often supplied as a placebo control in n-3 PUFA supplement studies (167,168,198,199). However, a significant body of evidence also highlights the potential cardiovascular effects of olive oil (see below).

1.4.2.1 MUFA supplementation on blood pressure

Supplementation with olive oil or nuts in normotensive and hypertensive populations have been shown to lower SBP (200–202), DBP (203), or both measures of BP across multiple studies (182,204–206). Overall, olive oil appears to reduce BP by 3-5 mmHg in normotensive subjects (182,201–203,206), with greater reductions of approximately 7-10 mmHg observed in hypertensive subjects (200,205,207). Other studies have shown either no effect of high MUFA intake on BP when compared to fatty acid sources such as fish oil, or no difference in the reduction in BP following dietary intervention of either fish oil or olive oil (167,168,208–211).

The potential mechanisms and pathways involved in mediating these reductions in BP have yet to be fully elucidated. Virgin olive oil treatment in rats resulted in a downregulation of
G proteins involved in inducing vasoconstriction in vascular smooth muscle. (184) In hypertensive women, olive oil or nut supplementation with a Mediterranean diet decreased endothelin-1 gene expression and increased circulating nitrate levels, coinciding with decreases in BP (212). Polyphenol rich olive oil increased circulating nitrate and nitrite levels along with concomitant reductions in BP (205). Acute olive oil consumption has been shown to have either no effect or impaired postprandial FMD in healthy individuals (213–215), whereas 4 weeks of Mediterranean diet with olive oil consumption improves postprandial FMD (216). Among studies of longer term olive oil supplementation or Mediterranean diet adherence in both healthy and clinical populations, many studies have found no effect on resting FMD (142,217–219), while others have shown improved FMD compared to n-3 PUFA’s or a low fat/control diet (202,220–222).

As mentioned previously, olive oil is a source of nitrated fatty acids which can act as NO donors (187). Oleuropein, a phenolic compound in olive oil, has also been shown to increase nitric oxide \textit{in vitro} via reduction of nitrite (223). As nitric oxide plays a role in inhibiting sympathetic outflow (115), it is possible that olive oil could elicit BP reducing effects through a central sympathoinhibitory mechanism. However, the only investigations into the potential involvement of the sympathetic nervous system have shown that olive oil does not reduce MSNA or circulating NE, although these studies did not show any changes in BP or report the specific biochemical content of the olive oil supplementation (167,168,208).

\textit{1.4.2.2 MUFA supplementation on heart rate}

Data from two large clinical trials investigating the health benefits of the Mediterranean diet have shown that resting heart rate is inversely related to adherence to the Mediterranean diet
(224,225). As these associations are based on scoring of overall adherence, which includes intake of olive oil and nuts but also of the other potentially beneficial components of the Mediterranean diet, it is difficult to attribute this relationship between heart rate and adherence solely to the effects of MUFA. In randomized trials of olive oil supplementation, the data regarding potential effects on heart rate in general do not support this relationship. Three studies have shown that 1-9 g/day of olive oil for 5-8 weeks does not affect resting heart rate in young healthy men and women, as well as hypertensive men (167,168,198,226). In addition, 12 weeks of olive oil was also shown to be ineffective in altering HRV in healthy men and women (179). Overall, MUFA from olive oil do not appear to have any appreciable effects on heart rate or its variability.

1.5 Differential cardiovascular effects of EPA and DHA

EPA and DHA are typically consumed in combination from food sources, with fatty fish generally providing relatively higher amounts of EPA. As well, most of the intervention studies examining n-3 PUFAs and cardiovascular outcomes have utilized supplements with a combination of EPA and DHA (123). As previously mentioned, the correlations with BP, cardiovascular risk, and mortality/morbidity are not always similar between these fatty acids, as EPA is inversely related to SBP in a small hypertensive population (33), while DHA but not EPA was more closely related to ambulatory BP in normotensive middle aged individuals (32). These differing associations have prompted several researchers to study these fatty acids separately to examine their individual effects on cardiovascular physiology.
1.5.1 EPA vs. DHA and the cardiovascular system

Since the mid 1990’s, several investigations have been conducted on the hemodynamic effects of EPA vs. DHA supplementation. In direct comparisons, DHA generally has more potent effects on hemodynamics, as 6-8 weeks of DHA but not EPA has been shown to reduce resting and 24 hour ambulatory BP and heart rate in healthy men and spontaneously hypertensive rats (198,227,228). In healthy men, EPA supplementation was accompanied by increases in resting heart rate despite increases in left ventricular filling time comparable to that observed with DHA (227). Subsequent standalone trials have replicated the finding of DHA’s ability to reduce resting heart rate and BP in healthy middle aged men and women (129,226), though not in all cases (127,128) or in clinical populations prescribed statins (229). Interestingly, one trial providing EPA alone in essential hypertension patients showed reductions in BP following 4 weeks of supplementation (230). Direct comparisons in other clinical populations have not been as promising, as neither DHA nor EPA altered BP or heart rate in men with dyslipidemia or diabetes despite similar increases in arterial compliance (199,231).

1.5.2 Mechanisms of action

Evidence highlighting the overlapping and separate physiological effects of EPA and DHA has emerged in recent years to explain the distinct clinical effects between these fatty acids. As mentioned, supplementation with a mixed formulation of EPA+DHA has generally been shown to result in reductions in central and peripheral arterial stiffness (149–151,153,154,156,232). When supplemented separately, both EPA (233–235) and DHA (229,236) reduce arterial stiffness. Direct comparisons show that acutely, DHA has more potent effects on arterial stiffness (236), whereas 7 weeks of supplementation with either n-3 elicits a similar reduction in arterial stiffness in dyslipidemic men (231). The beneficial effects on endothelial
function do not appear to differ significantly between EPA and DHA. Studies have shown increases in FMD with either EPA (237, 238) or DHA (239), and one study directly comparing them showed no improvement with either (218). Vascular reactivity appears to be differentially altered between the long chain n-3s. Specifically, DHA but not EPA attenuates vasoconstrictor responses to NE, whereas vasodilatory responses to acetylcholine are accentuated (240). Thus, there appear to be both shared and separate effects in terms of the peripheral vascular mechanisms of EPA and DHA.

Various animal and in vitro models have studied the separate effects of EPA and DHA directly on the heart. Isolated murine cardiomyocytes treated with either EPA or DHA display lower contractile rates and occurrence of arrhythmias compared to MUFA treatment (241, 242). This was attributed to a reduction in the electrical excitability of the cardiomyocytes (243). In healthy and diseased rat models, DHA but not EPA appears to elicit consistent reductions in resting heart rate and the QT interval (228, 244–246). Interestingly, despite these differences in heart rate, both EPA and DHA have been shown to reduce BP in renal and hyperinsulinemic experimental models of hypertension (244, 247), but were similarly ineffective in improving BP in other models (spontaneously hypertensive rat, congestive heart failure) (228, 246).

A relatively unexplored area that could account for diverging cardiovascular benefits of EPA and DHA is the ability to modulate the autonomic nervous system, specifically efferent sympathetic outflow. The only two human studies to have examined autonomic alteration with fish oil used a mixed supplement, with a ratio in favor of EPA (1.5:1), and showed no changes in blood pressure or MSNA (167, 168). Additionally, no other trials have studied the separate effects on other aspects of autonomic control of the cardiovascular system, such as HRV or arterial baroreflex sensitivity. Although both PUFAs can cross the blood-brain barrier (248, 249),
DHA tissue levels in the brain are significantly higher than EPA due to lower rates of beta-oxidation of DHA and higher levels of tissue incorporation (250). Given this, it is possible that the effects on neural regulation of the cardiovascular system, if any, differ between EPA and DHA. These could arise from structural differences between these fatty acids or from downstream effects on other signaling molecules. One example is the differing effects on NO metabolism. Although both EPA and DHA increase nitric oxide in vascular endothelial cells (251,252), a DHA rich diet increases brain nitric oxide synthase levels in rats, an effect not observed in EPA rich diets (253). Combined DHA+EPA, but not EPA alone, also increases urinary nitric oxide metabolites in humans (254). Nitric oxide inhibition results in increased resting sympathetic outflow (115), whereas increased nitric oxide bioavailability via dietary nitrates inhibits sympathetic outflow (255).

1.6 Conclusion

Both a high fish diet and the Mediterranean diet are associated with a reduced risk of cardiovascular morbidity and mortality (16,17,35). Supplementation with individual components of these diets, such n-3 PUFA rich fish oil, in comparison to population level data, has shown more inconsistent effects on cardiovascular risk and blood pressure (124,256). This may be due to mixed formulations of fish oil supplements, which provide both of the long chain n-3 PUFAs, EPA and DHA. Emerging evidence suggests that although there are some shared effects of these fatty acids, DHA may elicit more potent reductions in blood pressure and heart rate compared to EPA (198,227). It is unclear whether these differences arise from central effects, such as attenuation of sympathetic outflow, or peripheral vascular effects of DHA. Specifically, DHA increases central nitric oxide levels (253), a molecule which tonically inhibits sympathetic outflow (115). Reductions in sympathetic outflow to skeletal muscle could decrease blood
pressure by reducing total peripheral resistance due to lower vasoconstrictor tone. In contrast, DHA has been shown in humans to blunt vasoconstrictor responses to NE injections, and to increase vasodilation in response to acetylcholine (257), suggesting a peripheral pathway. The only studies which have examined whether fish oil acts through a central mechanism used a mixed formulation, and found no effects on either MSNA or BP (167,168). Thus, it is unknown whether the long chain n-3 PUFAs exert differential effects on sympathetic control of blood pressure.
Chapter 2. Purpose and Hypothesis

2.1 Purpose

The purpose of this study was to evaluate whether 12 weeks of EPA, DHA, or olive oil supplementation (3g/d) in young healthy men and women elicit differing effects on resting hemodynamics and muscle sympathetic outflow.

2.2 Hypothesis

DHA and olive oil will reduce resting blood pressure and muscle sympathetic nerve activity (MSNA) compared to EPA.

2.3 Individual contributions

I was involved in conceiving and designing the project, and was responsible for acquiring, analyzing, and interpreting data, as well as, writing the manuscript. Karam Notay was also involved in project conception and design, and data acquisition, analysis, and interpretation. Dr. Philip Millar performed all microneurography. Shannon Klingel screened, recruited, and randomized all participants.
This chapter is presented in manuscript format in preparation for submission to the American Journal of Clinical Nutrition
Chapter 3. Manuscript

Docosahexaenoic acid supplementation reduces resting blood pressure but increases muscle sympathetic outflow compared to eicosapentaenoic acid in healthy men and women: a randomized controlled trial

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Brief title: Fatty acid supplementation on blood pressure

Disclaimers: None

Abbreviations: BP, blood pressure; MSNA, muscle sympathetic nerve activity; n-3 PUFA, omega-3 polyunsaturated fatty acid

Number of tables/figures: 2/3

Clinical Trial Registration: URL: https://www.clinicaltrials.gov. Unique identifier: #NCT03378232

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Abstract

Background: Supplementation with monounsaturated or omega-3 polyunsaturated fatty acids (n-3 PUFA) can lower resting blood pressure (BP) and reduce the risk of cardiovascular events. However, the independent contributions of n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on BP, and mechanisms responsible, are less clear.

Objectives: To determine whether EPA and DHA have differential effects on heart rate, BP, and MSNA, (primary outcomes) as well as heart rate variability and both cardiac and sympathetic spontaneous arterial baroreflex sensitivity (secondary outcomes) in healthy young adults.

Methods: Ninety healthy young men and women were recruited to participate in a 12-week, randomized, double-blind trial examining the effects of orally supplementing ~3g/day of EPA (n=30), DHA (n=30), or olive oil (OO; n=30) on resting hemodynamics, and in a subset (n=43), muscle sympathetic nerve activity (MSNA; fibular nerve microneurography).

Results: The reductions in resting systolic BP were greater (adjusted intergroup mean difference [95% CI]) following DHA (-3.4 mmHg [-0.9, -5.9], p=0.008) and OO (-3.0 mmHg [-0.5, -5.4], P=0.01) compared to EPA supplementation; no differences were detected between DHA and OO (P=0.74). Similarly, the reductions in resting diastolic BP were greater following DHA (-3.4 mmHg [-1.3, -5.6], p=0.002) and OO (-2.2 mmHg [0.08, -4.3], P=0.04) compared to
EPA supplementation. Heart rate was higher with EPA compared to both DHA (4.2 beats/min, [-0.009, 8.4], \(P=0.05\)) and OO (4.2 beats/min, [0.08, 8.3], \(P=0.04\)). Resting MSNA burst frequency was higher following DHA (4 bursts/minute [0.5, 8.3], \(p=0.02\)) but not OO (-3 bursts/minute [-6, 0.6], \(P=0.2\)) compared to EPA supplementation.

Conclusions: Supplementation with DHA or OO evoke similar responses in resting BP compared to EPA; however, DHA but not OO was associated with higher peripheral vasoconstrictor outflow. These findings may have important implications for fatty acid supplementation in clinical populations characterized by both chronic high BP and sympathetic overactivation.

Key words: Blood pressure; Sympathetic nerve activity; Omega-3 polyunsaturated fatty acids; Diet

3.1 Introduction

Dietary modifications, such as reduced salt intake, moderate alcohol consumption, increased potassium intake, and consuming a healthy overall diet, consistently reduce resting blood pressure (BP) in normotensive and hypertensive populations (258,259). More controversial are the roles of dietary supplementation with monounsaturated or omega-3 polyunsaturated fatty acids (n-3 PUFA) (259). Olive oil (OO) supplementation can lower resting BP and reduce the risk of cardiovascular events (13,182,200). Likewise, fish oil supplementation, which is rich in the long chain n-3 PUFA eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), can also reduce resting BP, primarily in hypertensive patients (124,260,261), and reduce cardiovascular events (124,262). The overall reductions in resting BP with both
interventions appear modest (~3-5 mmHg) but would translate into important population-wide clinical benefits (263).

Despite a number of large trials investigating the clinical utility of n-3 PUFA supplementation (see (123)), less frequently considered is animal and human evidence that the cardiovascular responses to EPA and DHA supplementation may be distinct (198,227,228,257,264,265). For example, in middle-aged men, 7-weeks of 4g/day DHA supplementation decreased, while EPA increased resting heart rate, although neither EPA or DHA impacted BP (227). In a similar study, 6-weeks of supplementation with 4g/day DHA, but not EPA, reduced mean daytime and 24-hour systolic and diastolic ambulatory BP and heart rate (198). These results suggest that the hemodynamic benefits of fish oil supplementation may be mediated primarily by DHA. The mechanisms by which DHA could lower BP are not fully established. DHA can attenuate vasoconstrictor and enhance vasodilatory responses in the forearm (257), suggesting a peripheral vascular mechanism. Alternatively, in animal models, DHA can increase the activity of nitric oxide synthase and the concentration of tetrahydrobiopterin in the brain (252,253,266), which may subsequently increase nitric oxide availability, an important molecule that exerts tonic inhibition of central sympathetic outflow (115,267). Interestingly, OO has also been shown to increase circulating levels of nitric oxide metabolites, which coincide with reductions in BP (205).

To our knowledge, no studies have compared the independent effects of EPA and DHA on direct measurements of central sympathetic outflow. Two prior trials reported that fish oil supplementation (which contains both EPA and DHA in ~1.5:1 ratio, respectively) had no effect on microneurographic measures of resting muscle sympathetic nerve activity (MSNA) in young, healthy men and women after 4 weeks (168) or 8 weeks (167), though both studies did not detect
any changes in resting BP. Whether these results were obscured by using combined EPA and DHA supplements is unclear. Further, both prior studies failed to biochemically verify supplement compliance. Therefore, the purpose of this prospective, randomized, double-blind trial was to examine the effects of 12 weeks of EPA, DHA, or OO supplementation on resting heart rate, BP, and in a subset of participants, MSNA. We hypothesized that both DHA and OO supplementation would reduce resting BP and MSNA compared to EPA.

3.2 Methods

3.2.1 Subjects

Ninety healthy men and women aged 18-30 years (45 men and 45 women) were recruited to participate in the study. Exclusion criteria included use of fish oil supplements within the previous three months, >2 servings of fish/seafood per week, prescription of chronic pharmacological medication (with the exception of oral contraceptives), smoking, and history of cardiovascular disease. All women were studied during the early follicular phase (between days 1-5), or if taking oral contraception (n=16), during the initial five days of their placebo pills. All procedures were approved by the Research Ethics Board at the University of Guelph. Written informed consent was obtained prior to collection in all subjects. This study was registered at clinicaltrials.gov under the unique identifier #NCT03378232.

3.2.2 Study Design and Experimental Protocol

Participants completed a double-blind, randomized trial comparing the effects of supplementing ~3 g per day with EPA, DHA, or OO for 12 weeks. All participants completed a familiarization visit to undergo detailed verbal and visual explanation of all testing protocols and
requirements before undergoing randomization to group allocation. Participants were instructed to refrain from making any major lifestyle (e.g. diet or exercise) changes throughout the course of the study and to abstain from alcohol, caffeine, and vigorous exercise for 24 hours before any study visits.

Experimental data were collected during two study visits conducted at the same time of day (±2 h), i.e. once before and after 12 weeks of supplementation. During each visit, participants entered a light- and temperature-controlled laboratory following voiding and underwent anthropometric measurements. Next, participants were positioned in an upright seated position with their feet supported on an ottoman. Following instrumentation, participants underwent a 10-minute rest period and subsequent 10-minute epoch to collect continuous and discrete blood pressure, heart rate, and in a subset of participants, MSNA.

3.2.3 Measurements

Discrete measurements of BP were obtained from the left brachial artery using an automated oscillometric sphygmomanometer (BPTru Medical Devices, Coquitlam, BC, Canada). Continuous heart rate measurements were made using single lead electrocardiography (Lead II). Beat-to-beat BP was measured using a finger cuff placed on the right middle finger (Finometer MIDI, Finapress Medical Systems, The Netherlands). Due to the technical and time constraints of performing microneurography, only a subset of study participants were recruited (n=43) to undergo multiunit recordings of MSNA. As described previously (255,268), a 2mΩ tungsten microelectrode was inserted percutaneously into a motor fascicle of the right fibular nerve and adjusted until spontaneous bursts of integrated sympathetic activity could be observed from the background noise. To ensure selectivity of muscle sympathetic activity, we tested reflex
increases in response to an end expiratory apnea and lack of responsivity to unexpected clapping. The raw neural signal was amplified (75,000x), band-pass filtered (0.7–2.0 kHz), rectified, and integrated to obtain the mean voltage multiunit neurogram (model 662C-4, Nerve Traffic Analyzer, Absolute Design and Manufacturing Services, Salon, IA). The neural signal was monitored audibly and visually to ensure no changes in the microelectrode placement occurred during each visit. Our laboratory has demonstrated high inter-day reliability (intraclass correlation coefficient >0.76) for measures of resting MSNA (255). Continuous heart rate, BP, and integrated MSNA signals were recorded at a sampling frequency of 1,000 Hz, while the raw MSNA signal was sampled at 10,000 Hz.

3.2.4 Dietary Supplements and Compliance

All supplements were obtained from KD Pharma (Bexbach, Germany). Supplements consisted of softgel capsules that were identical in taste and appearance. Gas chromatography analysis determined that EPA and DHA supplements both had a purity of ~99%. EPA supplements contained 813 mg of EPA and 7 mg of DHA per capsule. DHA supplements contained 814 mg of DHA and 7.5 mg of EPA per capsule. OO supplements contained 818 mg oleic acid, and no detectable EPA or DHA per capsule. All capsules contained 0.2% tocopherol to prevent oxidation of fatty acids. Participants were instructed to ingest two capsules with food twice per day, and instructed to store supplements in the freezer. Participants were provided a one month supply of capsules at a time and returned to the lab to receive further supplements.

Supplement compliance was assessed by gas chromatography. Venous blood samples were collected at baseline and week 12 to analyze EPA and DHA content in erythrocytes, as previously described (269). Given significant increases in erythrocyte EPA and DHA levels
occur when supplementing with dosages lower than that used in the current study (270),
participants were deemed non-compliant if they showed either no change or a reduction in EPA
or DHA levels following EPA or DHA supplementation, respectively. Participants in the OO
group were excluded if they demonstrated significant changes in either EPA and/or DHA levels.

3.2.5 Data Analysis

All data were analyzed by investigators blinded to the supplement allocations. BP and
heart rate were calculated from the average of the last five minute-to-minute measurements.
MSNA was analyzed using a custom semi-automated LabVIEW program (National Instruments,
Austin, Texas, USA), wherein sympathetic bursts were quantified based on a 3:1 signal to noise
ratio and alignment with the time shift of the cardiac cycle (255,268). Sympathetic activity was
quantified as both burst frequency (bursts/minute) and burst incidence (bursts/100 heart beats);
the latter to normalize for inter-individual differences in heart rate. Spontaneous arterial
sympathetic baroreflex sensitivity was calculated by assessing the relationship between diastolic
BP and MSNA burst occurrence, as previously described (255,271). A weighted regression line
was fit between the likelihood of a MSNA burst within 2 mmHg bins of diastolic BP for each
participant, and the slope was taken as the sympathetic gain. The relationship was deemed
acceptable if the regression coefficient of the regression line was $\geq 0.5$; five participants did not
meet this criteria.

Time- and frequency-domain measures of heart rate variability (HRV) were assessed
during the last five minutes of resting baseline measurements (Kubios HRV 2.2). High and low
frequency spectral power were not normally distributed and all values underwent natural
logarithm transformation. The sequence method was used to calculate spontaneous cardiac
baroreflex sensitivity (LabChart v8, ADInstruments, Colorado Springs, CO). We identified three or more consecutive and concurrent increases or decreases in systolic BP and R-R interval with a minimum threshold of 1 mmHg and 6 ms, respectively (272). All sequences were reviewed, and cBRS was calculated by plotting the R-R interval over the systolic BP for each series, and averaging the slopes of all up and down sequences. Sequences were deemed acceptable if the regression coefficient was >0.8 and each participant had a minimum of three up and three down sequences; all participants met this criteria.

### 3.2.6 Statistical Analysis

The size of the study sample was estimated from previous work examining the effects of DHA, EPA, and OO on systolic BP (198), based on a power of 80% and α equal of 5%, we computed a required sample size of 22 participants per group. No comparable data was available to estimate the required sample size for detecting differences in MSNA (should they exist). All statistical analyses were performed using SPSS 24.0 (SPSS Chicago IL). Participant baseline characteristics were analyzed using one-way analysis of variance (ANOVA). Pre-post changes in anthropometric, hemodynamic, and neural variables were compared using one-way analysis of covariance (ANCOVA), with baseline values and sex as covariates. The use of an ANCOVA model adjusting for baseline values is recommended to reduce the inter-subject error and increase statistical power (273). Grubbs’ test was conducted on change scores for systolic blood pressure to detect outliers. Statistical significance was considered $P< 0.05$. 

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3.3 Results

Ninety participants were studied between January and June 2017. Of these, 88 completed the full study protocol, with one participant dropping out due to verbal non-compliance and another due to unavailability for post testing. One participant was deemed non-compliant in the EPA group due to lack of change in erythrocyte EPA content and consequently excluded from all analyses. Grubbs’ test detected one outlier for systolic blood pressure changes in the DHA group which was then excluded from all analyses. No participants reported any adverse outcomes associated with the intervention.

Baseline participant characteristics are presented in Table 1. All groups possessed similar baseline anthropometric and cardiovascular characteristics (All $P>0.05$). Body weight did not change following supplementation in any of the groups ($P>0.05$). Complete pre-post microneurographic recordings of MSNA were obtained in 32 of 43 participants (inability to secure a recording site at baseline [n=3] or visit 2 [n=4], or poor recording quality between visits [n=3]). Baseline characteristics of the MSNA subset were similar to the larger sample, with the exception that baseline systolic BP and MSNA differed between the groups. Specifically, systolic BP was higher in the OO group subset compared to the EPA subset ($P=0.01$), while MSNA burst frequency was higher in the DHA subset compared to the EPA subset ($P=0.03$). All pre-post analyses were adjusted for baseline values and sex.
Table 1. Baseline participant characteristics.

<table>
<thead>
<tr>
<th></th>
<th>EPA</th>
<th>DHA</th>
<th>OO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female, n</td>
<td>14/14</td>
<td>14/14</td>
<td>15/15</td>
</tr>
<tr>
<td>Age, years</td>
<td>21 ± 2</td>
<td>22 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Height, cm</td>
<td>173 ± 9</td>
<td>174 ± 9</td>
<td>168 ± 9</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>70 ± 12</td>
<td>73 ± 16</td>
<td>68 ± 11</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23 ± 3</td>
<td>24 ± 4</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>103 ± 9</td>
<td>104 ± 11</td>
<td>106 ± 9</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>64 ± 7</td>
<td>65 ± 7</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>67 ± 9</td>
<td>68 ± 10</td>
<td>68 ± 12</td>
</tr>
</tbody>
</table>

Mean ± SD. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; OO, olive oil.
Table 2. Effects of 12-weeks of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or olive oil (OO) supplementation on hemodynamic and neural variables.

<table>
<thead>
<tr>
<th></th>
<th>EPA Pre</th>
<th>EPA Post</th>
<th>DHA Pre</th>
<th>DHA Post</th>
<th>OO Pre</th>
<th>OO Post</th>
<th>EPA vs. OO Inter</th>
<th>DHA vs. OO Inter</th>
<th>EPA vs. DHA Inter</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>103.2±8.1</td>
<td>104.9±6.7</td>
<td>103.8±11.1</td>
<td>101.6±8.4</td>
<td>105.4±8.5</td>
<td>103.4±8.8</td>
<td>3.0 (0.5 to 5.4)</td>
<td>.01</td>
<td>-0.4 (-2.8 to 2.0)</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>63.8±6.5</td>
<td>65.9±6.1</td>
<td>65.0±7.3</td>
<td>63.1±5.7</td>
<td>65.2±8.0</td>
<td>64.8±7.5</td>
<td>2.2 (0.08 to 4.3)</td>
<td>.04</td>
<td>-1.2 (-3.3 to 0.8)</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>77.0±6.5</td>
<td>78.7±5.7</td>
<td>77.6±8.3</td>
<td>76.0±6.2</td>
<td>78.5±7.8</td>
<td>77.7±7.4</td>
<td>2.2 (0.2 to 4.3)</td>
<td>.02</td>
<td>-0.7 (-2.7 to 1.2)</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>66.9±9.4</td>
<td>71.3±10.3</td>
<td>67.7±9.7</td>
<td>67.5±7.6</td>
<td>67.9±11.8</td>
<td>67.4±10.1</td>
<td>4.2 (0.08 to 8.3)</td>
<td>.04</td>
<td>0.02 (-4.1 to 4.1)</td>
</tr>
<tr>
<td>SDNN, ms</td>
<td>65.8±24.4</td>
<td>62.7±24.4</td>
<td>62.1±26.7</td>
<td>64.8±21.0</td>
<td>69.6±27.8</td>
<td>67.2±21.0</td>
<td>-2.4 (-11.4 to 6.5)</td>
<td>.58</td>
<td>1.6 (-7.3 to 10.7)</td>
</tr>
<tr>
<td>RMSSD, ms</td>
<td>51.1±26.2</td>
<td>45.2±26.9</td>
<td>50.5±40.3</td>
<td>48.1±25.3</td>
<td>61.3±37.5</td>
<td>56.1±24.4</td>
<td>-6.1 (-16.3 to 4.1)</td>
<td>.23</td>
<td>-2.9 (-13.1 to 7.3)</td>
</tr>
<tr>
<td>LF, ln ms²</td>
<td>7.1±0.9</td>
<td>7.0±0.9</td>
<td>6.9±0.7</td>
<td>7.1±0.7</td>
<td>6.9±0.8</td>
<td>6.8±0.7</td>
<td>0.05 (-0.2 to 0.3)</td>
<td>.69</td>
<td>0.3 (0.04 to 0.6)</td>
</tr>
<tr>
<td>HF, ln ms²</td>
<td>6.6±0.9</td>
<td>6.3±1.0</td>
<td>6.5±1.0</td>
<td>6.5±0.9</td>
<td>6.9±1.3</td>
<td>6.9±0.9</td>
<td>-0.4 (-0.7 to -0.05)</td>
<td>.02</td>
<td>-0.1 (-0.5 to 0.2)</td>
</tr>
<tr>
<td>LF/HF</td>
<td>2.9±4.7</td>
<td>2.7±2.4</td>
<td>1.9±1.8</td>
<td>2.3±1.3</td>
<td>1.9±3.0</td>
<td>1.3±1.2</td>
<td>1.0 (0.4 to 1.7)</td>
<td>.002</td>
<td>.96 (0.293 to 1.6)</td>
</tr>
<tr>
<td>cBRS, ms/mmHg</td>
<td>18.3±7.8</td>
<td>16.4±8.9</td>
<td>17.2±8.7</td>
<td>17.3±7.2</td>
<td>20.5±11.2</td>
<td>20.3±11.8</td>
<td>-2.7 (-6.8 to 1.3)</td>
<td>.18</td>
<td>-1.2 (-5.3 to 2.8)</td>
</tr>
<tr>
<td>BF, bursts/min</td>
<td>18.7±6.1</td>
<td>19.2±5.4</td>
<td>24.4±6.2</td>
<td>26.5±5.2</td>
<td>22.2±6.4</td>
<td>17.9±4.7</td>
<td>2.9 (-0.6 to 6.4)</td>
<td>.1</td>
<td>7.3 (3.6 to 11.0)</td>
</tr>
<tr>
<td>BI, bursts/100hb</td>
<td>29.8±10.0</td>
<td>29.1±8.5</td>
<td>36.8±9.1</td>
<td>39.1±9.1</td>
<td>33.7±8.5</td>
<td>27.8±8.5</td>
<td>3.3 (-2.1 to 8.9)</td>
<td>.2</td>
<td>9.1 (3.4 to 14.9)</td>
</tr>
<tr>
<td>sBRS</td>
<td>-3.9±1.2</td>
<td>-3.8±1.6</td>
<td>-4.8±1.9</td>
<td>-4.5±0.6</td>
<td>-4.1±1.2</td>
<td>-3.6±1.2</td>
<td>-0.1 (-1.3 to 1.0)</td>
<td>.76</td>
<td>-0.6 (-1.8 to 0.5)</td>
</tr>
</tbody>
</table>

Mean ± SD. Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; OO, olive oil; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate; SDNN, standard deviation of R-R interval; RMSSD, root mean square of the sum of squares of differences of successive R-R intervals; LF, low frequency power; ln, natural log; HF, high frequency power; LF/HF, ratio of low frequency to high frequency power; cBRS, cardiac baroreflex sensitivity; BF, MSNA burst frequency; BI, MSNA burst incidence; sBRS, sympathetic baroreflex sensitivity. *, adjusted for baseline values and sex. Sample size for hemodynamic, heart rate variability, and cBRS data (EPA: n=28; DHA: n=28; and OO: n=30); MSNA data (EPA: n=11; DHA: n=9; and OO: n=11); sBRS data (EPA: n=8; DHA: n=8; and OO: n=10).
Figure 2. Consolidated Standards of Reporting Trials flow chart of participant recruitment.
Figure 3. Adjusted change in resting systolic and diastolic blood pressure and heart rate following 12 weeks of ~3 g per day supplementation with eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or olive oil (OO). Values presented as mean ± SEM and adjusted for baseline and sex.
Figure 4. Adjusted change in resting muscle sympathetic nerve activity (MSNA) burst frequency and burst incidence following 12 weeks of ~3 g per day supplementation with eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or olive oil (OO). Values presented as mean ± SEM and adjusted for baseline and sex.
The effects of supplementation on resting hemodynamic and neural variables are presented in Table 2. Resting heart rate was higher following EPA supplementation compared to OO \((P=0.04)\) and DHA \((P=0.05)\). In contrast, resting systolic BP (Figure 3) was lower with DHA or OO supplementation compared to EPA (both \(P<0.05\)), though changes with DHA and OO did not differ \((P=0.74)\). Similarly, resting diastolic BP (Figure 3) was lower with DHA and OO (both \(P<0.05\)) compared to EPA, though changes were not different between OO and DHA \((P=0.23)\). With respect to indices of cardiac autonomic modulation, the LF/HF ratio was higher with both EPA and DHA compared to OO (both \(P<0.01\)), while the low frequency power was higher following DHA supplementation compared to both OO \((P=0.02)\) and EPA \((P=0.07)\). The high frequency power was higher following OO than EPA \((P=0.02)\). Cardiac baroreflex sensitivity was not different between groups \((P>0.05)\). Changes in resting MSNA burst frequency (Figure 4) were higher with DHA \((P=0.02)\) but not OO \((P=0.1)\) compared to EPA, with a significant difference also found between DHA and OO supplementation \((P<0.001)\).

When expressed as MSNA burst incidence (Figure 4), sympathetic outflow was trending towards being higher with DHA \((P=0.058)\) but not OO \((P=0.2)\) compared to EPA. MSNA burst incidence was also higher with DHA compared to OO \((P=0.003)\). Sympathetic baroreflex sensitivity was not different between groups \((P>0.05)\).

3.4 Discussion

The present study adds to a growing body of evidence demonstrating that n-3 PUFA supplementation can reduce resting BP, and more specifically, that DHA is likely responsible for reductions in BP following the consumption of mixed EPA/DHA supplements. The primary novel finding is that although resting BP was similarly lower following DHA or OO compared to
EPA supplementation, the changes in MSNA were quantitatively opposite. A similar finding was observed for changes in the LF/HF ratio of heart rate variability. Together, these results suggest that the mechanisms responsible for changes in resting BP differ between DHA and OO and that the formulation of fatty acid supplements may be better tailored to maximize cardiovascular benefits.

Prior work has shown that DHA elicits more potent reductions in BP, heart rate, and total peripheral resistance compared to EPA (198,227,228,257,265), and that DHA but not EPA can upregulate central levels of nitric oxide in rats (253), a known sympatholytic molecule (267). These data led us to hypothesize that DHA supplementation would lead to greater reductions in resting BP and MSNA compared to EPA. Such evidence would demonstrate that prior null studies directly assessing central sympathetic outflow following n-3 PUFA supplementation (167,168) were impacted by formulations containing higher levels of EPA relative to DHA. Our study also utilized OO as a positive control. It is important to acknowledge that a large number of studies have employed OO as a placebo control when studying the effects of n-3 PUFA supplementation on hemodynamics and sympathetic outflow (167,168,198,257). However, a similar body of evidence has shown the positive effects of a Mediterranean-style diet on resting BP (182), and more specifically the hypotensive effects of OO,(182,200,201,205) which are mediated through oleic acid (184). Additionally, OO contains several plant polyphenols which may also influence BP (274,275). For example, OO containing high polyphenols compared to polyphenol-depleted OO was found to reduce resting BP in hypertensives (205). Our findings suggest that OO is not appropriate to use as a placebo due to its effects on BP.

Although we confirmed our hypothesis that DHA and OO both lower resting systolic BP compared to EPA supplementation, the spectral heart rate variability and MSNA responses to
DHA and OO supplementation were directionally opposite. This observation offers unique insight into the neural mechanisms responsible for the BP reductions with both fatty acids. For example, a reduction in resting BP would be expected to evoke a reflex increase in sympathetic outflow; we observed higher MSNA and markers of cardiac sympathetic modulation (LF power and LF/HF ratio). As a result, we speculate that DHA acts to lower BP primarily through a peripheral vasodilator mechanism and that increases in MSNA are the result of arterial baroreflex engagement to prevent changes in perfusion pressure in this population of normotensive participants. DHA can directly influence vascular smooth muscle cells and increase activation of calcium-dependent potassium ion channels involved in vasodilation (12,276). Prior evidence of increased forearm blood flow responses to co-infusion of acetylcholine and L-NMMA also suggest the involvement of nitric oxide-independent mechanisms (135,257,277). This may explain the observation that 1.6g/day of DHA for 16 weeks lowered resting BP in young healthy men concomitant with a reduction in brachial artery endothelium-dependent vasodilation (220). In contrast, OO exerted a parallel reduction in BP and MSNA, without a change in sympathetic baroreflex sensitivity, suggesting a resetting of central autonomic control. Such a response may yield additional clinical benefits as elevated sympathetic activity is associated with left ventricular hypertrophy (278), insulin resistance (279,280), and arrhythmogenesis (281).

The American Heart Association recommends that both the general and cardiovascular patient population consume at least two meals of fish per week or supplement their diet with n-3 PUFA (123). However, a recent meta-analysis of large prospective clinical trials has questioned the efficacy of n-3 PUFA supplementation to reduce fatal or non-fatal coronary heart disease or major vascular events in those with high cardiovascular risk (256). Unfortunately, the majority of
clinical trials have tested both low daily n-3 PUFA doses (~1g/day), much lower than that tested in the present study, and employed supplements that contain ~60% EPA (123,256). Our results suggest that altering the specific ratio of EPA:DHA in n-3 PUFA supplements or adding concurrent supplementation with OO may be able to increase the clinical benefit derived by both reducing BP and sympathetic activity. Specific recommendations may also be made on a population level. For example, in patients with heart failure with reduced ejection fraction, central sympathetic outflow is a strong predictor of mortality (103). These patients may exert greater benefit from OO supplementation. A Mediterranean-style diet supplemented with extra-virgin OO has been shown to reduce the incidence of major cardiovascular events in high risk participants (13).

It is important to consider that while the primary outcomes of the current study were BP and MSNA, EPA supplementation has been shown to be beneficial in improving other cardiovascular risk factors, such as blood lipid profiles (264). Further, circulating EPA may also be more strongly associated with a reduced risk of non-fatal cardiovascular events, compared to circulating DHA (282,283). Given that EPA supplementation increased resting heart rate by approximately 4 BPM relative to both OO and DHA and appeared to decrease HF power, a marker of cardiac parasympathetic modulation, it is possible that EPA exerts cardiovascular benefits through BP and autonomic nervous system-independent mechanisms.

We acknowledge several limitations. We were not able to collect MSNA data in the entire sample due to the technical demands and time intensive nature required for completing microneurography recordings. In particular, we purposefully sought to limit the window for initiating the study to ~6 weeks to reduce any variations in BP or MSNA due to seasonal variability following the 12 week supplementation period (284). We have previously
demonstrated that the inter-day reproducibility of resting MSNA is higher than that of BP, permitting the use of smaller sample sizes (255). We did not assess ambulatory BP, though our results demonstrating reductions in BP with DHA but not EPA agree with prior results (198). Finally, the present work was conducted in a population of young healthy adults and further work is needed to translate these observations to relevant clinical populations (including those on concurrent anti-hypertensive medications or statins) and larger sample sizes.

In conclusion, 12 weeks of supplementing 3g/day of DHA or OO reduced resting systolic and diastolic BP compared to EPA treatment in young healthy men and women. These findings support the concept (198) that the hypotensive effects of n-3 PUFA supplementation are mediated primarily by DHA. Direct microneurographic recordings of MSNA demonstrated that DHA and OO supplementation were accompanied by opposite changes in central sympathetic outflow (MSNA), revealing that the hemodynamic responses are likely to be mediated through different mechanisms. These results have implications for determining formulations of EPA:DHA for future clinical trials and raise the possibility that OO supplementation may be beneficial in populations characterized by increased BP and central sympathetic activity.
Chapter 4. Extended discussion

4.1 Summary of findings

In the present study, both DHA and OO were observed to similarly reduce resting BP, although the diverging effects on muscle sympathetic outflow suggest separate mechanisms of action. There is prior evidence that DHA can alter vascular reactivity, blunting vasoconstrictor responses following NE and promoting greater vasodilation following acetylcholine (257). Alternatively, animal data showing increases in central NOS levels with DHA (253) led us to hypothesize that sympathoinhibitory effects could be involved in the cardiovascular benefits of DHA. Our data do not align with this later hypothesis, and instead support a peripheral mechanism of action as the primary target, which is likely accompanied by compensatory arterial baroreflex-mediated sympathoexcitation. The ability of olive oil to reduce BP without concomitant sympathoexcitation suggests that this may have been elicited by a direct inhibitory effect on the SNS, potentially mediated by an increase in NO from either olive polyphenols or nitro fatty acids (188,205).

4.2 Clinical significance

The present study examined the effects of fatty acid supplementation on young healthy men and women with normal resting BP, however, the extension of our results may be clinically relevant. It is known that elevated sympathetic outflow contributes to increased morbidity and mortality in several clinical cardiovascular populations, including heart failure and hypertension (103,281). Whether DHA supplementation in a clinical cardiovascular population would further increase the already elevated sympathetic outflow in these groups, and whether this translates to a worsening of the deleterious effects of sympathoexcitation, remains an interesting question that warrants further research. If so, it is possible that the peripheral vascular effects of DHA, such as
increased vasodilation due to greater NO bioavailability (11,253,257,285), could offset the effects of higher sympathetic outflow in these populations (286,287) and explain prior results of null effects of n-3 supplementation on clinical outcomes (24–28).

The uncertainty of the balance between potential benefits and contraindications of DHA warrants additional research to expand upon its clinical utility. Considering the current findings, it is also interesting to note that fish and n-3 intake is not always associated with improvements in the risk of cardiovascular morbidity and mortality. For example, although fish oil supplementation and higher n-3 concentrations are generally associated with antiarrhythmic benefits in cardiovascular patients (288,289), those in the highest quartiles (290) and quintiles (289) of erythrocyte n-3 levels may actually have an increased incidence of both atrial and ventricular fibrillation (289,290), suggesting that there is a threshold after which increased n-3’s could result in deleterious effects. In the Diet and Angina Randomized Trial 2 (DART 2), which followed approximately 3000 men being treated for angina, the occurrence of sudden cardiac death was greater in men not on beta-adrenergic blockade given advice to consume fish or fish oil capsules (291). A smaller trial later found that fish oil supplementation increased ventricular arrhythmia occurrence in patients with implantable defibrillators (292). The mechanisms underlying these findings are unknown, but it has been speculated that in chronic disease states, cardiac ion equilibrium is adapted to maintain function, and that a bolus of n-3’s (such as from fish oil) perturbs the established ion equilibrium in high risk patients (293). These findings of potential pro-arrhythmic effects of fish oil and n-3’s have not been consistently shown (294), likely due to differences in supplements, baseline n-3 levels, participant health status, and so overall, there remains a need to further explore the use of DHA supplementation in clinical populations and the benefits and contraindications.
Unlike DHA, OO’s hypotensive effects were not accompanied by reflex sympathoexcitation, which provides insight into the mechanisms which could underlie the benefits seen in previous studies. Large scale studies show that olive oil is beneficial in the prevention of cardiovascular disease and mortality (13,34,36), and that this effect is mediated in part by reductions in BP (36,182,205,206). Our data suggests that olive oil lowered BP by resetting autonomic control to a lower set point. This reduction in BP without sympathoexcitation could be important for the treatment of clinical populations characterized by sympathetic overactivity, as exaggerated sympathetic outflow is a risk factor for left ventricular hypertrophy (278), insulin resistance (279,280), and arrhythmogenesis (281). In support of this, olive oil has also been found to improve insulin sensitivity in both healthy and insulin resistant animals and humans (295–297), as well as reduce the risk of atrial fibrillation in humans (298).

4.3 Limitations

4.3.1 Microneurography and sympathetic transduction

Although MSNA is considered the gold-standard measure of central sympathetic outflow, there are limitations to the technique which warrant consideration. Specifically, while the technique measures postganglionic action potentials transmitted to the sympathetic terminals, it does not provide information regarding end-organ responses in the vasculature (i.e. vasoconstriction). At rest, spontaneous MSNA bursts are generally accompanied by decreases in vascular conductance, which indicate a vasoconstrictor response to this outflow (76,83). However, there are instances where increased sympathetic outflow does not translate to increased vasoconstriction, such as during exercise where the transduction of muscle sympathetic outflow to vasoconstriction is attenuated by metabolically-induced vasodilation and reduced
vascular adrenergic sensitivity despite increased sympathetic outflow (299). Fish oil supplementation in rats and primates reduces the vascular and cardiac adrenergic response to NE and electrical stimulation (131,300–302). In humans, higher resting MSNA is associated with reduced adrenergic sensitivity (286), and so in our study the increase in resting MSNA with DHA could be reflective of a change in adrenergic receptor responsiveness. MSNA does not quantify the amount of NE or other neurotransmitters released from the postganglionic sympathetic nerves, nor does it account for NE reuptake. In addition, the pattern of sympathetic nerve firing may also influence the amount of NE released from sympathetic nerves, with bursts of activity releasing more NE than continuous stimulation, independent of the overall level of sympathetic outflow (63).

4.3.2 Dietary olive oil intake and quantification of olive oil compounds

OO has been used in some research n-3 PUFA studies as a placebo control (167,168,198), whereas in other studies it has been the primary supplement of interest for reducing BP (182,200,201,203,205,207,211). OO supplements vary widely in their amounts of oleic acid (194), polyphenols (195,196), and possibly nitrated fatty acids (188). Although we used gas chromatography to quantify the oleic acid present in our OO supplement, which has not been typically reported in previous literature (167,168,182,198), we do not have any information on polyphenol or nitrated fatty acid content in our supplement.

One animal study has suggested that oleic acid is the primary constituent responsible for the hypotensive effects of OO (184), whereas other evidence shows that polyphenol rich OO elicits significant reductions in BP, which were not seen with processed OO low in polyphenols (205). Recent work in animals also shows that nitro fatty acids protect against the development
of hypertension (191,192). The targets these constituents act upon differ, as oleic acid regulates G protein signalling (184), whereas polyphenols and nitro fatty acids likely reduce BP through NO-mediated mechanisms (188,192,205), which could be acting peripherally or centrally. NO and its by-products nitrate and nitrite appear to be a significant factor involved in mediating the cardiovascular effects of these supplements (11,205,303). Quantification of circulating nitrate/nitrite levels and the potential changes occurring with supplementation would allow us to better understand the underlying mechanisms responsible for the reduction in BP with OO.

4.4 Future directions

Further research is warranted to explore the generalizability of these findings to clinical populations, especially those with elevated resting sympathetic outflow, such as healthy ageing or patients with heart failure, and whether DHA alters the sympathoexcitation present in these conditions. As n-3 PUFA consumption has shown both positive and null effects in improving outcomes in cardiovascular patients, future work should examine the interactions between DHA’s activation of the SNS and its peripheral effects to understand whether the latter negates or offsets the potentially harmful effects of increased sympathetic outflow. Additionally, the pathways by which OO reduces blood pressure without sympathoexcitation require more investigation, as well as to elaborate on which components in OO are responsible for these benefits.

4.5 Conclusions

DHA and OO supplementation were shown to similarly reduce resting BP in young healthy men and women compared to EPA. In contrast, the changes in MSNA with DHA and
OO were directly opposite, with DHA eliciting a small increase in MSNA. The exact mechanisms underlying this increase are unknown, but may involve arterial baroreflex-mediated increases in sympathetic outflow to counter peripherally-mediated vasodilation (12,257,276). Conversely, the hypotensive effects of olive oil in our study may be due to centrally-mediated pathways involving NO (188,205). Given the role of the SNS in the development and progression of hypertension and other cardiovascular diseases (63,103,304), addressing sympathetic over-activity remains an important issue since the majority of therapies target peripheral adrenergic receptors as opposed to central sympathetic outflow (305). Despite recommendations from the American Heart Association regarding n-3 PUFA consumption and cardiovascular health, the use of fish oil supplements in the prevention and treatment of cardiovascular disease remains a controversial topic due to the inconsistent benefits associated with their consumption (123,256,258,259). Many of the studies have examined supplements containing higher quantities of EPA compared to DHA (124,256), while data from our study and others (198,227,257) suggest that the cardiovascular effects of these fatty acids are not equivalent, and that the reductions in BP with fish oil are primarily due to DHA. These findings highlight the need to study the separate cardiovascular effects of EPA and DHA in order to understand the optimal ratio of the n-3’s and tailor fatty acid supplements to maximize the benefit to specific clinical populations.
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Appendix 1: Ethics Approval

The members of the University of Guelph Research Ethics Board have examined the protocol which describes the participation of the human participants in the above-named research project and considers the procedures, as described by the applicant, to conform to the University’s ethical standards and the Tri-Council Policy Statement, 2nd Edition.

The REB requires that researchers:

- Adhere to the protocol as last reviewed and approved by the REB.
- Receive approval from the REB for any modifications before they can be implemented.
- Report any change in the source of funding.
- Report unexpected events or incidental findings to the REB as soon as possible with an indication of how these events affect, in the view of the Principal Investigator, the safety of the participants, and the continuation of the protocol.
- Are responsible for ascertaining and complying with all applicable legal and regulatory requirements with respect to consent and the protection of privacy of participants in the jurisdiction of the research project.

The Principal Investigator must:

- Ensure that the ethical guidelines and approvals of facilities or institutions involved in the research are obtained and filed with the REB prior to the initiation of any research protocols.
- Submit a Annual Renewal to the REB upon completion of the project. If the research is a multi-year project, a status report must be submitted annually prior to the expiry date. Failure to submit an annual renewal will lead to your research being suspended and potentially terminated.

The approval for this protocol terminates on the EXPIRY DATE, or the end of your appointment or employment at the University of Guelph, whichever comes first.
Appendix 2: Figure 1 permissions

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