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Effects of Dietary Components on Cardiometabolic Health in Postmenopausal Women

By

SUN JUNG YIM
DISSERTATION

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Abstract

Cardiovascular disease (CVD) is the leading cause of death worldwide. Menopause represents a key cardiometabolic shift for women. Estrogen loss leads to visceral fat accumulation, dyslipidemia, insulin resistance, and vascular dysfunction, accelerating CVD risk. The aim of this dissertation was to examine how specific Mediterranean dietary components influence cardiometabolic outcomes in postmenopausal women. I integrated clinical, mechanistic, and translational perspectives.

Chapter 1 reviews CVD pathophysiology, vascular function, and menopause as a sex-specific CVD risk factor. It details surrogate vascular outcomes, such as flow-mediated dilation (FMD) and peripheral arterial tonometry (PAT), alongside epidemiological and clinical evidence for Mediterranean diet (MedDiet) adherence in CVD prevention. Mechanistic pathways, including oxylipin signaling, flavonoid metabolism, and gut microbiome interactions, are also discussed.

Chapters 2 and 3 focus on walnuts as a Mediterranean nut source rich in alpha-linolenic acid (ALA) and ellagitannins. Chapter 2 reports a short-term intervention showing that walnut consumption improved microvascular function and altered oxylipin profiles. Particularly, epoxides support endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilation. Chapter 3 demonstrates that walnuts modify lipoprotein composition to suppress tumor necrosis factor (TNF)- α -stimulated cytokine production in diabetic adipocytes. This study suggests that dietary modification may attenuate adipose tissue-driven inflammation.

Chapter 4 reports randomized, crossover trials of Oligonol, a flavanol-rich lychee and green tea extract, in postmenopausal women (100-600 mg/day for 2 weeks) and healthy adults

(acute 2 g). No significant improvements were observed in any measured outcomes, likely due to factors such as healthy baseline vascular status and lower extract epicatechin content than reported.

Chapter 5 integrates observational and clinical findings in a narrative review of Mediterranean style dietary components related to menopausal staging and women-specific cardiometabolic changes. It highlights specific diet-responsive pathways, including oxylipin formation, urolithin production, short-chain fatty acid production, and estrobolome, with emphasis on individual gut microbiome-driven variability.

Collectively, the studies in this dissertation demonstrate that Mediterranean dietary components, particularly walnuts, can benefit microvascular function and lipoprotein-associated inflammation, whereas polyphenol extracts show limited short-term vascular effects in low-risk groups. Mediterranean dietary patterns emerge as adaptable strategies for postmenopausal CVD prevention. Future work should prioritize menopause-staged trials with multi-omics to identify responders and refine precision nutrition strategies.

Chapter 1:
Literature Review

1. Cardiovascular Disease and the Endothelium

Cardiovascular disease (CVD) affects the blood vasculature and heart and includes conditions such as coronary heart disease (CHD or ischemic heart disease), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, and pulmonary embolism. Traditionally thought as a disease of affluence, CVD impacts individuals in both developed and developing countries, and is the leading cause of global mortality with an estimate of 19 million dying from CVD-related causes in 2020, representing more than 30 % of all deaths worldwide¹. In the United States (US), the death rate from CVD has decreased, yet both the health and financial burdens of CVD continue to grow¹.

The endothelium is the inner lining of blood vessels and is an active paracrine organ that regulates and maintains vascular tone through a variety of physical and chemical mediators. This dynamic balance is achieved with vasodilators such as nitric oxide (NO), prostacyclin, and endothelial-derived hyperpolarizing factor (EDHF) and vasoconstrictors such as endothelin-1, angiotensin-2, and thromboxane. Moreover, these endothelial-derived mediators regulate platelet reactivity, vascular smooth muscle cell proliferation, and inflammation. In healthy blood vessels, laminar blood flow produces shear stress that stimulates endothelial nitric oxide synthase (eNOS) to produce NO. Subsequently, NO diffuses to vascular smooth muscle cells and stimulates cyclic guanosine monophosphate (cGMP)-mediated smooth muscle relaxation in what is known as NO-dependent vasodilation. In addition, EDHF mediates vasodilation through a NO-independent pathway, primarily by activating small- and intermediate-conductance calcium-activated potassium channels. This activation induces potassium efflux, leading to hyperpolarization of endothelial and adjacent vascular smooth muscle cells, thereby promoting vasodilation²⁻⁴. This

NO-independent signaling pathway predominates in regions with reduced NO bioavailability and within resistance vessels of the microcirculation^{2,3}.

Cardiovascular risk factors can deleteriously alter the endothelium, resulting in non-laminar blood flow that decreases NO production. In addition to its role as a vasodilator, NO is also an anti-inflammatory mediator through its inhibition of the proinflammatory transcription factor nuclear factor (NF)- κ B pathway⁵. This pathway promotes gene expressions of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , Interleukin (IL)-1 and IL-6, chemokines such as monocyte chemoattractant protein (MCP)-1 and adhesion molecules such as intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin⁵. These cytokines and chemokines attract monocytes and T-cells and allow monocytes to travel across the endothelium and to mature into macrophages that ultimately become foam cells. Foam cells release additional inflammatory mediators and cytokines that promote fatty streak formation. Over time, the fatty streak evolves into a fibrous plaque that, upon rupture, results in cardiovascular events.

Platelets are small anucleate cells that primarily function to maintain homeostasis and facilitate healing. Platelets are also recognized as a key player in the inflammatory processes underlying CVD⁶. Under normal conditions, platelets circulate in a non-activated state, however, endothelial damage that exposes the subendothelial extracellular matrix will initiate platelet activation. Platelet adhesion occurs through the interaction between platelet receptors and collagen-bound von Willebrand factor (vWF) present in the subendothelial extracellular matrix⁷. Platelet adhesion amplifies platelet activation, leading to a series of biochemical changes that result in increased levels of inflammatory mediators and cytokines such as growth factor, IL-1 β and TNF- α ⁸. Activated platelets recruit and interact with monocytes via adhesion molecules

expressed on platelets, such as P-selectin and CD40 ligand, facilitating migration into the vascular wall and transformation into macrophages consuming oxidized low-density lipoprotein (ox-LDL), which accumulates in the intima. The uncontrolled uptake of oxLDL by macrophages leads to an overload of cholesterol within macrophages, transforming them into foam cells and ultimately plaque^{9,10}. Thus, platelet adhesion and activation, release of inflammatory mediators, recruitment of and interaction with monocytes and their conversion into foam cells and plaque are an early step in the development of CVD.

2. Hypercholesterolemia as a CVD risk factor

Hypercholesterolemia is a major risk factor for CVD and is a target in the prevention of CVD¹¹⁻¹³. In a recent systematic review including 14 independent reports, individuals with the highest levels of low-density lipoprotein cholesterol (LDL-C) have a 25 % increased risk in cardiovascular outcomes such as CVD death and sudden cardiac death (hazard ratio (HR) 1.25, 95% CI, 1.11-1/41) when compared to those with the lowest levels of LDL-C¹⁴. LDL-C is a major transporter of cholesterol and consists of a hydrophobic core of triglycerides and cholesterol esters in a hydrophilic surface membrane composed of phospholipids, unesterified cholesterol, and apolipoproteins¹⁵. Elevated levels of LDL-C infiltrate the intima and become trapped by proteoglycans within this layer. Within the intima, LDL-C undergoes oxidation, transforming it to ox-LDL. A number of adverse events are promoted by ox-LDL, including endothelium damage, inflammation, plaque formation, and ultimately the clinical event of plaque rupture¹⁶.

Familial hypercholesterolemia is a genetic disorder that causes an abnormal increase in plasma LDL-C levels; however, for many, lifestyle choices, including poor diet and low physical

activity, increase the risk of developing elevated LDL-C levels¹⁷. Dietary factors that increase LDL-C levels include high intakes of saturated fat, trans fat and added sugars¹⁸. Therefore, dietary modification remains the foundation for lowering LDL-C levels, with accumulating evidence strongly suggesting that healthy dietary patterns such as the Mediterranean (Med) diet and Dietary Approaches to Stop Hypertension (DASH) diet help reduce LDL-C levels^{19,20}. Common components of these healthy dietary patterns include the frequent intake of fruits, vegetables, whole grain, nuts, and beans, a moderate intake of fish and leaner cuts of meat, and limited consumption of refined grains and sweets^{18,21}.

3. Menopause as a CVD risk factor

A woman undergoes significant hormonal and metabolic changes during the menopausal transition (MT) that can influence her health outcomes, particularly concerning cardiovascular disease (CVD)²². During this time, ovarian function declines, leading to reduced production of estrogen and progesterone²³. These hormonal shifts can disrupt critical physiological balances such as lipid metabolism, glucose regulation, and body fat distribution. Moreover, as a woman reaches menopause, she can experience insulin resistance and dyslipidemia that heighten her risk for CVD and type 2 diabetes²⁴. The metabolic changes across the menopausal transition and with the onset of menopause underscore the necessity for tailored strategies, such as diet modification, to mitigate associated health risks²⁵.

The MT encompasses three phases: perimenopause, menopause, and postmenopause²³. Perimenopause begins several years before menopause and is characterized by irregular menstrual cycles and fluctuating estrogen levels. Menopause is defined retrospectively, occurring 12 months after a woman's final menstrual period, marking the cessation of ovarian function.

Postmenopause begins immediately after menopause and continues for the rest of a woman's life. During this phase, estrogen levels stabilize to very low levels, which can lead to long-term health implications, particularly concerning bone density, cardiovascular health, and metabolic function^{23,24,26}.

Cardiometabolic health and estrogen's protective role

Estrogen plays a critical role in cardiometabolic health, exerting protective effects on the lipid metabolism, glucose regulation, and body fat distribution^{27,28}. Its decline during the MT and postmenopause directly contribute to increased susceptibility to cardiometabolic disorders, including increased visceral fat dyslipidemia, and insulin resistance²⁹⁻³³. Notably, shifts in body composition can occur in women during midlife, characterized by increased fat mass and reduced lean body mass³⁴. The decline in estrogen levels is a critical factor driving these changes, as estrogen influences fat distribution and energy metabolism²⁸. Postmenopausal women often experience a redistribution of fat from peripheral to central regions, leading to increased visceral adiposity³⁵. This central fat accumulation is particularly concerning due to its strong association with metabolic disturbances and cardiovascular risk³⁶. The reduction in lean muscle mass during menopause contributes to a decline in basal metabolic rate, further predisposing women to weight gain³⁷.

Estrogen regulates hepatic lipid metabolism and lipoprotein function that helps maintain favorable lipid profiles by decreasing LDL-C and increasing high-density lipoprotein cholesterol (HDL-C)³³. With declining estrogen levels, a number of significant changes in lipid metabolism occur including an increase in LDL-C due to reduced hepatic LDL receptor activity and diminished clearance of LDL from circulation³⁸. Additionally, elevated triglyceride levels reflect

altered hepatic lipid synthesis and impaired lipoprotein metabolism³⁹. Although reported changes in HDL-C during the MT and postmenopause have been inconsistent, emerging evidence suggests a reduction in the quality and functionality of HDL particles⁴⁰. These changes collectively contribute to a pro-atherogenic lipid profile, increasing the risk of arterial plaque formation and subsequent cardiovascular events.

Estrogen enhances insulin sensitivity and regulates adipocyte function. The decline in estrogen levels impairs insulin sensitivity through several mechanisms. Estrogen plays a critical role in glucose homeostasis by enhancing insulin signaling pathways, promoting glucose uptake in skeletal muscles, and suppressing hepatic gluconeogenesis³⁰. Thus, reduced estrogen levels disrupt insulin signaling pathways, leading to impaired glucose metabolism and, consequently, increased insulin resistance, a key feature of type 2 diabetes and dyslipidemia⁴¹. Moreover, the increase in visceral adiposity that is commonly observed in menopause further worsens insulin resistance by releasing adipokines and pro-inflammatory cytokines, such as TNF- α and IL-6^{35,42,43}. These metabolic deficits can be further aggravated with a decline in physical activity which can also contribute to reduced glucose utilization by skeletal muscles⁴⁴.

Vascular function and blood pressure regulation

In addition to unfavorable shifts in metabolism, the MT and postmenopause significantly affect cardiovascular health due to the loss of estrogen's protective effects on the vasculature⁴⁵. During this period, women experience an accelerated increase in blood pressure, and vascular dysfunction that elevates CVD risk⁴⁶. Additionally, the pro-inflammatory state associated with menopause further exacerbates vascular damage⁴⁷. Estrogen helps maintain vascular homeostasis by promoting NO production, which aids in vasodilation, reduces inflammation, and prevents the

buildup of atherosclerotic plaques⁴⁸. Impairments in NO production that occur with reduced circulating estrogen levels leads to reduced vasodilation and increased vascular resistance⁴⁹. Arterial stiffness, characterized by the loss of arterial elasticity and compliance, becomes more pronounced after menopause⁵⁰. Arterial stiffness is attributed to structural changes within the arterial wall, including increased collagen, decreased elastin content, and increased intima-media thickness (IMT), and functional changes in smooth muscle cells⁵¹. Increased arterial stiffness further exacerbates increased systolic blood pressure by reducing the compliance of large arteries⁵¹. Moreover, reduced estrogen levels can disrupt the regulation of the renin-angiotensin-aldosterone system (RAAS), resulting in heightened vasoconstriction and fluid retention⁵². Taken together these changes in vascular homeostasis in the presence of declining estrogen levels can collectively contribute to an increased prevalence of hypertension in postmenopausal women. ⁵³.

In conclusion, MT and postmenopause are considered a period of significant cardiovascular risk for women. The dramatic decline in estrogen levels significantly affects cardiometabolic factors, including lipid and glucose metabolism, insulin sensitivity, and body composition. Concurrently, the loss of estrogen's protective effects accelerates arterial stiffness and dysregulation of blood pressure. As a result, these changes likely make women more susceptible to cardiovascular events. Therefore, it is important to assess outcomes associated with cardiovascular health and educate women on preventive strategies such as dietary habits and lifestyle modifications.

4. Assessment of Vascular Health

Clinical meaningful endpoints such cardiac death, stroke, and ischemia are the gold standard in determining the impact of treatments or dietary change on cardiovascular health in the population, however, many of these clinical endpoints need study designs that allow for the capture of data over a number of years within large population groups⁵⁴. Indeed large, long-term interventional studies, such as the Prevención con Dieta Mediterránea (PREDIMED) and Cocoa Supplement and Multivitamin Outcomes Study (COSMOS), used direct measures of cardiac events to test the influence of diet and dietary components towards the primary prevention of CVD ^{54,55}. Alternately, indirect measures such as biomarkers or surrogate outcomes that include measures of physiological function and biochemical analyses are often employed to predict clinical endpoints. These indirect measures are widely used in dietary interventions as they reduce size, duration and study costs, while allowing for the accumulation of an evidentiary base for the conduct trials using clinical outcomes ⁵⁶. However, it is important to consider whether selected biomarkers or surrogates provide reliable and interpretable evidence on intervention benefits related to clinical endpoints⁵⁷.

Vascular Tone

The assessment of vascular tone has been well documented in its association with clinical outcomes and can be measured in a relatively short period of time. Given that endothelial dysfunction is an early physiological feature in the development of CVD, assessment of vascular endothelial function may serve as a useful biomarker to evaluate cardiovascular health^{58,59}. Since the discovery of NO by Furchgott and Zawadski, an improved understanding of vascular biology has emerged that has led to the development of tests to evaluate vascular function⁶⁰. Non-invasive methods to assess vascular function in the peripheral circulation have been shown

associated with coronary endothelial function, cardiovascular risk factors and cardiovascular events^{61,62}. Therefore, endothelial function has been generally accepted as an early indicator of the development of CVD^{58-60,62}. Moreover, changes in these vascular measures can also be influenced by diet. For example, changes in flow mediated dilation (FMD), a physiological outcome to assess endothelial function, have been consistently shown to improve two hours after intake of a cocoa product rich in flavanols^{61,63}. Thus, the assessment of vascular tone regulated by the endothelium can be reliable to interpret potential dietary effects on cardiovascular health.

An ultrasound-based test, FMD measures changes in brachial artery diameter in response to reactive hyperemia following a brief period of ischemia (about 5 minutes) by inflating a blood pressure cuff on the forearm. The increase in blood flow following cuff release leads to shear stress that stimulates the endothelium to produce and secrete and vessel dilation⁵⁹. Endothelial function assessed by FMD correlates with coronary endothelial function, with several studies reporting an association between FMD and cardiovascular risk⁶⁴⁻⁷⁰. Impaired FMD has been observed in individuals with hypertension, dyslipidemia, diabetes mellitus and metabolic syndrome, those who smoke, have obesity, and older adults⁷¹⁻⁷⁷. Meta-analyses have also shown that an increase in FMD is significantly correlated to a lower risk of CV events⁷⁸⁻⁸¹. Moreover, improvements in FMD response have been reported in dietary intervention trials for selected foods or nutrients^{63,82,83}. Despite its popularity and advantages as a non-invasive method its use is limited to labs that specialize in its use as extensive training extensive training using standardized procedures are critical for the collection of quality data⁸⁴.

Peripheral arterial tonometry (PAT) is another technique for the assessment of vascular function. Similar to FMD, PAT uses reactive hyperemia to induce shear stress. Peripheral arterial tonometry measures the change in arterial pulsatile volume using fingertip probes. Simultaneous

recordings are made in both right and left index fingers with the contralateral arm used as an internal control. The ratio between hyperemic and baseline pulse wave amplitude is calculated and normalized for the same ratio in the contralateral arm to obtain the reactive hyperemia index (RHI). An alternative reactive hyperemia index, the Framingham RHI (fRHI), uses the natural logarithmic transformation of the RHI ratio⁸⁵. RHI and fRHI strongly correlate to one another, and an inverse relation of fRHI to CVD risk factors was reported^{85,86}. In a recent large cohort study, Rajai et al. demonstrated optimal RHI cut-off values for predicting cardiovascular events as 1.83 for the overall population, 1.61 for females, and 1.80 for males⁸⁷. In addition, adding peripheral microvascular dysfunction status based on the cut-off values to traditional assessment models, including Framingham risk score and CHA2-DS2-Vasc score, can provide additional prognostic value⁸⁷.

A measure of arterial stiffness, the augmentation index (AIx), is calculated via the pulse waveform obtained as the ratio of the pulse pressure at the second systolic peak to that at the first systolic peak⁸⁸. Several studies have demonstrated that arterial stiffness is independently predictive of cardiovascular morbidity and mortality⁸⁹⁻⁹¹. While carotid-femoral pulse wave velocity (PWV) measured by applanation tonometry has been a traditional standard for assessing arterial stiffness, the AtCor Medical SphygmoCor system offers a non-invasive alternative^{92,93}. The AtCor measures the brachial pulse waveform by using a cuff, employing a generalized transfer function to derive central aortic pressure and AIx. Since AIx is inversely related to heart rate, the AIx values are adjusted at a standard heart rate of 75 beats per minutes (AI@75)⁹⁴. Studies show that PAT-derived AIx is associated with ventricular-vascular coupling and correlated with cardiovascular risk factors, the presence of coronary artery disease, and AIx measured by other conventional devices⁹⁵.

Although FMD and PAT utilize the principle of reactive hyperemia to assess vascular tone, FMD and PAT may not always correlate and can reflect different aspects of vascular function^{96,97}, as they can have distinct physiological mechanisms influencing either technique⁹⁸. In general, FMD primarily assesses NO-mediated dilation in conduit arteries, while PAT reflects a combination of NO and EDHF effects in resistance vessels of the microcirculation^{99,100}. EDHF-mediated vasodilation is particularly important in microcirculation, where resistance arteries (e.g., arterioles and capillaries) play a critical role in regulating blood flow at the tissue level¹⁰¹. Therefore PAT, which assesses vascular tone in these small vessels, is influenced by both NO and EDHF-dependent mechanisms⁹⁹. EDHFs include epoxyeicosatrienoic acids (EETs), potassium ions (K⁺), and the electrical coupling between endothelial and smooth muscle cells through gap junctions¹⁰². Indeed, we have demonstrated that omega-3 fatty acids-rich foods like walnuts modulate EDHF pathways, leading to improvement in RHI¹⁰³.

This may explain studies that report that FMD and PAT are not significantly correlated, and that these two techniques might not be substituted for each other due to the different vascular beds evaluated^{104,105}. However, other studies have demonstrated a modest but significant correlation between FMD and PAT when assessing endothelial responses driven by NO^{106,107}. These results suggest that dietary interventions can influence endothelial response in both macro- and microvascular function in a similar direction¹⁰⁸. For example, Schroeter et al. investigated the acute effects of consuming flavanol-rich cocoa on vascular function in healthy male adults using both FMD and PAT, suggesting that acute consumption of a flavanol-rich cocoa beverage can improve both conduit and resistance artery endothelial function in humans by increasing NO bioavailability⁶³.

Given the above, changes in FMD and PAT in response to a dietary intervention may not always occur simultaneously. However, a change in one measure without a corresponding change in the other does not necessarily imply a lack of effect. Instead, it can highlight the specific vascular bed or underlying mechanism targeted by the intervention. A dietary component enhancing NO bioavailability might show a greater effect on FMD, whereas an intervention affecting EDHF pathways might predominately change PAT responses. Thus, assessment using both FMD and PAT can offer a more comprehensive understanding of how diet influences vascular health across different vascular systems.

5. Mediterranean diet and Cardiovascular Health

With advances in nutrition science, dietary recommendations have shifted from specific or isolated nutrients to overall dietary patterns. This is due to data from well-designed, large prospective cohorts and randomized clinical studies that have provided robust evidence that a food-based and dietary pattern approach reduces the risk for the development of cardiometabolic disease¹⁰⁹. Moreover, there is an increasing body of literature that lends support to the potential health benefits of specific dietary patterns, such as the Med diet. Adherence to a Med diet not only includes a healthy dietary pattern but also other known cardioprotective lifestyle factors such as moderate physical activity¹¹⁰. Key components of the Med diet include a high intake of plant foods such as fruits, vegetables, legumes, unrefined cereals, whole grains, nuts and seeds, virgin olive oil as a main source of fat, a frequent intake of fish and seafood, moderate consumption of alcohol, particularly red wine, moderate intake of eggs, poultry and dairy products (mostly cheese and yogurt), and low consumption of red meat¹¹¹. This dietary pattern

for the prevention of CVD has been emphasized in guidelines such as the 2020 Dietary Guidelines for Americans and the European Society of Cardiology^{112,113}.

Foods within a Med diet pattern provide a diverse array of nutrients and bioactive compounds that have consistently demonstrated cardioprotective effects with increasing intake in a number of epidemiological studies, including the landmark Seven Countries Study, European Prospective Investigation into Cancer and Nutrition (EPIC) study, the Nurses' Health Study (NHS) and Health Professionals Follow-up Study (HPFS)¹¹⁴⁻¹¹⁹. Examples include olive oil, a fat source of the Med diet, which has been associated with improvement in lipid profiles and endothelial function, while abundant intake of fruits, vegetable, nuts and legumes provide antioxidants and anti-inflammatory phytochemicals attenuate oxidative stress and inflammation. Moreover, intake of whole grains promotes glycemic control. The diverse array of foods in the Med diet works synergistically to promote metabolic regulation and cardiovascular health. Hence, by prioritizing this dietary pattern, various risk factors for CVD can be managed simultaneously; sustainable eating habits can be encouraged; and ultimately, cardiovascular health can be improved over the long term.

Epidemiological studies and clinical trials have widely adopted various Meddiet adherence scores to evaluate the association between diet adherence and cardiovascular outcomes (Table 1.1). The Mediterranean Diet Score (MDS), initially developed by Trichopoulou et al. and first utilized in the EPIC-Greece cohort, provided the definition of the Mediterranean diet^{120,121}. Although subsequent modified versions of this original scoring approach have emerged, each one has significantly contributed to the understanding of how dietary adherence influences cardiovascular health. Despite similarities in their underlying dietary principles, differences in scoring methods and included dietary components enhance the precision and

applicability of these tools across diverse populations. Consistently, greater adherence to the Med diet, as measured by these various scoring methods, has been associated with a decreased risk of cardiovascular disease across geographical regions and study designs^{114,115,120-123}.

Table 1.1. Comparison of Mediterranean diet adherence scores

Score Name	Components Included	Scoring Method	Score Range	First Use	Study Population
Trichopoulou Mediterranean Diet Score (MDS) ^{120,121}	Higher intake beneficial: vegetables (excluding potatoes), legumes, fruits & nuts, cereals, fish, MUFA:SFA. Lower intake beneficial: meat/meat products and dairy. Moderate intake beneficial: Alcohol (sex-specific ranges).	Binary (0 or 1) per component based on sex-specific median cutoffs. Points given for intake at/above median for beneficial components (1 point), below median for unhealthy components (0 point), and within range for alcohol.	0–9	EPIC-Greece	Greek adults
Alternate Mediterranean Diet Score (aMED) ¹¹⁵	Higher intake beneficial: vegetables (excluding potatoes), fruits, nuts, whole grains (excluding refined), legumes, fish, MUFA:SFA ratio. Lower intake beneficial: red/processed meat. Moderate intake beneficial: alcohol (sex-	Binary (0 or 1) per component based on sex-specific median cutoff. Points given for intake at/above median beneficial components, below median for red/processed meat, and within range for alcohol.	0–9	Nurses' Health Study	U.S. cohorts

	specific ranges). Dairy excluded				
Panagiotakos MedDietScore ¹ ₂₃	Higher intake beneficial: non-refined cereals, fruits, vegetables, potatoes, legumes, olive oil, fish. Lower intake beneficial: red meat, poultry, full-fat dairy products. Moderate intake beneficial: alcohol (<300 mL/day)	Frequency-based scoring (0–5) per component based on consumption (e.g. servings/week). Reversed scoring for unhealthy foods. Higher frequency gets more points for beneficial items; lower frequency gets more points for unhealthy items. Moderate alcohol gets 5 points.	0–55	ATTIC A study	Greek adults
PREDIMED Med Diet Screener (MEDAS) ¹²²	14 specific items assessing frequency/use of: olive oil (main fat, ≥ 4 tbsp/d), vegetables (≥ 2 /d), fruits (≥ 3 /d), red/processed meat (<1/d), butter/margarine/cream (<1/d), sweetened/carbonated beverages (<1/d), wine (≥ 7 glasses/wk), legumes (≥ 3 /wk), fish/seafood (≥ 3 /wk), commercial pastries/sweets (<2/wk), nuts (≥ 3 /wk), white meat preference (≥ 2 /wk).	Binary yes/no per item. One point for meeting criteria, 0 for not meeting per item based on consumption frequency.	0–14	PREDIMED trial	Spanish adults
Relative Mediterranean	Higher intake beneficial:	Scored 0, 1 or 2 per component based	0–18	EPIC cohorts	European populations

Diet Score (rMED) ¹¹⁴	vegetables (excluding potatoes), fruits, nuts, whole grains (excluding refined), legumes, fish, MUFA:SFA ratio. Lower intake beneficial: red/processed meat. Moderate intake beneficial: alcohol (sex-specific ranges). Dairy excluded	on tertiles of intake. Higher tertiles gets more points for healthy components; lower tertiles get more points for unhealthy components. Moderate alcohol gets 2 points.			
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Epidemiological studies on the influence of the Mediterranean Diet on CVD

Epidemiological evidence consistently shows a strong association between adherence to a Med diet and a reduced risk of CVD and mortality. This association was initially highlighted by the landmark Seven Countries Study, which observed lower CVD mortality in Southern Europe compared to Northern Europe or the US, and has been extensively supported by numerous large-scale prospective cohort studies and meta-analyses^{114,115,117,118,120,124-127}. Studies across Europe consistently report significant cardiovascular benefits with greater Med diet adherence. The EPIC study, for example, found a 33% (HR 0.67, 95% CI 0.47, 0.94) lower risk of CHD mortality for every two-point increase in a Med diet score, with similar protective associations observed among elderly participants and those with existing CHD^{120,126}. Likewise, the Spanish EPIC cohort reported a 6% decrease in CHD risk per unit increase in a related score¹¹⁴. Going beyond baseline adherence, the Greek ATTICA study not only showed a dramatically lower risk of CVD events over 10 years for those with the highest adherence but also demonstrated that individuals improving their adherence during follow-up experienced a fourfold risk reduction

compared to those maintaining low adherence¹²⁶. While a limitation in some analyses is to rely on dietary data at baseline only, the overall consistency observed across these European cohorts, despite using varied scoring methodologies, suggests a protective effect of the Med diet^{120,124,125}.

Similar protective associations have been observed in large US cohorts, bolstering the generalizability of these findings beyond Mediterranean populations. The NHS found that women with the highest adherence to an adapted Mediterranean diet score (aMED) had a 29% (relative ratio (RR) 0.71, 95% CI 0.62, 0.82) and 13 % of stroke (RR 0.87, 95% CI 0.73, 1.02) lower risk of CHD and a 13% (RR 0.87, 95% CI 0.73, 1.02) lower risk of stroke compared to those with the lowest adherence after multivariate adjustments for confounding factors¹¹⁵. Extending this to include male participants from the HPFS, showed that improvements in diet quality towards a Med diet pattern over a 4-year period were associated with a lower short-term CVD risk. Individuals who significantly improved their diet quality showed a 7% reduction in CVD risk (HR 0.93, 95% CI 0.85, 1.02) over four years, compared to those whose diet quality remained stable at baseline.¹¹⁸ Furthermore, achieving higher adherence scores was linked to significantly reduced CVD risk by 9 % (HR 0.91, 95% CI 0.86, 0.97) over the subsequent two decades, suggesting both short- and long-term benefits from adopting and maintaining the Med diet pattern¹¹⁸. A cross-sectional study within the NHS explored the relationship between the aMED and cardiometabolic biomarkers. Participants with the highest aMED scores exhibited 16% lower triglycerides. Although adjusting for body mass index (BMI) slightly weakened this association, it remained statistically significant¹²⁴.

It is important to note that studies employ various scoring tools (e.g., MDS, aMED, PREDIMED screener) to assess Med diet adherence, often adapted for specific populations (e.g., excluding potatoes and dairy in the US-based aMED)^{115,121}. An individual's score was based on

the quality of the dietary pattern and its components (Table1). Despite these variations in dietary components and scoring methods, the overall conclusion remains consistent: higher scores, representing greater adherence to the core principles of the Med diet, are associated with better cardiovascular outcomes.

Multiple meta-analyses further confirm the cardioprotective effects of the Med diet by pooling data from numerous observational studies and clinical trials. One analysis reported a 10% (RR = 0.90, 95% CI 0.87, 0.92) reduction in CVD risk for every 2-point increase in adherence score¹²⁸. In this analysis, the authors formulated a new Med diet adherence score for literature-based research and calculated it based on mean or median food group intake reported. Thus, the adherence scores were adjusted for variations in sample size and mean or median food group intake¹²⁸. Another comprehensive meta-analysis, encompassing nearly 900,000 participants, confirmed a significant inverse relationship between the Med diet adherence and CVD incidence, specifically highlighting the consumption of olive oil, vegetables, fruits, and legumes as key contributing components associated with reduced CVD risk (RR 0.83, 95% CI 0.77, 0.89 for olive oil; 0.87, 95% CI 0.77, 0.89 for vegetables; 0.88 95% CI 0.81, 0.96 for fruits; and 0.91 95% CI 0.83, 0.98 for legumes). This analysis also noted significant risk reductions by up to 40% for Myocardial Infarction (MI) and stroke from randomized controlled trials (RCTs) in high-risk patients adhering to the Med diet (RR 0.60, 95% CI 0.44, 0.82 for MI; and 0.64, 95% CI 0.47, 0.86 for stroke ¹²⁹. In another meta-analysis, based on 11 studies, the pooled RR comparing the highest to the lowest score of the Med diet was 0.81 (95% CI 0.74, 0.88) for unspecified CVD and 0.70 (95% CI 0.62, 0.80) for the CHD/AMI risk¹³⁰. It is noted that this inverse relationship was consistent across strata of study design, end point, sex, geographic area and the Med diet score used. While the association was significant for overall stroke risk (RR

0.73, 95% CI 0.59, 0.91), this analysis did not find a significant link specifically for ischemic or hemorrhagic stroke subtypes when analyzed separately¹³⁰.

Collectively, the body of epidemiological evidence from cohorts and meta-analyses confirms the role of the Med diet in primary and secondary prevention of CVD. The consistent findings highlight the benefits of the Mediterranean dietary pattern for cardiovascular health.

Randomized Clinical Trials

Beyond observational data, several RCTs support evidence on the effects of adopting a Med diet or Med-style diet on cardiovascular outcomes, particularly in populations with existing CVD (secondary prevention) or high risk (primary prevention).

Two notable secondary prevention trials focused on the effect of the Med diet high in alpha-linolenic acid (ALA) in patients with MI. The pioneering Lyon Diet Heart Study demonstrated a remarkable risk reduction after 27 months¹³¹. The intervention group was provided dietary guidance by the research cardiologist and dietitian, whereas the control group received no dietary advice. In an intermediate analysis, a protective effect was shown after 27 months of follow-up. The intervention group experienced a 73 % (RR 0.27, 95% CI 0.12, 0.59) lower risk of the combined primary endpoints (cardiovascular death and non-fatal MI) compared to the control group. The adjusted mortality risk was also reduced by 70 % (RR 0.30, 95% CI 0.11, 0.82) in the intervention group¹³¹. These protective effects were consistently supported by showing significant reductions across composite cardiovascular outcomes¹³². To examine the relationship with traditional risk factors, RR were calculated based on three defined composite outcomes: composite outcome (CO)1 (cardiac death and nonfatal MI), CO2 (CO1 plus major secondary endpoints), and CO3 (CO2 plus minor events requiring hospitalization). Compared

with the control, the intervention group showed a significant reduction of 72 % for CO1 (RR 0.28, 95% CI 0.15, 0.53), 67 % for CO2 (RR 0.33, 95% CI 0.21, 0.52), and 47 % for CO3 (RR 0.53, 95% CI 0.38, 0.74). While this trial showed substantial benefits, the results must be interpreted cautiously. As participants were concurrently using cardiovascular medications, including anticoagulants, antiplatelet agents, beta-blockers, calcium channel blockers, ACE inhibitors, and lipid-lowering drugs, the reduction of up to 72% could not be solely attributed to the Med diet¹³². Similarly, the Indo-Mediterranean Diet Heart Study, conducted in an Indian population, was a randomized, single-blind trial with a two-year follow-up involving 1,000 individuals with CAD risk factors, angina pectoris, and/or MI¹³³. Compared to a control group following an NCEP Step 1 Prudent diet, participants assigned to an ALA-rich Mediterranean-style diet demonstrated significantly reduced adjusted risks for nonfatal MI (RR 0.47, 95% CI 0.28-0.79), sudden cardiac death (RR 0.33, 95% CI 0.13-0.86), and total cardiac endpoints (RR 0.48, 95% CI 0.33-0.71), along with lower mean serum cholesterol levels (5.04 vs. 5.59 mmol/l). The results from this study may have limited generalization to women, as the majority of participants were male and relied on self-reported dietary intake¹³³.

The most recent and largest dietary intervention study, the PREDIMED, tested the effects of a Med diet on CVD prevention among individuals at increased risk for cardiometabolic disease⁵⁴. Participants were randomly allocated to one of three diets: Med diet supplemented with extra-virgin olive oil (EVOO), Med diet supplemented with mixed nuts or a control diet (low-fat diet). The results were significant: compared to the control, the Med diet with EVOO reduced major cardiovascular events by 31% (HR 0.69, 95% CI 0.53, 0.91), and the Med diet with nuts reduced events by 28% (HR 0.72, 95% CI 0.54, 0.95). These findings remained consistent even after reanalysis to address randomization issues⁵⁴.

Numerous sub-studies conducted within the PREDIMED study explored potential mechanisms to explain the Med diet's benefits. Compared to the control group (low-fat diet), both Med diet interventions (EVOO and nuts) led to favorable changes in blood pressure, insulin resistance, and lipid profiles. Furthermore, significant anti-inflammatory effects were observed, with reductions in key circulating and cellular biomarkers including C-reactive protein (CRP), IL-6, ICAM-1, and VCAM-1 within three months^{134,135}. The anti-inflammatory effects of the diet were further supported by other sub-studies¹³⁶. Urpi-Sarda and colleagues observed that participants consuming the Med diet supplemented with either EVOO or nuts experienced significant reductions in circulating inflammatory biomarkers, including ICAM-1, IL-6, and 2 TNF receptors (TNFR60 and TNFR80), after one year compared to the low-fat control group¹³⁷. Moreover, a reduction in inflammatory biomarkers like VCAM-1, ICAM-1, IL-6, TNF- α , and monocyte chemotactic protein 1 (MCP-1) corresponded with increased total urinary polyphenol excretion (TPE), a marker of intake from the Med diet interventions (EVOO and nuts). Interestingly, when participants were stratified based on the change in TPE levels over one year, those in the highest tertile of increase showed the most pronounced reduction in VCAM-1, ICAM-1, IL-6, and TNF- α compared to those in the lowest tertile¹³⁸. An additional pilot study examined gene expression involved in inflammation and initiation of atherosclerosis in a small subset of participants. Compared to baseline, after 3 years both intervention groups (Med diet supplement with either EVOO or nuts) showed significantly reduced plasma levels of pro-inflammatory biomarkers. Some of the decreased levels of inflammatory biomarkers, including IL-1 β , IL-6, IL-8, and TNF- α , were significantly different from the control group (low-fat diet). No significant changes were observed at the gene level after both Med diet interventions¹³⁹.

Another sub-study conducted by Martinez-Gonzalez et al. analyzed data from the PREDIMED study to investigate the impact of the Med diet interventions on the incidence of new-onset arterial fibrillation (AF)¹⁴⁰. The analysis included 6705 participants who were free of AF at randomization and followed them for a median of 4.7 years. Those in the Med diet group supplemented with EVOO showed a 38% risk reduction in incident AF compared to the control group, whereas the group assigned to the Med diet supplemented with nuts did not show a significant difference in AF incidence compared to the control group¹⁴⁰. Another study with a subset of 164 PREDIMED participants aimed to determine whether the Med diet interventions could beneficially affect carotid IMT, a well-established structural marker of cardiovascular events¹⁴¹. In addition to IMT, the study assessed plaque height in the internal carotid artery, carotid bifurcation, and common carotid artery at baseline and after a mean follow-up of 2.4 years. The finding was that consumption of a Med diet supplemented with nuts was significantly associated with favorable changes in carotid artery structure compared to the control group. The beneficial changes in the group of the Med diet supplemented with EVOO were not observed¹⁴¹. These two sub-studies showed contrasting findings, suggesting that while both EVOO and nuts contribute to the overall cardiovascular benefits of the Med diet, beneficial components in EVOO and nuts may act through different pathways of cardiovascular health, such as arrhythmia risk versus structural atherosclerosis progression.

The results suggest that the benefits related to dietary components, such as EVOO and nuts, include not only improvement in traditional CVD risk factors but also the modulation of inflammatory responses. Therefore, the combination of a variety of dietary components may enhance overall cardiovascular health, as different components may affect heart health in distinct pathways, yet contribute to cardiovascular protection. In conclusion, numerous RCTs,

particularly the large-scale PREDEMED study and its sub-studies, provide evidence strongly supporting epidemiological findings on the Med diet's benefits for both primary and secondary CVD prevention.

6. Polyphenols, fatty acids and the Mediterranean diet

Polyphenols and the Med Diet

A major characteristic of the Med diet is high consumption of a variety array of plant-based food items rich in phytonutrients, such as phenolics and polyphenols. Dietary intakes high in polyphenols have been suggested to have beneficial effects on cardiometabolic health¹⁴². While numerous chemical libraries and databases contain information on a diverse range of dietary phytochemicals, their properties and bioavailability vary¹⁴³. Dietary polyphenols are categorized into five main groups based on their chemical structure: phenolic acids, flavonoids (e.g., anthocyanins, flavones, flavonols, flavanones, and flavan-3-ols), stilbenes, lignans, and tannins (Table 1.2)¹⁴⁴. Their chemical structure is characterized by the presence of one or more phenol structural units consisting of an aromatic ring with one or more hydroxyl groups attached¹⁴⁵.

Table 1.2. Classification of polyphenols and food sources^{146,147}

Polyphenols	Subgroup of Polyphenols	Examples of Compounds	Food Sources
Flavonoids	Flavonols	Quercetin, Kaempferol, Myricetin, Isorhamnetin	Onions, apples, berries, kale, broccoli, tea, red wine, grapes, citrus fruits
	Flavones	Luteolin, Apigenin	Celery, parsley, thyme, chili peppers, chamomile, peppermint

	Flavanones	Hesperidin, Naringenin, Eriocitrin	Citrus fruits (oranges, lemons, grapefruit), tomatoes
	Flavanols (Flavan-3-ols)	Catechins (epicatechin, epigallocatechin gallate), Proanthocyanidin s (condensed tannins)	Green tea, cocoa, apples, grapes, berries, red wine, chocolate
	Anthocyanins	Cyanidin, Delphinidin, Malvidin, Pelargonidin	Berries (blueberries, strawberries, raspberries), red grapes, cherries, plums, red cabbage, blackcurrants
	Isoflavones	Genistein, Daidzein	Soybeans and soy products (tofu, tempeh, soy milk), legumes
Phenolic Acids	Hydroxybenzoic acids	Gallic acid, Protocatechuic acid, p- Hydroxybenzoic acid	Tea, berries, grapes, red wine, coffee, plums, cherries
	Hydroxycinnamic acids	Caffeic acid, Ferulic acid, Coumaric acid, Chlorogenic acid	Coffee, whole grains (oats, wheat, rice), apples, pears, blueberries, carrots, potatoes
Stilbenes		Resveratrol	Grapes (especially red grape skins), red wine, peanuts, berries
Lignans		Secoisolariciresinol, Matairesinol, Lariciresinol	Flaxseeds, sesame seeds, whole grains, berries, cruciferous vegetables
Tannins	Hydrolyzable tannins	Gallotannins, Ellagitannins (e.g., ellagic acid, punicalagin)	Pomegranates, walnuts, pecans, berries (raspberries, blackberries), oak-aged wines
	Condensed tannins (Proanthocyanidins)	Procyanidins	Cocoa, apples, grapes, berries (cranberries, blueberries), cinnamon, tea, red wine

Dietary polyphenols, secondary metabolites abundantly found in vegetables, fruits, cereals, tea, red wine, and their derived foods and beverages¹⁴⁸. These compounds exhibit a

spectrum of bioactivities that can offer many health advantages to consumers, including but not limited to antioxidant, anti-inflammatory, anticancer, antimicrobial, and neuroprotective effects¹⁴⁹. As a plant-based diet, the Med diet can provide a wide array of polyphenols. In the forementioned PREDIMED trial, the authors used the Phenol-Explorer database (www.phenol-explorer.eu), a comprehensive database on 502 polyphenols contained in 452 foods^{150,151} to assess associations between the cardiovascular outcomes and polyphenol intake. For the 7200 participants completing a validated 1-year food frequency questionnaire (FFQ) at baseline, the mean total polyphenol intake was 820 ± 323 mg/day, of which 443 ± 218 mg were flavonoids, 304 ± 156 mg phenolic acids, and 73 mg belonged to other polyphenol groups¹⁵¹. Therefore, of the polyphenol classes, flavonoids were the greatest contributor to polyphenol intake, mainly provided by fruits, vegetables, and red wine.

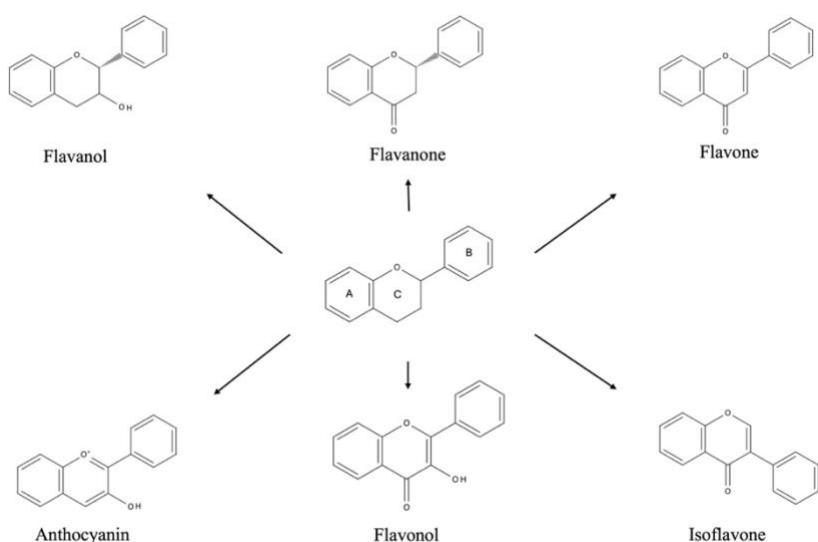


Figure 1.1. Basic structure of flavonoids.

Flavonoids are an integral component of the diet with their general structure consisting of a 15-carbon skeleton that includes two phenyl rings, the A- and B-ring, and a heterocyclic C-ring (Figure 1.1)¹⁵². Flavonoid subgroups include flavones (e.g. luteolin, apigenin), flavonols (e.g.

quercetin, kaemferol, myricetin), flavanones (e.g. naringenin, hesperidin), flavanols (e.g. catechin, epicatechin, epigallocatechin gallate (EGCG)), isoflavones (e.g. genistein, daidzein) and anthocyanins (e.g. cyanidin, delphinidin, malvidin, pelargonidin). Food sources rich in flavonoids are shown in Table 1.2.

Due to different chemical structures, it is important to note that the nature of each flavonoid (absorption, metabolism, excretion and bioavailability) can vary across the subclasses; therefore, they can have different biological activities¹⁵³. Ottaviani et al. demonstrated that flavanol isomers are variable in their absorption from the small intestine and are physiologically distinct¹⁵⁴. Any specific food may provide several flavonoid subclasses however, typically one subclass is predominant. For example, flavanols are mainly present in cocoa and tea whereas anthocyanidins can be found commonly in berries¹⁵⁵. In addition, the amount of flavonoids provided can be dependent on several factors such as food storage and processing, agricultural practices and cooking¹⁵³.

An essential step to clearly understanding the relation between flavonoid consumption and positive health outcomes is an accurate estimate of dietary flavonoid intake; however, an accurate assessment of flavonoid intake is a challenge. Common methods of estimating flavonoid intake at the population level mostly rely on dietary assessment using repeated 24-hour diet recalls, diet history interviews, or food frequency questionnaires. These are cross-referenced with food composition databases for flavonoids. However, large variations, such as a limited list of food items or any factors (e.g., harvesting and processing conditions) that can affect flavonoid contents in estimates of flavonoid intake, can occur with these databases. Thus, several strategies across studies should be considered. Collection of data on food consumption to evaluate the intake of flavonoids can be achieved through multiple 24-hour recalls and valid food frequency

questionnaires (FFQs) throughout the year or a period of the study^{156,157}. Establishing uniform protocols for the extraction and determination of flavonoids is necessary for accurate analysis as their metabolites and physiologically active forms in systemic circulation vary^{158,159}. In addition, the bioavailability of flavonoids after consumption is a critical issue, and biomarkers measured in blood and urine should be used to better understand flavonoids' impact on outcomes¹⁶⁰.

Biomarkers can particularly account for individual differences in bioavailability and metabolism and provide a more accurate reflection of the body's actual exposure to flavonoids. Biomarker analysis is commonly performed using urine and plasma samples¹⁶¹. Urine biomarkers provide insight into dietary intake over a short period of time, typically 24 hours¹⁶¹. In contrast, biomarkers found in plasma give information about the levels of flavonoids and their metabolites in the bloodstream, which helps understand potential exposure at the tissue level¹⁶². For example, biological samples (e.g., urine and plasma) of epicatechin and its metabolites have been identified as biomarkers of flavanol intake, particularly sources like cocoa and its products¹⁶³. A cross-sectional analysis in the EPIC Norfolk cohort examined the relationship between flavan-3-ol intake, estimated through biomarkers, and cardiovascular health. This study found that high flavan-3-ol intake was significantly associated with lower systolic and diastolic blood pressure, particularly in hypertensive individuals¹⁶⁴. Importantly, each compound of flavonoids is different in absorption, activity, metabolism, bioavailability, and excretion.

Bioavailability refers to the extent and rate at which a substance becomes available to its biological destination such as the systemic circulation or targeted organs/tissues. Flavonoids generally have low bioavailability due to limited absorption, extensive metabolism, and rapid excretion (Figure 1.2)¹⁶⁵. The metabolic fate of flavonoids varies depending on the different subclass, glycosylation, molecular weight, and esterification. Flavanol monomers are rapidly

absorbed in the small intestine and diffused into enterocytes, whereas glycosylated flavonoids (e.g., flavonol glycosides) are enzymatically hydrolyzed and absorbed in the small intestine; both are conjugated to their glucuronide, sulfate, or methylated forms by phase 2 enzymes in epithelial cells and the liver. In an enterocyte, aglycones are released from absorbed flavonoids by lactase phlorizin hydrolase (LPH) or beta-glucosidase and undergo formation of glucuronides, sulfates, and methylated conjugates¹⁶⁵. Then, they are transported to the liver, where they undergo either phase 1 or phase 2 metabolism prior to excretion into the bile and return to the intestine through enterohepatic circulation. Unabsorbed flavonoids pass intact to the colon, where they are degraded by colonial resident microbiota for further metabolism or excretion¹⁶⁶.

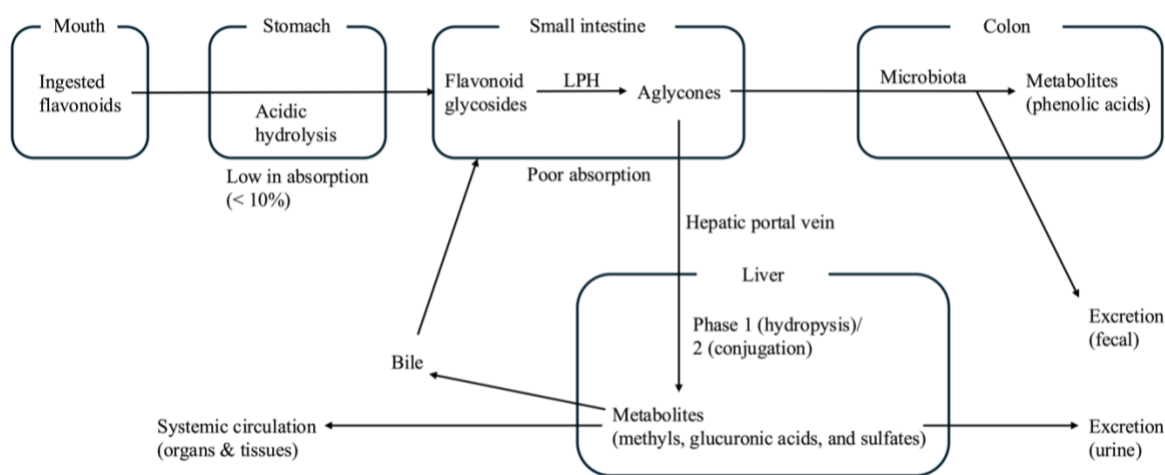


Figure 1.2. Metabolism of flavonoids in the intestine and liver.

Microbial metabolism of flavonoids in the colon produces smaller molecules, which can be absorbed and systemically distributed in the body. Moreover, large molecular weight flavonoid polymers such as proanthocyanidins which are poorly absorbed in the small intestine are degraded and metabolized by the colonic microbiota to bioactive forms such as phenolic

acids and valerolactones¹⁶⁷. Given that, the gut microbiota plays a critical role in flavonoid bioavailability.

The gut microbiota is an important part of the intestinal ecosystem and plays a significant role in human health. It helps protect the gut barrier, contributes to the development and maturation of the immune system, and regulates nutrient metabolism and absorption¹⁶⁸. Its effect extends beyond the gut, with cumulative evidence highlighting its significant role in cardiovascular health. The “diet-gut-heart axis” is an emerging concept emphasizing the interplay between dietary components, gut microbiota, and cardiovascular health. This is based on the understanding that diet not only provides essential nutrients but also influences the composition, diversity, and functionality of gut microbiota, which in turn modulates overall health¹⁶⁹. The gut microbiota acts as a mediator between dietary intake and cardiovascular outcomes by metabolizing bioactive compounds in food, such as polyphenols and fibers, into metabolites with physiological effects¹⁷⁰.

Key metabolites produced by gut microbiota, including short-chain fatty acids (SCFAs) and microbial derivatives of polyphenols, which have anti-inflammatory and cardio-protective effects¹⁷¹. SCFAs such as acetate, butyrate, and propionate modulate blood pressure, lipid metabolism, and endothelial activation^{172,173}. In contrast, the imbalance of microbial composition, known as dysbiosis, has been implicated in CVDs. This imbalance often involves a shift from a diverse and beneficial microbial community towards one that promotes the generation of harmful metabolites such as trimethylamine N-oxide (TMAO)¹⁷⁴. Studies have shown that plasma levels of TMAO are significantly associated with the risk of cardiovascular events^{171,175}.

The gut microbiota composition can influence systemic inflammation, with dysbiosis leading to gut barrier dysfunction, allowing the translocation of microbial components like lipopolysaccharides (LPS) into the circulation that triggers chronic low-grade inflammation¹⁷⁶. This systemic inflammatory state is closely linked to disrupted cardiometabolic outcomes such as high blood pressure and insulin resistance^{177,178}. Collectively, the gut microbiota serves as an important mediator in the diet-gut-heart axis.

As mentioned earlier, flavonoids can undergo extensive metabolism by gut microbiota in the colon rather than being directly absorbed in the upper gastrointestinal tract¹⁶⁵. Microbial enzymes catalyze the breakdown of flavonoids into smaller bioactive metabolites, such as phenolic acids, which can have enhanced bioavailability and biological activity compared to their parent compounds. Importantly, flavonoids influence the composition and functionality of gut microbiota¹⁷⁹. Several studies have demonstrated their prebiotic-like effects by promoting the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* while inhibiting the proliferation of pathogenic species¹⁷⁹⁻¹⁸¹. These changes in microbial diversity and abundance aid in restoring gut homeostasis, improving barrier function, and decreasing inflammatory signaling^{182,183}.

In summary, polyphenols, particularly flavonoids, are abundant bioactive compounds in the Med diet, primarily derived from plant foods. Flavonoids have been known for antioxidant, anti-inflammatory, and cardioprotective effects. However, their bioavailability and activity are likely affected by chemical structure, food processing, and metabolism. The gut microbiota plays an important role in metabolizing and transforming flavonoids and, therefore, enhances bioavailability of their metabolites. Furthermore, flavonoids can positively change the gut

microbiota composition. This provides a beneficial feedback loop along the diet-gut-heart axis, supporting consumption of flavonoids as a promising way for cardiovascular health.

Fatty Acids and Med Diet

The Med diet is characterized by a high intake of unsaturated fatty acids, especially those found in olive oils, nuts, seeds, and fish. This dietary pattern is consistently associated with improved cardiovascular health outcomes, reduced incidence of cardiovascular diseases (CVD), and overall mortality¹⁸⁴. Unsaturated fatty acids are primarily classified into two categories: monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs)¹⁸⁵. Unsaturated fatty acids are chemically characterized by the presence of one (monounsaturated) or multiple (polyunsaturated) carbon-carbon double bonds within their hydrocarbon chains. MUFAs contain a single double bond, while PUFAs contain two or more double bonds and are further categorized into omega-3 (ω -3) and omega-6 (ω -6) fatty acids based on the position of the first double bond from the methyl end of the fatty acid chain. Some PUFAs, such as ALA and LA, are classified as essential fatty acids because they cannot be synthesized endogenously by the human body and must be obtained through dietary intake¹⁸⁶.

Structural characteristics of unsaturated fatty acids have been linked to their potential health benefits¹⁸⁷. The presence of double bond(s) can create a bend or slight curve in the fatty acid. Unsaturated fatty acids have two main forms: cis and trans. Cis isomers have a more pronounced bend, while trans isomers are straight, like saturated fatty acids¹⁸⁶. The presence of cis double bonds helps maintain cell membrane integrity and function because they maintain membrane fluidity, which impacts membrane protein (e.g., receptors and enzymes) function and permeability, and thus cellular signaling and metabolic activities^{187,188}.

MUFAs are abundant in olive oil, avocados, nuts and seeds, whereas omega-3 fatty acids are found in fatty fish (e.g., salmon, sardines), flaxseeds, chia seeds, and walnuts. While olive oil, rich in MUFA oleic acid, has been discussed, other dietary sources of unsaturated fatty acids also contribute significantly to cardiovascular health. For example, walnuts are a rich source of omega-3 fatty acid ALA. Studies suggest that consuming walnuts can lead to improvement in cardiometabolic health. In addition to ALA, walnuts also contain oleic acid, LA, and various vitamins and minerals, further contributing to their nutritional profile. Furthermore, the Food Drug and Administration (FDA) and European Food Safety Authority (EFSA) recognize the health benefits of consuming oleic acid and omega-3 fatty acids (ALA, EPA and DHA) in relation to cardiovascular health¹⁸⁹⁻¹⁹².

Numerous epidemiological studies and clinical trials have linked high intake of unsaturated fatty acids to significant reductions in cardiovascular events, including myocardial infarction, stroke, and cardiovascular mortality^{193,194}. A great example is the PREDIMED trial, which demonstrated that increased consumption of unsaturated fats, particularly MUFAs from EVOO and omega-3 fatty acids from nuts, was associated with improvements in cardiovascular risk factors such as improvements in lipid profiles, reduced blood pressure, improved endothelial function, and decreased systemic inflammation⁵⁴.

In small intestine, ingested fats, mainly in the form of triglycerides, are broken down by bile and pancreatic lipases into monoglycerides and free fatty acids, which are then absorbed into intestinal cells¹⁹⁵. These are reassembled into triglycerides and packaged into chylomicrons, which transport lipids via the lymphatic system into the bloodstream¹⁹⁶. In circulation, lipoprotein lipase (LPL) hydrolyzes chylomicron triglycerides, releasing free fatty acids for cellular uptake. Then, free fatty acids taken up by cells are used for immediate energy or storage¹⁹⁷.

Beyond energy source, PUFAs, both omega-3 (ALA, EPA, and DHA) and omega-6 (LA and AA), are transformed into important signaling molecules known as oxylipins, a class of bioactive molecules that originate from the oxidation of PUFAs¹⁹⁸. Oxylipins are produced by specific enzymes, including cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome (CYP) P450 enzymes¹⁹⁹. Oxylipins include a variety of molecules, including prostaglandins, thromboxanes, leukotrienes, lipoxins, and resolvins, which act as local hormones²⁰⁰. These molecules play roles in regulating various physiological processes, including inflammation, endothelial function, and blood coagulation^{199,200}. PUFAs serve as a precursor for oxylipins that have been shown to have anti-inflammatory and other beneficial cardiovascular effects.

Particularly, ALA is the parent omega-3 PUFA which can be converted in the body, albeit inefficiently in humans, to longer-chain omega-3 PUFAs such as EPA and DHA. ALA is metabolized to hydroperoxy-, epoxy-, mono- and dihydroxylated oxylipins through COX, LOX, and CYP450²⁰¹. While many of well-studied anti-inflammatory and cardioprotective oxylipins are derived from EPA and EHA, which are synthesized from ALA or consumed directly, ALA itself and some of its oxylipins contribute to cardiovascular health by improving inflammation, and vascular and platelet function²⁰¹. Supplementation of flaxseed reduces pro-inflammatory dihydroxy-octadecenoic acids (DiHOMEs) levels, along with pro-inflammatory omega-6 oxylipins such as 5-HETE and trihydroxy-octadecenoic acids (TriHOMEs)²⁰². Walnuts are another significant source of ALA. In a four-week dietary intervention of 40 g of daily walnut intake confirm these results, ALA-derived oxylipins such as 9-hydroxy-octadecatrienoic acid (9-HOTrE), 13-HOTrE, and 12, 13-epoxy-octadecadienoic acid (12,13-EpODE) were increased, alongside reductions in pro-inflammatory oxylipins such as 5HETE and 19-HETE. Walnut consumption has also been linked to increased plasma levels of ALA epoxides (9(10)-, 12(13)-,

15(16)-EpODE) and DHA epoxide (16(17)-EpODE), which correlate with improved microvascular function¹⁰³. Studies showed a correlation between dietary PUFA intake and concentration of these fatty acids in tissues, directly impacting the availability of oxylipin precursors²⁰³. Given that, dietary unsaturated fatty acids profoundly influence the endogenous oxylipin profile within tissues^{103,204,205}.

7. Summary

Epidemiological studies and clinical trials have consistently support cardiovascular benefits of the Med diet rich in plant-based foods. Key components contributing to protective effects include fruits and vegetables rich in polyphenols, particularly flavanols, and olive oil and walnuts rich in both polyphenols and unsaturated MUFA and PUFA. Evidence suggests foods rich in flavanols and walnuts improve vascular function and anti-inflammation. Given that the Med diet's benefits from compounds like flavanols and PUFA from walnuts show promoting cardiovascular health, this dissertation will focus on the effects of flavanols and walnuts on cardiometabolic outcomes, particularly in postmenopausal women.

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Chapter 2:

**Effects of short-term walnut consumption on human microvascular function
and its relationship to plasma epoxide content (J Nutr Biochem. 2015 Dec;26(12):145)**

Effects of short-term walnut consumption on human microvascular function and its relationship to plasma epoxide content[☆]

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Abstract

Improved vascular function after the incorporation of walnuts into controlled or high-fat diets has been reported; however, the mechanism(s) underlying this effect of walnuts is(are) poorly defined. The objective of the current study was to evaluate the acute and short-term effects of walnut intake on changes in microvascular function and the relationship of these effects to plasma epoxides, the cytochrome-P450-derived metabolites of fatty acids. Thirty-eight hypercholesterolemic postmenopausal women were randomized to 4 weeks of 5 g or 40 g of daily walnut intake. All outcomes were measured after an overnight fast and 4 h after walnut intake. Microvascular function, assessed as the reactive hyperemia index (RHI), was the primary outcome measure, with serum lipids and plasma epoxides as secondary measures. Compared to 5 g of daily walnut intake, consuming 40 g/d of walnuts for 4 weeks increased the RHI and Framingham RHI. Total cholesterol and low- and high-density cholesterol did not significantly change after walnut intake. The change in RHI after 4 weeks of walnut intake was associated with the change in the sum of plasma epoxides ($r=0.65$, $P=.002$) but not with the change in the sum of plasma hydroxyeicosatetraenoic acids. Of the individual plasma epoxides, arachidonic-acid-derived 14(15)-epoxyeicosatrienoic acid was most strongly associated with the change in microvascular function ($r=0.72$, $P<.001$). These data support the concept that the intake of walnut-derived fatty acids can favorably affect plasma epoxide production, resulting in improved microvascular function.

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1. Introduction

Epidemiological evidence suggests that the intake of tree nuts can provide significant cardiovascular benefits [1,2]. Numerous investigators have reported that acute and short-term intake of walnuts can be associated with reductions in total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels, reductions in blood pressure and improved vascular function [3–8]. However, the mechanism(s) underlying the above physiological changes has(have) not been well

characterized. Measures of vascular function, such as flow-mediated dilation (FMD) and peripheral arterial tonometry (PAT), are used as noninvasive surrogate outcome measures, the responses to which can be associated with a number of cardiovascular risk factors [9]. Improvements in vascular function have been reported in several dietary interventional trials after walnut intake [1,4,6,7,10]. Notably, a variety of study designs have been used that include the assessment of vascular function after an overnight fast [7,10] or during the postprandial period with the walnuts incorporated or consumed with a high-fat meal [3,4,11]. The latter studies have explored the potential of walnuts to reduce lipidemia-induced vascular dysfunction, an effect of walnuts that may represent a distinct mechanism of action that is separate from the vascular effects induced during the fasted state [12].

Walnuts can be a substantial source of the essential fatty acids omega-3 alpha-linolenic acid (ALA) and omega-6 linoleic acid (LA) [1], as well as several other nutrients that have been postulated as being valuable for reducing the risk for cardiovascular disease (CVD) [1].

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Only a small number of studies have examined the relationships between specific components within walnuts and vascular function in human subjects [5,6]. An enhanced FMD response, which is a measure of macrovascular function, has been reported when walnuts, walnut oil and/or ALA-rich flaxseed oil were added to the diet for 6 weeks [5]. An acute (single-feeding) noted a greater microvascular response, as measured by PAT, after intake of walnut oil compared to walnut skin consumption [6]. Both studies suggest that components within the oil fraction of walnuts can influence the vascular response [5,6].

The endothelium plays a critical role in maintaining vascular homeostasis and produces a number of mediators involved in the regulation of vascular tone. These include vasodilators such as nitric oxide (NO), prostacyclin and endothelial-derived hyperpolarizing factor (EDHF), and vasoconstrictors such as endothelin-1. These mediators can have profound influences on platelet reactivity, inflammatory responses and vascular smooth muscle cell remodeling. Cardiovascular risk factors, particularly lifestyle choices such as diet, are thought to modulate the balance of these mediators. The mechanisms by which walnut intake influences vascular function have not been defined, but they may include lipoprotein-induced modulation of vascular hemostasis, particularly with regards to a large group of bioactive lipids collectively termed oxylipins [13]. Known bioactive oxylipins are the cyclooxygenase (COX)-, lipoxygenase (LOX)- and cytochrome P450 (CYP)-derived metabolites of arachidonic acid (AA), and include thromboxane and prostacyclin, known modulators of platelet and vascular reactivity, and epoxyeicosatrienoic acids (EpETEs or EETs), which have been proposed as putative EDHFs that regulate microvascular tone [13,14]. In addition to EpETE formation by CYP epoxygenase, CYP ω/ω -1 hydrolases produce hydroxyeicosatetraenoic acids (HETEs) that are considered vasoconstrictive and inflammatory [15]. Furthermore, soluble epoxide hydrolase (sEH) metabolizes EpETEs to form dihydroxyeicosatrienoic acids, which are generally thought to be less vasoactive. Dysregulation of CYP epoxygenases and hydrolases has been observed in diabetes [16], in hypertension [17,18] and in patients with stable CVD [19,20].

While epoxides derived from AA are the most extensively studied and are commonly inferred to be the principal bioactive agents, most studies have not been designed to address the potential bioactivity of other fatty-acid-derived epoxides. Considerable interest has been given to the potential discovery of sEH inhibitors that may potentiate the beneficial vascular effects of epoxides [21]. Recent studies have suggested that ALA-rich flaxseed may be of benefit to those with hypertension, as a decrease in sEH metabolites was associated with improved blood pressure after 6 months of flaxseed intake [18]. Given the above, the primary aim of the current study was to evaluate the acute and short-term effects of walnut intake on microvascular function in a population at increased cardiovascular risk; secondly, we sought to assess the effects of walnut intake on lipid profiles including an examination of relationships between microvascular function and plasma epoxides.

2. Methods

2.1. Participants

Thirty-eight postmenopausal women (50–70 years of age) were recruited from the greater Sacramento area via public advertisements. After providing written informed consent, potential participants were screened by a multiple-phase process that included a telephone interview, a lipid and metabolic panel, and PAT. Inclusion criteria were a body mass index (BMI) <35 kg/m², postmenopausal state (defined as the absence of a menstrual cycle for at least 1 year and a follicle-stimulating hormone level between 23 and 116.3 IU/L) and a fasting TC level $\geq$ 15.18 mmol/L. Exclusion criteria included allergies to walnuts or other nuts, use of prescription medications except for treating

hypothyroidism, use of dietary supplements except standard multivitamin/mineral formulas, cigarette smoking, and a history of CVD or other chronic diseases. This dietary intervention trial was registered (NCT01235390) at ClinicalTrials.gov, with the study protocol approved by the Institutional Review Board of the University of California, Davis.

2.2. Study design

Participants were randomized into a 4-week, parallel-arm dietary intervention trial by block design to consume 5 g or 40 g of walnuts daily for 4 weeks. Randomization was performed by the study coordinator following a predetermined plan formulated via a Web-based random number generator (www.randomization.com). One week prior to and during the intervention period, participants were instructed to refrain from consuming any type of nuts, except for the walnuts provided, as well as to refrain from the use of aspirin and other over-the-counter pain relievers. To assess the short-term (4 weeks) effects of walnut intake, assessments were made prior to the initiation of daily walnut intake and at the end of the 4-week intake period. For each assessment, participants arrived at the facility in the morning after an overnight fast. After a 30-min rest period, a PAT measurement was performed, followed by blood collection from the antecubital vein. After this initial set of baseline measurements, the acute (single intake) response of walnut consumption was also determined, with the subjects consuming their assigned amount of walnuts, followed by an additional PAT measurement and blood draw 4 h later.

Each participant received walnuts prepackaged into 5- or 40-g daily servings and were instructed to consume one serving daily for 4 weeks. The 40-g level of daily walnut intake was chosen based on the level of daily intake suggested in the 2004 Food and Drug Administration qualified health claim for walnuts and cardiovascular health [22], and also reflected the level and intake period (4 weeks) previously observed to improve postprandial vascular dysfunction induced by a high-fat meal [3]. The goal of the current study was to determine the vascular effects of walnuts when incorporated into the participant's habitual diet as a whole food. As this study design makes blinding difficult without significantly changing the form of the walnut (i.e., making it into a powder, baking, etc.), we chose to utilize a dose-response design, asking the subjects to consume either 40 or 5 g/d of walnuts as either a snack or incorporating the walnuts, without cooking them, into their meal.

Compliance was monitored by a self-administered log. Food intake was assessed from the compliance log as well as two 3-day food records that included two weekdays and one weekend day, consecutively, that were completed before each study day visit. Food records were analyzed using The Food Processor SQL (version 10.1.0; ESHA Research, Salem, OR, USA), which provides nutrient information for 26,000 food items compiled from the USDA nutrient database for standard reference, manufacturer and restaurant nutrition information, and other source information. Compared to values obtained from laboratory analysis, previous versions of this software have a reported accuracy within 5% of the measured macronutrient values to include the fatty acid classes [23]. In addition, nutrient estimates have strongly correlated to nutrient data from the First National Health and Nutrition Examination Survey [24].

2.3. Assessment of microvascular function and augmentation index

Microvascular function, assessed as reactive hyperemia index (RHI), was measured using PAT (EndoPAT 2000; Itamar Medical, Caesaria, Israel). Before each RHI measurement, participants relaxed in a supine position for at least 30 min in a quiet, temperature-controlled room, with speaking and movement kept to a minimum. A plethysmographic-like biosensor was placed on the middle finger of

both hands, and a blood pressure cuff was placed on the proximal forearm of the nondominant arm. The RHI testing consisted of a 5–10-min baseline collection period followed by 5 min during which the blood pressure cuff was continuously inflated to 60 mmHg above systolic blood pressure, after which time the pressure was released and the resulting reactive hyperemia response was collected for 3–5 min. The RHI was automatically calculated by the system software as the average ratio of pulse wave amplitude (PWA) during 1 min of reactive hyperemia to the average baseline PWA. A valid RHI test was set at a postocclusion signal of 70% or greater.

The augmentation index (AIx) was automatically calculated by the system software using a computerized algorithm to identify the systolic peak pressures created from the inflection point of forward systolic pressure and the reflected systolic peak, with the difference between the two expressed as a percentage of the pulse pressure. AIx@75 was obtained through normalization of AIx to a heart rate of 75 beats per minute. The software also calculated a Framingham RHI (FRHI), which is the log of RHI measured 90–120 s following cuff deflation; the FRHI has been reported to be negatively associated with a number of cardiovascular risk factors in the Framingham Third-Generation Cohort [25].

2.4. Assessment of lipoproteins, and plasma fatty acids and oxylipins

Plasma samples were analyzed at the Department of Pathology and Laboratory Medicine at the University of California, Davis Medical Center. In a subset of individuals (40-g/d group, $n=15$; 5-g/d, group $n=5$), plasma fatty acids and epoxides were measured. Esterified plasma particle lipids were quantified by mass spectrometry after total lipid extraction in the presence of fatty acid, cholesteryl ester, triglyceride and phospholipid surrogates. Esterified fatty acids were transformed to fatty acid methyl esters and quantified by gas chromatography–mass spectrometry [26]. Free plasma oxylipins [27] were quantified by ultraperformance tandem mass spectrometry after solid phase extraction. Briefly, whole plasma (150 μ l) was enriched with deuterated oxylipin free acid surrogates in 5 μ l methanol (see Supplement Table 1 for compound list). Plasma free oxylipins were isolated on 10-mg Oasis HLB SPE columns, and analytes were separated on an Acquity BEH column (Waters, Milford, MA, USA; see Supplement Table 2). Oxylipins were detected on an API 4000 QTrap (AB Sciex, Framingham, MA, USA). Acquisition parameters and estimated detection limits are presented in Supplement Table 3, and the criteria for reporting were as follows: data >3:1 signal to noise; method blanks contribution <25% of signal; values within calibrated range.

2.5. Statistical analysis

Data were checked for normality and homogeneity of variance, and log transformed as necessary, with potential outliers assessed using z-scores. Potential between group differences at baseline was assessed by two-tailed *t* tests. Short-term (4-week) microvascular function data after an overnight fast were analyzed with analysis of covariance (ANCOVA) using baseline as the covariate, with results presented as estimated marginal means $\pm$ S.E.M. (SPSS version 22; IBM Corp., Armonk, NY, USA). The 4-week change in plasma epoxides (i.e., $Epoxide_{4\text{ weeks}} - Epoxide_{baseline}$) was analyzed using two-tailed *t* tests, with a 95% bootstrapped confidence interval (CI) obtained using 1000 bootstrapped samples to confirm statistical significance set at $P<0.05$. Acute responses to walnut intake were expressed as the change 4 h post- and pre-walnut intake for the variable of interest (i.e., $Variable_{4\text{ h post walnut intake}} - Variable_{baseline\text{ in the fasted state prior to walnut intake}}$) and subsequently analyzed using ANCOVA or a univariate general linear model (GLM). Nutrient intake values were log transformed as necessary prior to assessment of differences between treatment and time using a GLM. Differences in means with $\alpha<0.05$ were determined significant after multiple comparison adjustment for false positives

using a $q=0.05$ [28] and are presented as the mean $\pm$ S.D. or the back-transformed mean and the 95% CI. Sample size was calculated from previous data that observed diet-induced changes in RHI in healthy adults [29] using an estimated standard deviation of 0.5 and the two-tailed alpha set at 0.05. An $n=18$ per group was determined to provide sufficient power at 0.8 to detect a significant difference between two groups with analysis of variance.

3. Results

3.1. Population characteristics

Thirty-six individuals completed the study (Fig. 1). Baseline characteristics between groups were similar (Table 1) and, except for RHI, did not significantly change over the 4-week period for either group of walnut intake (data not shown). The average age of the study participants was 60 years, with borderline-high cholesterol levels. Within the 5-g/d group, six individuals were excluded from the final analysis due to noncompliance with the PAT protocol ($n=1$), statistical outlier in the primary outcome measure ($n=2$) and an invalid PAT signal ($n=3$); therefore, a total of 12 subjects in the 5-g/d group and 18 subjects in the 40-g/d group were used in the final statistical analysis. Due to equipment or collection failure, complete data sets were not obtained for all acute measures with final group totals reported in the results.

Based on self-reports and investigator tally of empty packages, the participants maintained a compliance greater than 80%. Over the 4-week walnut intake period, the subjects primarily consumed their walnuts in the morning prior to noon (5-g/d group: 70% of the time, 40-g/d group: 56% of the time), followed by afternoon intake (5-g/d group: 23% of the time, and 40-g/d group: 28% of the time), with the remainder of intakes reported in the evening after 6 p.m. Table 2 presents the study participants' baseline dietary intake prior to the start of walnut intake, as well as their intake during week 4 of walnut intake. Complete 3-day food records were obtained from all but one subject in the 5-g/d group. Reported baseline energy and macro- and micronutrient intakes were similar between the intervention groups. At baseline, total energy intake was 6.8 (5.7–7.9) MJ in the 5-g/d group and 7.2 (6.2–8.3) MJ in the 40-g/d group, with total fat intake at 31.8% and 31.1% of total caloric intake for those in the 5-g/d and 40-g/d group, respectively. Total fat intake did not significantly change with walnut intake compared to baseline within the 5-g/d group, while incorporation of 40 g/d of walnuts within the diet significantly increased total fat intake to 84.5 g/d at 38.8% of total energy intake. No significant changes from baseline were noted for saturated and monounsaturated fat intake for either group throughout the study. The incorporation of 40 g/d of walnuts into the diet significantly increased the intake of polyunsaturated fat (PUFA), including both n-3 and n-6 fatty acids. No other significant changes were observed in the nutrients analyzed. Forty grams per day of walnuts provides 1.1 MJ/d and 13% of total energy from fat. This level of walnut intake was readily incorporated into the diet without substitution, as no significant change in the measured macronutrients was observed with removal of walnut intake from the analysis.

3.2. Changes in microvascular function

At baseline, fasting microvascular function for the study participants was at a level associated with low cardiovascular risk [30] and was not significantly different between groups (5-g/d group: 2.65 ± 0.48 versus 40 g/d group: 2.37 ± 0.38 , $P=.087$; Table 1). After 4 weeks of daily intake of 40 g/d of walnuts, fasting RHI was significantly increased compared to the 5-g/d group (2.63 ± 0.10 versus 2.23 ± 0.13 , respectively; $F=5.63$; $P=.025$; Fig. 2A). A similar response was observed for the FRHI ($F=4.30$, $P=.048$, Fig. 2B). No significant changes in the measures of AIx were observed (data not shown).

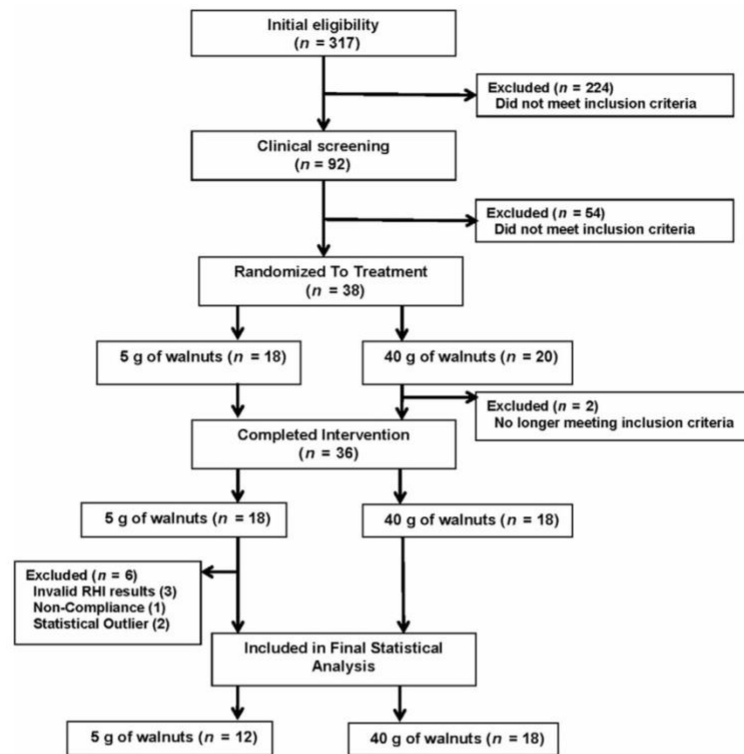


Fig. 1. Participant flow diagram. Thirty-eight postmenopausal women were enrolled based on inclusion and exclusion criteria and randomly assigned to consume either 5 g/d or 40 g/d of walnuts for 4 weeks. Thirty-six women completed the study. After initial examination of the data for completion of study protocols as prescribed, confirmation of a valid RHI measurement and outlier analysis, an $n=12$ in the 5-g group and a $n=18$ in the 40-g group were used in the statistical analysis.

As previous studies have focused on the postprandial effect of walnut intake on vascular function when consumed in conjunction with a high-fat diet, we were particularly interested in the specific walnut-induced acute response on vascular function apart from its potential to counteract the negative postprandial vascular effects of a lipidemic diet. Toward this end, we measured the change in acute RHI ($RHI_{4 \text{ weeks of intake}} - RHI_{\text{baseline}}$). The acute change in RHI to walnut

intake was not significantly different between groups during day 1 of walnut intake [5-g/d group ($n=9$): 0.19 ± 0.46 versus 40-g/d group ($n=18$): 0.25 ± 0.69 , $P=.831$]. However, after 4 weeks of walnut intake, the acute 4-h change in RHI to 40 g of walnut intake was reduced compared to those individuals who consumed 5 g/d of walnuts [-0.04 ± 0.15 ($n=15$) versus 0.71 ± 0.19 ($n=9$), respectively; $F=9.38$; $P=.006$; Fig. 3A]. This reduction in acute microvascular response after 4 weeks of walnut intake could represent a negative effect of walnut intake or that the acute RHI response to walnut intake was not further improved due to an already improved baseline (i.e., fasting) RHI observed after 4 weeks of daily walnut consumption, as prior studies have observed a negative relationship between baseline vascular function and treatment response [31]. In the current dataset, an examination between the acute change in RHI and baseline RHI revealed a highly significant negative association ($r=-0.492$, $P<.001$) between the change in acute RHI response and microvascular function measured after an overnight fast prior to acute walnut intake (Fig. 3B), suggesting that the reduced acute RHI response was due to an already improved baseline microvascular function after 4 weeks of 40 g/d of walnut intake.

3.3. Influence of walnut intake on circulating lipids

No significant differences were observed after 4 weeks of walnut intake for fasting plasma concentrations of TC, high-density lipoprotein-cholesterol (HDL-C), LDL-C or triglycerides (data not shown). Moreover,

Table 1
Baseline characteristics of participants

	5-g/d group ($n=12$)	40-g/d group ($n=18$)
Age (years)	60 ± 5.6	60 ± 3.8
BMI (kg/m^2)	24.1 ± 2.7	25.1 ± 4.1
WC (cm)	82 ± 10.9	83 ± 11.1
SBP (mmHg)	124 ± 17.2	125 ± 14.7
DBP (mmHg)	70 ± 6.7	74 ± 8.9
HR (beats/min)	65 ± 9.5	69 ± 7.3
MAP (mmHg)	88 ± 8.7	91 ± 10.0
Glucose (mmol/L)	5.33 ± 0.28	5.25 ± 0.49
TC (mmol/L)	5.96 ± 0.39	6.02 ± 0.62
HDL-C (mmol/L)	1.70 ± 0.25	1.65 ± 0.26
LDL-C (mmol/L)	3.72 ± 0.30	3.94 ± 0.56
TG (mmol/L)	2.04 ± 2.97	0.94 ± 0.46
RHI	2.65 ± 0.48	2.37 ± 0.38

Baseline variables prior to 5 g/d ($n=12$) and 40 g/d ($n=18$) of walnut intake were not significantly different between the two groups; two-tailed t test. Values are presented as mean $\pm$ S.D. WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; TG, triglyceride.

Table 2
Estimated dietary nutrient intake

	5-g/d group (n=11)		40-g/d group (n=18)		P
	Baseline	Week 4	Baseline	Week 4	
Energy (MJ)	6.8 (5.7, 7.9)	7.7 (6.2, 9.1)	7.2 (6.2, 8.3)	8.4 (7.8, 9.0)	.107
Protein (g)	78.3 (66.2, 90.5)	73.8 (62.2, 85.4)	65.3 (56.0, 74.6)	73.9 (64.9, 83.0)	.339
Carbohydrates (g)	199.3 (155.2, 243.3)	235.3 (193.3, 277.3)	216.0 (182.2, 249.7)	223.9 (191.0, 256.7)	.675
Fat (% energy)	31.8 (27.5, 36.0) ^a	28.8 (24.7, 32.9) ^a	31.1 (27.9, 34.4) ^a	38.8 (35.6, 41.9) ^b	.001
Fat (g) [†]	54.6 (36.9, 80.8) ^a	56.6 (41.9, 76.6) ^a	56.6 (41.9, 76.6) ^a	84.5 (63.1, 113.2) ^b	<.001
SFA (% energy)	10.0 (7.6, 12.5)	7.8 (5.4, 10.1)	10.7 (8.8, 12.6)	9.9 (8.1, 11.8)	.278
SFA (g) [†]	15.6 (8.6, 28.5)	14.2 (8.0, 25.2)	18.7 (11.8, 29.7)	20.8 (13.3, 32.6)	.158
MUFA (% energy)	7.1 (5.0, 9.3)	6.5 (4.5, 8.5)	7.8 (6.2, 9.4)	7.8 (6.3, 9.4)	.703
MUFA (g)	13.5 (8.8, 18.2)	13.5 (9.0, 18.0)	14.5 (10.9, 18.1)	17.2 (13.7, 20.7)	.487
PUFA (% energy)	3.6 (2.5, 4.8) ^a	4.4 (3.4, 5.5) ^a	3.3 (2.4, 4.1) ^a	11.5 (11.6, 12.3) ^b	<.001
PUFA (g) [†]	5.9 (3.9, 10.2) ^a	8.0 (4.7, 13.5) ^a	5.5 (3.6, 8.5) ^a	24.9 (16.5, 37.4) ^b	<.001
n-3 (g) [*]	0.7 (0.3, 1.0) ^a	1.2 (0.8, 1.6) ^a	0.5 (0.3, 0.6) ^a	4.3 (4.1, 4.5) ^b	<.001
n-6 (g) [†]	4.4 (2.2, 8.6) ^{a,b}	6.2 (3.3, 11.8) ^a	3.8 (2.3, 6.4) ^b	19.5 (11.8, 32.3) ^c	<.001

Values are the mean or the back transformed mean[†] of the log data (95% CI) obtained from 3-day food records recorded the week prior to the baseline visit and 4 weeks post 5 g/d (n=11) and 40 g/d (n=12) of walnut intake. ^{a,b}Means in a row with different superscript letters differ with a bolded *P* value <.05 GLM and a false discovery rate set at *q*=0.05. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids.

^{*} Kruskal–Wallis test with all pairwise comparisons.

there were no significant changes in TC, HDL-C and LDL-C levels 4 h after walnut intake (data not shown). The acute change in plasma triglycerides 4 h after walnut intake did not significantly differ within treatment groups either on day 1 of the study or after 4 weeks of walnut intake [5-g/d group (n=12): -0.05 ± 0.13 mmol/L versus -0.03 ± 0.11 mmol/L, respectively;

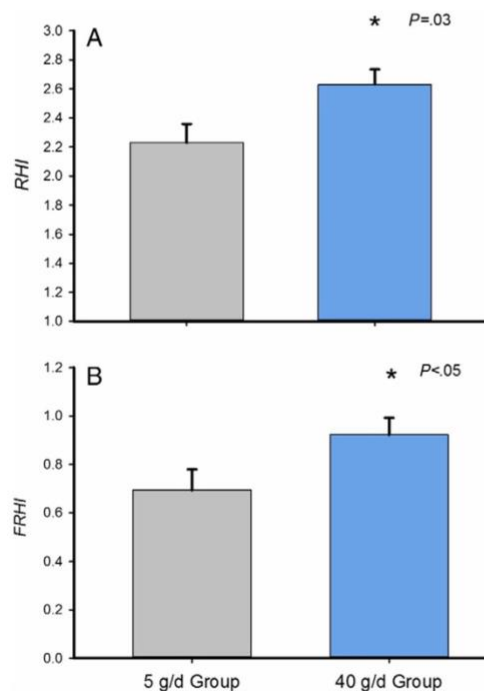


Fig. 2. Four weeks of walnut intake increases microvascular function. Microvascular function as measured by both RHI (A) and FRHI (B) was significantly increased after 4 weeks of 40 g/d of walnut intake compared to the response after 5 g/d of walnut intake. Gray and blue bars represent the 5-g (n=12) and 40-g (n=18) walnut groups, respectively; statistical analysis performed by ANCOVA, adjusted for baseline; data are estimated marginal means $\pm$ S.E.M.

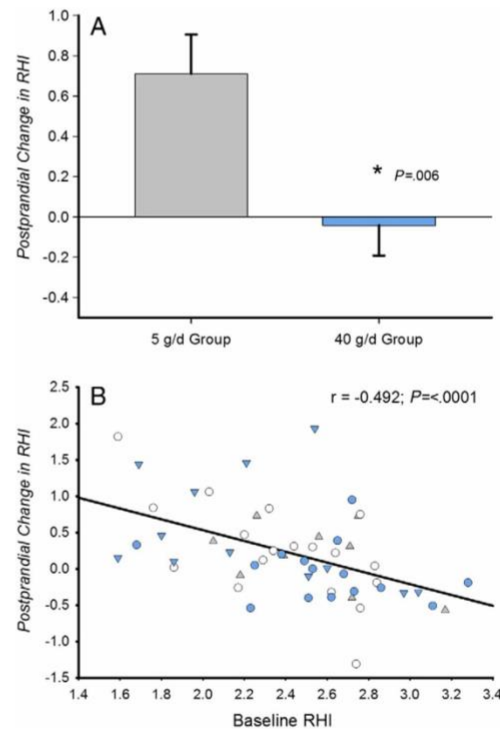


Fig. 3. Inverse association between acute RHI response and baseline RHI. A significant reduction in the acute change in RHI to walnut intake (i.e., $RHI_{4h \text{ post walnut intake}} - RHI_{\text{baseline in the fasted state prior to walnut intake}}$) was observed after 40 g/d of walnut intake for 4 weeks (A); ANCOVA, adjusted for the acute change in RHI during the first day of walnut intake. Data are estimated marginal means $\pm$ S.E.M. with the gray and blue bars representing the 5-g (n=9) and 40-g (n=15) walnut groups, respectively. The postprandial response to walnut intake was inversely associated with baseline (i.e., fasting) RHI (B), suggesting that the significant reduction in postprandial response in the 40-g group after 4 weeks of walnut intake is related to the increase in baseline RHI after short-term walnut consumption. Gray triangle, 5-g group at baseline; white circle, 40-g group at baseline; blue triangle, 5-g group at 4 weeks; blue circle, 40-g group at 4 weeks.

$P=.814$; 40-g/d group ($n=16$): 0.23 ± 0.20 mmol/L versus 0.30 ± 0.26 mmol/L, respectively; $P=.331$]. The between-group acute change in triglyceride levels was significantly increased with 40 g compared to that of 5 g of walnut intake on both the first day of the study and after 4 weeks of walnut intake ($P=.001$ and $P<.001$, respectively). The acute change in triglyceride levels with walnut intake was not associated with the acute change in RHI (Pearson correlation, $P=.831$).

Plasma levels of unesterified ALA, LA, AA, eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA) did not significantly change among the assessed individuals that consumed either 5 g/d or 40 g/d of walnuts for 4 weeks. However, a significant increase in the ratio of plasma ALA to LA was observed after the 4-week intake period (0.10 ± 0.01 $\mu\text{mol/L}$ at baseline versus 0.14 ± 0.01 $\mu\text{mol/L}$ at 4 weeks, $F=12.0$, $P=.003$). No significant relationships were observed between the 4-week change in RHI versus the 4-week change in plasma ALA, LA, AA, EPA and DHA levels (i.e. $\text{Fatty Acid}_{4\text{ weeks of intake}} - \text{Fatty Acid}_{\text{baseline}}$) as assessed, where appropriate, via Pearson or Spearman correlations.

3.4. Relationship of microvascular function to changes in unesterified ALA, LA, AA, EPA and DHA epoxides

ALA and LA are metabolized, in part, by CYP epoxygenases to form epoxyoctadecadienoic acid (EpODE) and epoxyoctadecamonoenoic acid (EpOME), respectively. While these metabolites are distributed among plasma compartments, one important pool is the metabolites circulating as “free” epoxides, meaning that they are unesterified and not associated with plasma lipoproteins. Compared to 5 g of daily walnut intake, 4 weeks of 40 g/d of walnuts significantly increased plasma levels of the free ALA epoxides 12(13)-, 15(16)- and 9(10)-EpODE; the LA epoxides 9(10)- and 12(13)-EpOME; and the DHA epoxide 16(17)-epoxydocosapentaenoic acid (EpODE, Table 3). In contrast, no significant between-group differences were observed for free AA, EPA and DHA plasma epoxides (Table 3). An examination of the relationship between the change in RHI and the change in the sum of plasma ALA, LA, AA, EPA and DHA epoxides showed a strong positive association after 4 weeks of walnut intake ($r=0.65$, $P=.002$, Fig. 4A) but not for the change in the sum of 5- and 12/15-lipoxygenase metabolites or the $\omega/\omega-1$ hydroxylase product 20-hydroxyeicosatetraenoic acid (data not shown).

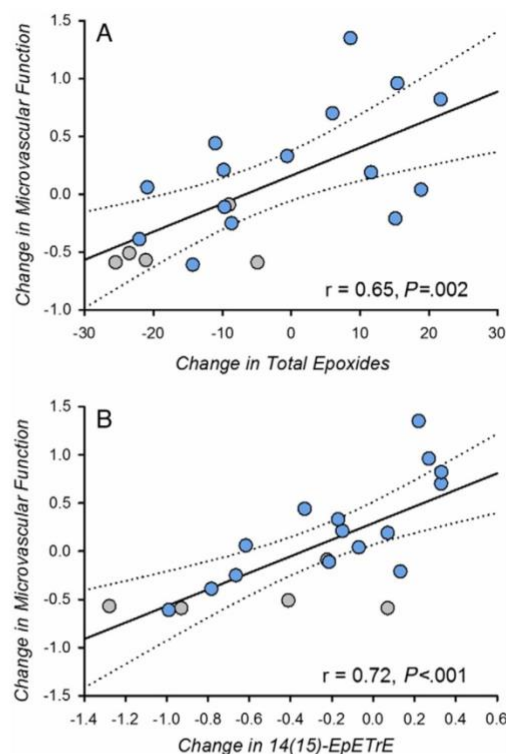


Fig. 4. Positive association between plasma epoxides and RHI response. Scatter plots and 95% CI (dotted lines) of the linear relationship between the change in microvascular function, as measured by RHI (i.e., $\text{RHI}_{4\text{ weeks}} - \text{RHI}_{\text{baseline}}$), after 4 weeks of 5 g (gray circle, $n=5$) or 40 g (blue circle, $n=15$) of daily walnut intake and (A) the change in the sum of ALA, LA, AA, EPA and DHA plasma epoxides ($\text{Epoxide}_{4\text{ weeks}} - \text{Epoxide}_{\text{baseline}}$) and (B) the change in the AA plasma epoxide 14(15)-EpETE.

Table 3
Change in plasma epoxide levels (nmol/L) after 4 weeks of daily walnut intake

	Baseline		4-week change		Mean difference (95% CI) *	P value
	5 g/d	40 g/d	5 g/d	40 g/d		
ALA epoxides						
9(10)-EpODE	1.72±0.52	1.68±0.71	-0.80±0.57	0.26±1.05	-1.07±0.36 (-1.78, -0.39)	.008
12(13)-EpODE	0.49±0.15	0.50±0.23	-0.26±0.23	0.11±0.35	-0.37±0.14 (-0.63, -0.08)	.019
15(16)-EpODE	2.37±0.86	2.57±1.04	-0.78±1.04	1.72±2.85	-2.50±0.83 (-4.21, -1.00)	.045
LA epoxides						
9(10)-EpOME	8.39±2.99	7.52±3.78	-4.42±1.60	-0.51±3.45	-3.91±1.11 (-6.24, -1.77)	.009
12(13)-EpOME	15.02±4.15	13.67±5.83	-6.85±3.79	-0.73±6.10	-6.12±2.31 (-10.64, -1.38)	.020
AA epoxides						
8(9)-EpETE	0.79±0.32	0.77±0.52	-0.37±0.36	-0.10±0.30	-0.28±0.18 (-0.65, 0.04)	.123
11(12)-EpETE	2.41±0.94	2.48±1.54	0.22±1.61	-0.78±2.18	1.01±0.88 (-0.56, 2.76)	.252
14(15)-EpETE	1.27±0.41	1.31±0.82	0.09±0.95	-0.39±1.18	0.48±0.51 (-0.38, 1.59)	.359
EPA epoxides						
11(12)-EpETE	0.34±0.26	0.24±0.20	-0.15±0.30	0.01±0.15	-0.16±0.14 (-0.48, 0.07)	.271
17(18)-EpETE	0.64±0.55	0.39±0.29	-0.32±0.58	0.01±0.21	-0.32±0.28 (-0.97, 0.09)	.314
DHA epoxides						
16(17)-EpDPE	0.73±0.13	0.53±0.33	-0.30±0.33	0.06±0.40	-0.36±0.17 (-0.73, -0.03)	.049
19(20)-EpDPE	2.15±0.76	1.57±0.72	-0.86±0.92	-0.30±0.53	-0.56±0.43 (-1.42, 0.31)	.218

Baseline plasma levels of epoxides prior to as well as the change in plasma epoxides (i.e., $\text{Epoxide}_{4\text{ weeks}} - \text{Epoxide}_{\text{baseline}}$) after 5 g ($n=5$) or 40 g ($n=15$) of daily walnut intake for 4 weeks. Values are means $\pm$ SD. EpETE, epoxyeicosatetraenoic acid.

* Between-group changes in plasma epoxides after 4 weeks of walnut intake were analyzed using two-tailed t tests, with a 95% bootstrapped CI obtained using 1000 bootstrapped samples to confirm statistical significance set at a $P<.05$, with results reported as the between-group mean difference $\pm$ S.E.M. (bootstrapped 95% CI) and P value.

The relationship between the changes in individual plasma epoxides to the change in microvascular function 4 weeks after daily walnut intake was assessed using a Pearson correlation matrix. The change in microvascular function was positively and strongly associated with the change in 9(10)- and 12(13)- EpODEs and EpOMEs, and 8(9)-, 11(12)- and 14(15)-EpETrE regioisomers ($|r|=0.56$, $P<.01$, Fig. 5). Weaker associations were observed between the change in RHI and 15(16)-EpODE and the DHA epoxide 19(20)-epoxydocosapentaenoic acid (EpDPE) ($|r|=0.44$, $P<.05$), while a highly significant correlation ($|r|=0.68$, $P<.001$) to the change in RHI was observed for the change in 14(15)-EpETrE ($r=0.72$, $P<.001$, Figs. 4B and 5).

4. Discussion

Epidemiological observations support the concept that high intakes of certain tree nuts can be associated with a reduced risk for CVD [1]. A recent meta-analysis found a 19% decrease in the risk of coronary heart disease and a 34% reduction in hypertension for each 28.4-g daily serving increase in nuts [32]. A 28% reduction in cardiovascular events was reported when 30 g/d of nuts (50% walnuts, 25% almonds, 25% hazelnuts) was incorporated into a Mediterranean dietary pattern [33]. In addition to clinical end points, the potential of specific nut types to alter physiologically relevant end points of vascular disease, such as FMD and RHI, has been explored because these end points have been associated with cardiovascular risk factors and considered early event indicators of CVD etiology [34]. Our study confirms, and extends, observations that short-term walnut intake can improve vascular function [3,4,6,7]. Moreover, the potential influence of bioactive fatty acid metabolites on microvascular function is suggested from the strong associations between improved microvascular response and the increase in ALA-, LA-, AA-, EPA- and DHA-derived plasma epoxides.

RHI is inversely associated with a number of CVD risk factors, attenuated in ischemic heart disease in women and predictive of secondary events [25,35,36]. The significant increase in RHI in the current study is consistent with previous reports of improved fasting FMD responses after 56 g/d of walnuts was incorporated into the habitual diets of individuals with type 2 diabetes or metabolic

syndrome for 8 weeks [7,10]. In these studies, FMD was induced with reactive hyperemia using an upper arm occlusion protocol, which is thought to increase the influence of other vascular dilators, such as EDHF and prostacyclin, relative to NO [37]. In addition, both studies examined the relationship of FMD to reactive hyperemia response, with one study showing an increase in this ratio [7] and the other observing no change [10]. Whether or not this indicates a significant change in the reactive hyperemia stimulus after walnut intake is unknown with the data provided; however, it is thought that the reactive hyperemia response reflects the dilation of resistance vessels of the microvasculature and has been associated with cardiovascular risk burden [38,39].

Walnuts contain a number of phytochemicals, lipids, fiber and minerals that may affect vascular function. As a number of studies have reported increased vascular function 2–6 h after the consumption of plant foods that are high in specific flavonoids [40–42], potentially through improvements in NO availability [29], we were particularly interested whether walnut intake acutely improves vascular function. In the current study population, we did not observe further improvement in the acute response to walnut intake, with an apparent reduction in vascular function after 4 weeks of continuous walnut intake. This is in contrast to previous reports of improved postprandial vascular function when walnuts were incorporated into or consumed with a high-fat meal in those with hypercholesterolemia but not for those with normal cholesterol levels [3,4]. Vascular dysfunction during the postprandial period has been explored as a potential mechanism of the proatherogenic nature of hypertriglyceridemia [12]. The current study was not designed to specifically examine this effect, as the acute increase in triglyceride levels was approximately sixfold lower than triglyceride levels previously observed to cause a vascular deficit [12]. Instead, the reduction in the acute response within this subject population is most likely due to an already improved baseline response after 4 weeks of walnut intake. This result is similar to previous reports of an inverse association between baseline vascular function and acute response after short-term flavanol or resveratrol intake [31,43] and suggests that, in a population with normal vascular function, acute walnut intake does not further improve microvascular response [43].

That walnut fatty acids may beneficially influence vascular function is suggested by data from Berryman et al., who reported a positive acute vascular response after acute walnut oil intake compared to the consumption of skins, nut meat and the whole walnut [6]. Based on USDA food composition data, the 40-g portion of walnuts used in the present study provided approximately 15 g and 4 g of the essential PUFAs n-6 LA and n-3 ALA, respectively [44]. While the walnuts used in the current study were not characterized for their specific LA and ALA level, a significant increase in the ratio of ALA to LA and their respective epoxides within plasma was noted in the 40-g/d group after 4 weeks of intake. Studies on the potential health effects of PUFA and cardiovascular health have been focused, in part, on the conversion of LA and ALA to EPA and DHA. However, as high ALA intakes may also be associated with a reduction in CVD risk [45], it is possible that ALA influences cardiovascular function through a mechanism apart from its formation of EPA and DHA.

PUFAs undergo bioactivation by COX, LOX and CYP enzyme systems to produce a myriad of lipid products, collectively called oxylipins. Both PUFAs and many oxylipins can be esterified within cellular phospholipids and, after phospholipase activation, participate in autocrine and paracrine signaling. Of particular importance to the current study is the fact that endothelial cell phospholipases can be activated by vasodilatory stimuli such as shear stress to produce both AA-derived eicosanoids as well as EpETrE by CYP epoxygenases, which are considered cardioprotective through their anti-inflammatory, antiatherogenic and EDHF effects [13,14,46]. The inhibition of CYP epoxygenases in humans has demonstrated a role for EpETrE in

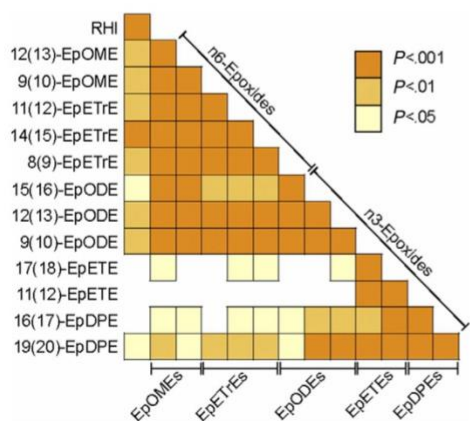


Fig. 5. Pearson correlation matrix of the change in microvascular function ($RHI_{4weeks} - RHI_{baseline}$) and the change in plasma Epoxides ($Epoxide_{4weeks} - Epoxide_{baseline}$) after 4 weeks of 5 g/d ($n=5$) and 40 g/d ($n=15$) of walnuts. Correlation coefficients (r) within yellow boxes are at the $P<.05$ level, light orange boxes at the $P<.01$ and dark orange boxes at $P<.001$.

regulating vascular tone [47,48]. The results from the current study strongly suggest the influence of plasma epoxides on microvascular function after walnut intake. It is thought that approximately 90% of plasma epoxides, such as EpETRE, are associated with lipoprotein phospholipids [49]. However, we are reporting the “free” fraction, which is not associated with lipoprotein. Whether the change in plasma epoxides after walnut intake represents a source of potentially bioactive lipids or epoxides produced from the vasculature will require further exploration.

The direct vascular effects of LA or ALA CYP epoxygenases (i.e., EpOME and EpODE, respectively) are not well understood. Higher plasma levels of EpOMEs relative to their DiHOME metabolites have been inversely correlated to the occurrence of aortic lesions in a mouse model [46]. Recently, it has been proposed that ALA intake can inhibit sEH and reduce the production of deleterious vascular metabolites to improve blood pressure [18]. Conversely, sEH inhibition may increase vascular reactive epoxide levels, as demonstrated by the current data. Our results support the need to further define the physiological role between dietary PUFA and their bioactive metabolites, particularly given the inconsistencies in the relationship between ALA intake, plasma ALA levels and cardiovascular end points [45,50,51].

In conclusion, our study confirms and extends observations that a relatively short-term intake of walnuts can be associated with improvements in vascular function. While the lack of a standardized, controlled diet over the test period can be considered a limitation of our study, our goal was to observe whether or not the addition of walnuts into the habitual diet could result in changes in outcome measures, which is a model consistent with a free-living population. Overall, data from this study suggest that the microvascular response from walnut intake was influenced by changes in LA and ALA composition within the diet. While the potential health effects of n-3 fatty acids on cardiovascular health have been focused, in part, on the role of LA and ALA as precursors for EPA and DHA, given the current results and the distinct epoxide profile provided, further research is warranted to better define the role of these fatty acids and their oxylipins in vascular homeostasis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jnutbio.2015.07.012>.

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Chapter 3:

Walnuts change lipoprotein composition suppressing TNF α -stimulated cytokine production by diabetic adipocyte (J Nutr Biochem. 2019 Jun;68:51-58)



Walnuts change lipoprotein composition suppressing TNF α -stimulated cytokine production by diabetic adipocyte

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Abstract

Walnut consumption can provide both vascular and metabolic health benefits, and walnut-induced changes in lipoprotein particle chemical payloads may be responsible for these health benefits. To explore this possibility with a focus on metabolic health, this study investigated the impact of walnut consumption on lipoprotein lipid composition and changes in LDL anti-inflammatory properties, as reported by inflamed adipocyte. Hypercholesterolemic, postmenopausal females were treated with 40 g/day (i.e., 1.6 servings/day; $n=15$) of walnuts for 4 weeks. Fatty acids and their oxygenated metabolites, i.e., oxylipins, were quantified in isolated lipoproteins. Human primary adipocytes were exposed to LDL and TNF α -stimulated adipokine production was measured. Walnut treatment elevated α -linolenic acid and its epoxides in all lipoproteins and depleted mid-chain alcohols in VLDL and LDL, but not HDL. Walnuts also reduced TNF α -induced diabetic adipocyte production of IL-6 (-48% , $P=.0006$) and IL-8 (-30% , $P=.01$), changes inversely correlated with levels of α -linolenic acid-derived epoxides but not α -linolenic acid itself. In conclusion, modest walnut consumption can alter lipoprotein lipid profiles and enhance their ability to inhibit TNF α -dependent pro-inflammatory responses in human diabetic primary adipocytes. Moreover, this study suggests the oxylipins, rather than the parent fatty acids, mediate LDL action of adipocytes.

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Keywords: Adipocytes; LDL; Diet; Lipid mediators; Dietary lipids; Lipoprotein metabolism

1. Introduction

The consumption of walnuts and other tree nuts has been associated with a lower risk for cardiovascular disease [1]. Specifically, walnut intake has been associated with lower type-II diabetes risk [2], while tree nuts can lower LDL cholesterol and triglycerides [1,3] and attenuate endothelial dysfunctions induced by hypercholesterolemia [4] and high-fat meals [5]. In subjects with hypercholesterolemia, walnut interventions can also decrease the production of the pro-inflammatory cytokines interleukin-6 (IL-6), IL-1b and TNF α by peripheral blood mononuclear cells [6]. Adipose inflammation is a significant consequence of obesity that negatively impacts tissue glucose handling and insulin sensitivity [7]. Importantly, elevated oxidized LDL (ox-LDL) are associated with type II diabetes [8], adipocytes actively take up ox-LDL [9,10], the plasma levels of ox-LDL has been negatively correlated with plasma levels of the adipose generated cytokine adiponectin in humans [11] and walnut consump-

tion can reduce LDL oxidation [4,12,13]. Walnuts are a rich source of linoleic acid (LA), α -linolenic acid (aLA) [14] and numerous bioactive phenolics [1,15–19]. While each of these components have bioactive potential, it has been postulated that aLA, an omega-3 fatty acid, is a major factor responsible for the anti-inflammatory effects in a manner analogous to the anti-inflammatory effects of fish oil being attributed to the long chain omega-3 fatty acids [1].

The bioactivity of PUFAs can be associated with their direct action, as well as through the action of their metabolic products. Major products of PUFA metabolism are the oxylipins, a superclass of oxygenated metabolites. These compounds are formed by: cyclooxygenases (COX)-producing prostaglandins and thromboxanes; lipoxygenases (LOX)-producing hydroperoxides and their associated downstream products (e.g., mid-chain alcohols); cytochrome P450 (CYP)-producing epoxides and omega-hydroxides; auto-oxidation, producing peroxide associated products including isoprostanes and certain mid-chain alcohols [20,21]. The structures of representative alkaline-stable arachidonic acid-derived oxylipins are presented in the Fig. 1. Oxylipins are the primary mediators of PUFA action, regulating processes including inflammation, glucose metabolism and cardiovascular homeostasis (broadly reviewed in [22–29]). Different classes of oxylipins manifest distinct properties, and these effects are

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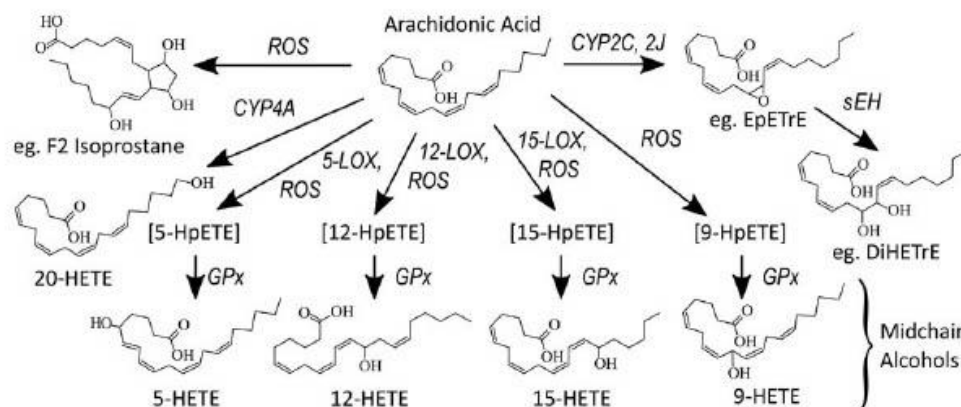


Fig. 1. Representative alkaline-stable arachidonic acid-derived oxylipins which can be released from and measured in esterified oxylipin pools. Unsaturated fatty acids (e.g., arachidonic acid) can be transformed into a variety of structures through interactions with cytochrome P450s (CYPs), epoxide hydrolases (EH), lipoxygenases (LOX), glutathione peroxidases (Gpx), and reactive oxygen species (ROS), as well as other enzymes. These reactions result in the formation of epoxides (e.g., epoxyeicosatrienoic acids (EpETREs), vicinal or 1,2-diols (e.g., dihydroxyeicosatrienoic acids (DiHETREs)), hydroperoxides (e.g., hydroperoxyeicosatrienoic acids (HpETREs)), mono-alcohols (e.g., hydroxyeicosatrienoic acids (HETREs)), and isomeric prostaglandins (e.g., F2 isoprostanes). These pathways and additional structures are well reviewed elsewhere.

influenced by the parent fatty acid from which they are derived [22]. For instance, epoxides are generally anti-inflammatory [23,24] and can suppress the production of pro-inflammatory cytokines [25,26]. Moreover, elevated plasma concentrations of epoxides have been linked with better cardiovascular outcomes in diverse animal models [26]. On the other hand, many mid-chain alcohols are pro-inflammatory [27], with links to low-grade systemic inflammation and pro-inflammatory cytokine production in adipose tissue [30]. While much is known regarding the action of these compounds in general, their sources and mechanisms of production and delivery *in vivo* are as of yet poorly understood.

While oxylipins are generally considered to be autocrine and paracrine in nature, they are transported through plasma in esterified forms within lipoprotein particles at high abundances [31,32]. The trafficking of these particle associated metabolites, and their impact on lipoprotein-metabolizing tissues are not well understood [28,29]. Clinical trials have demonstrated that PUFA intake influences the oxylipin composition of lipoproteins. Supplementation with marine omega-3 PUFAs results in increased docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)-derived oxylipins at the expense of arachidonic acid (AA) metabolites [31–33]. Moreover, marine-PUFAs induce changes in oxylipin composition differently among different lipoproteins classes [31]. While the effect of fish oil supplementation on the oxylipin profile has been the subject of several studies [31–33], to the best of our knowledge, the impact of tree nuts on the lipoprotein esterified oxylipin profiles has not been reported.

Previously, in a randomized controlled trial walnut treatment improved endothelial dysfunction in hyper-triglyceridemic women, an effect associated with increases in plasma non-esterified fatty acid epoxides [34]. In the current study, using samples from the study above, we investigated walnut-induced changes in the lipoprotein oxylipin and fatty acid composition. Given the known pathological association of ox-LDL, we also tested the impact of walnut-induced changes to LDL on human primary adipocytes, a model lipoprotein-metabolizing tissue which actively takes up LDL and ox-LDL [9,10]. The production of pro- and anti-inflammatory adipokines were assessed as the primary outcome. Since walnuts have been reported to have beneficial effect in pathological conditions associated with the metabolic syndrome [45,34] and elevated ox-LDL [4,12,13], adipocytes with diabetic and non-diabetic phenotypes were used. Moreover, since low-grade systemic inflammation accompanies the development of obesity and diabetes and is associated with

elevated pro-inflammatory cytokine production, cell culture experiments were performed with or without TNF α , as a surrogate for an inflammatory environment.

Previous studies have clearly demonstrated that dietary PUFAs can improve select health outcomes [32,35,36], and alter lipoprotein particles composition [32]. Moreover, lipoprotein composition has been associated with diverse physiological outcomes [37]. In the present study, we used a dietary intervention to link and examine the food-lipoprotein-inflammatory physiology axis.

2. Materials and methods

2.1. Study design and analysis strategy

This study is an ancillary study of a randomized controlled trial on the impact of walnut consumption on vascular function in hypercholesterolemic postmenopausal women [34] after 4 weeks of 40 g/day (i.e., 1.6 servings/day; $n=18$) or 5 g/day (i.e., 0.2 servings/day; $n=12$). In a subset of individuals (40 g/day, $n=15$ and 5 g/day $n=5$) samples were probed for potential changes in plasma lipids and lipoproteins (the baseline subject characteristics are presented in the Table S1). The study was registered with [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=NCT01235390) (NCT01235390) with the study protocol approved by the Institutional Review Board of the University of California, Davis. Subject inclusion and exclusion criteria and the feeding protocol were previously described [34]. The original study observed improvements in microvascular function with the intervention of 4 weeks of 40 g/day of walnuts compared to 5 g/day. The 5 g/day level was chosen in order to provide a “placebo” comparative group that was predicted to not improve the primary outcome of microvascular function. However, in the current subset of individuals, 5 g/day of walnut showed a lipoprotein lipidomic response when analyzed as a change from baseline (Fig. S1 and Table S2). Moreover, the low and variable number of subject in the treatment groups significantly limits the power of the analysis and the placebo-controlled approach does not allow for control of the subject effect. For those reasons the current study has been analyzed using a change from baseline approach.

2.2. Lipoprotein isolation

Lipoproteins were isolated by size exclusion chromatography as described by [38] using a 10 $\times$ 300 cm Superose 6 (GE Healthcare; Uppsala, Sweden). The isocratic mobile phase, 20 mM Tris+0.1% EDTA+0.1% BHT, was degassed with helium and run at 0.5 mL/min. A 200 μ L aliquot of plasma were injected and 500 μ L fractions were collected as shown in Fig. S2 for: VLDL, fractions 16–18 (3 total; 8.0 to 9.0 mL); LDL, fractions 23–26 (4 total; 11.5 to 13.0 mL); HDL, fractions 31–34 (4 total; 15.5 to 17 mL).

2.3. Lipoprotein fatty acids and oxylipins analysis

Total fatty acids and alkaline-stable oxylipins were isolated using a liquid-liquid extraction and quantified using GC-MS for fatty acids and ultra-performance LC-MS/MS for oxylipins. Lipids were isolated using a liquid-liquid extraction with cyclohexane/isopropanol/ammonium acetate after enrichment with a suite of deuterated and rare compounds used as analytical surrogates, [39,40]. Fatty acids were transformed into methyl

esters using methanolic transesterification. The fatty acid methyl esters were separated on 30 m×0.25 mm, 0.25 µm DB-225 ms (Agilent Technologies; Santa Clara, CA, USA), detected with a 5973 A mass selective detector (Agilent) using electron impact ionization and selected ion monitoring, and quantified against authentic standards. Results were corrected for the recoveries of analytical surrogates as previously described [40]. Esterified oxylipins were transformed into oxylipin free acids by base hydrolysis, and isolated by subsequent hydrophilic/lipophilic interaction solid phase extraction. Alkaline stable oxylipins were separated on a 2.1×150, 1.7 µm Acquity BEH column (Waters Corp, Milford, MA, USA) and detected by negative mode electrospray ionization and multi-reaction monitoring on a Sciex 4000 QTRAP [40]. Concentrations were calibrated using analytical standards as previously reported [40,41].

2.4. Adipocyte culture

Primary human diabetic and non-diabetic subcutaneous pre-adipocytes as well as culture media and hormones used for differentiation were purchased from Lonza (Basel, Switzerland). Both diabetic and non-diabetic cells were purchased as a single batch. Pre-adipocytes were grown in a 96 well plate format in PGM-2 Basal Medium (Lonza; Basel, Switzerland) supplemented with 10% FBS, 50 µg/mL of gentamicin and 37 ng/mL of amphotericin. Confluent cells were differentiated for 14 days in a PGM-2 Basal Medium (Lonza; Basel, Switzerland) supplemented with differentiation hormone mixture (PGM™-2 SingleQuot™; Lonza; Basel, Switzerland) containing insulin, indomethacin, 3-Isobutyl-1-methylxanthine and dexamethasone. All assays were performed on differentiated cells, after 6 h of treatment with 30 µg/mL of LDL (based on the protein content) from the walnut study ($n=5$ for each treatment group) [34] or phosphate buffered saline vehicle, in the presence or absence of 3 ng/mL TNF α [42]. Each replication in the cell culture experiment represents LDL isolated from a different subject.

2.5. Measurement of adipokines levels

The concentrations of IL-6, IL-8, MCP-1, PAI-1, IL-1b, leptin, adiponectin, and resistin were assessed in culture media using a Luminex multiplexing platform (ThermoFisher; Waltham, MA, USA) with kits purchased from BMD Millipore.

2.6. Generation of DIL-LDL and LDL uptake assay

Isolated LDL were combined with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DIL; Molecular Probes, Eugene, OR, USA) according to [43].

Generated DIL-LDL (30 µg/mL) were incorporated into culture media without FBS and applied on cells for 1 h. Next, the culture media was discarded and the amount of DIL dye in the culture dish was measured at 580 nm using Synergy MX plate reader (Biotek; Winooski, VT, USA).

2.7. Intracellular lipid accumulation

Intracellular lipid accumulation was measured using Oil Red O staining (Lonza; Basel, Switzerland). Briefly, cells were fixed with 10% formalin for 30 min. Next, the formalin was removed and the cells were stained with 2 mg/mL Oil Red O in 60% isopropanol for 1 h. Further excess Oil Red O was discarded and cells washed three times with distilled water. Bound Oil Red O was measured at 500 nm using Synergy MX plate reader (Biotek; Winooski, VT, USA).

2.8. Statistical analysis

All statistical tests were performed using JMP Pro 13.1 (JMP, SAS Institute, Cary, NC, USA). Prior to analysis outliers were removed using the robust Huber M test and missing data were imputed using multivariate normal imputation. Data imputation was performed to facilitate multivariate data analysis and did not significantly change univariate analysis results like t -tests. Both treatment groups (5 g/day and 40 g/day) were analyzed independently as change from the baseline. Treatment effects on lipoprotein composition were evaluated by t -test, multivariate ANOVA (MANOVA) and partial least square discriminant analysis (PLS-DA). The t -test was performed separately for each metabolite with the visit as a fixed effect, adjusted for subject effect. For MANOVAs, variables were auto scaled [44] and grouped by hierarchical clustering using the Ward minimal variance method. MANOVA model was built with the metabolite in each cluster as a fixed effect and subject as a random effect. MANOVA and t -test P values cutoffs were corrected for multiple comparisons using the false discovery rate (FDR) approach of Benjamini and Hochberg [45] with $q=0.1$. PLS-DA was used to facilitate visualization of the treatment effects across all lipoprotein particles. PLS-DA model was built using the nonlinear iterative partial least squares algorithm with K-Fold variation method ($k=7$) and included 204 variables (i.e., 68 detected fatty acids and oxylipins across the HDL, LDL and VLDL). Prior to the PLS-DA the paired data for each subject at baseline and after 4 weeks of treatment were scaled such that their means were centered on zero (i.e., subject centered) to reduce the subject effect and emphasizes the

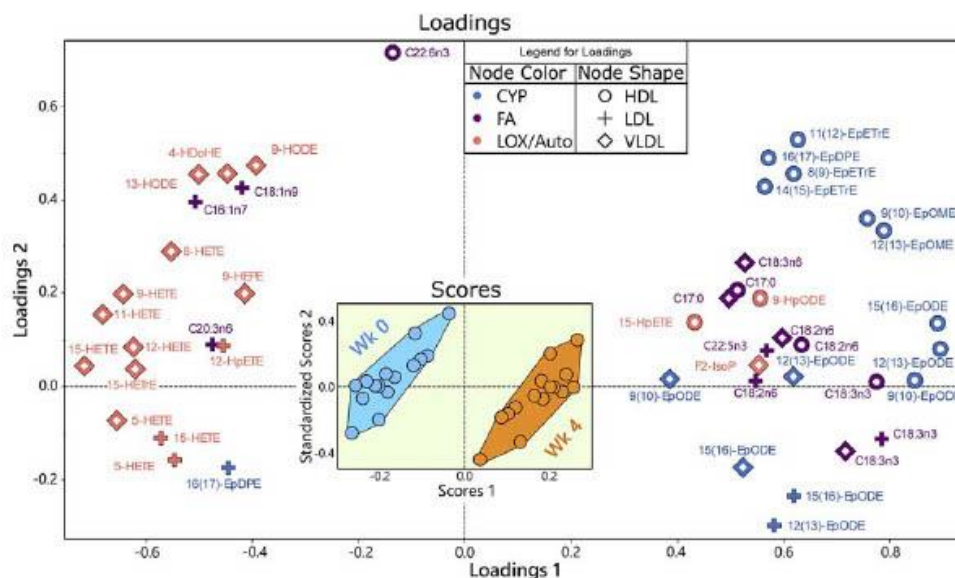


Fig. 2. Partial least squares-discriminant analysis of the impact of the 40 g/day walnut treatment on the level of fatty acids and oxylipins in VLDL, LDL and HDL. Treatment group discrimination is shown by the Scores Plot (inset), while metabolite weighting in group discrimination is shown by the Loadings Plot. Loading node colors indicate enzymatic pathways, while shapes indicate the lipoprotein particle in which the metabolite was found. Variables with Standardized Loading 1 values >0 are elevated at week 4 relative to baseline. Analysis was performed with all measured variables but only those with variable importance in projection (i.e., VIP) ≥ 1 are displayed. CYP, cytochrome P450; FA, fatty acid; LOX/Auto, lipoxygenase/auto-oxidation; sEH, soluble epoxide hydrolase; Wk 0, subject scores at baseline; Wk 4, subject scores after 4 weeks of walnut treatment.

Table 1
HDL fatty acids and lipid mediator before and after 40 g/day walnut treatment. Only affected clusters ($P < .1$) are presented, with unaffected clusters shown in Table S3a

Cluster	Unit	Metabolite	Baseline (Mean $\pm$ SD)	4 weeks (Mean $\pm$ SD)	t-test P value	MANOVA P value
HDL 1	nM	12(13)-EpODE	0.199 $\pm$ 0.113	0.397 $\pm$ 0.173	.0001	0.0001
C:18 Epoxides	nM	15(16)-EpODE	0.665 $\pm$ 0.448	1.69 $\pm$ 0.856	.0001	
	nM	9(10)-EpODE	0.658 $\pm$ 0.407	1.5 $\pm$ 0.817	.0001	
	nM	12(13)-EpOME	30.8 $\pm$ 11.8	45.9 $\pm$ 19.3	.0002	
	nM	9(10)-EpOME	28.5 $\pm$ 11.2	41.2 $\pm$ 18.4	.0005	
HDL 2	μ M	C18:3n3	0.878 $\pm$ 0.262	1.75 $\pm$ 0.77	.0001	0.0001
C:18 FA	μ M	C18:2n6	75.3 $\pm$ 22.1	96.9 $\pm$ 28.4	.0007	
HDL 3	%	9-HpODE	2.04 $\pm$ 0.703	2.78 $\pm$ 1.13	.0028	0.011
C:18-OOH	%	13-HpODE	2.05 $\pm$ 1.09	2.58 $\pm$ 1.06	.049	
HDL 4	nM	8(9)-EpETE	6.03 $\pm$ 2.46	7.2 $\pm$ 2.4	.012	0.012
AA Epoxides	nM	14(15)-EpETE	9.69 $\pm$ 3.71	12.1 $\pm$ 5.33	.019	
	nM	11(12)-EpETE	12.4 $\pm$ 4.91	14.9 $\pm$ 5.63	.022	
HDL 5	nM	5-KETE	0.919 $\pm$ 0.454	1.12 $\pm$ 0.522	.038	0.038

% – Percent composition. Semi-quantitative data generated based on MS area count, without standard curve.

treatment effect using the following equations:

$$\text{baseline}_{\text{Subjected Centered}} = \text{baseline} - \left(\frac{\text{baseline} + \text{final}}{2} \right)$$

$$\text{final}_{\text{Subjected Centered}} = \text{final} - \left(\frac{\text{baseline} + \text{final}}{2} \right)$$

Low density lipoprotein-dependent effects on adipocyte adipokine production were evaluated using MANOVA. Prior to analysis, variables were clustered as above and a Tukey post-hoc test allowed multiple comparisons adjustment. Correlations between culture media adipokine levels and LDL metabolite concentrations were analyzed separately for TNF α exposed and non-exposed cells using either Pearson's or Spearman's analysis as indicated on associated figures. Data from both treatments as well as final and baseline time points were used for the analysis.

3. Results

3.1. Walnuts impact lipoprotein composition

While 40 g/day of walnuts did not alter the lipoproteins triglycerides, cholesterol, phospholipids (Fig. S2) or protein levels, it significantly and differentially altered the lipoprotein fatty acid and oxylipin compositions. These changes are shown in aggregate in Fig. 2, and individually in tabular form for HDL (Table 1 and Table S3a), LDL (Table 2 and Table S3b) and VLDL (Table 3 and Table S3c). Of the fatty acids, aLA (C18:3n3), and LA (C18:2n6) were increased in all lipoproteins, while C22:5n3 was increased in both LDL and VLDL and

C18:3n6 was increased in VLDL but decreased in LDL. The saturated fatty acids stearic acid (C18:0) and palmitic acid (C16:0) were elevated only in VLDL, while the monounsaturated palmitoleic acid (C16:1n7) and oleic acid (C18:1n9) were decreased only in LDL. Of the oxylipins, mid-chain alcohols from all parent fatty acids, apart from aLA, were decreased in VLDL (VLDL clusters 1, 4 and 7 in the Table 3). On the other hand, in LDL only AA and DGLA-derived mid-chain alcohols decreased (LDL cluster 3 in the Table 2), whereas walnuts increased AA and DHA derived epoxides specifically in the HDL compartment (Fig. 3). It is important to mention that while walnut treatment did not affect fasting complex lipid levels, it significantly decreased postprandial cholesterol (-14% , $P = .0007$) and phospholipids (-16% , $P = .009$) in LDL (Data not shown).

3.2. Walnuts consumption changes functionality of low density lipoproteins in the context of adipocyte response to TNF α

Human primary adipocytes with and without TNF α stimulation, provided a model system to probe the impact of lipoprotein particle structure on inflammatory signaling. LDL isolated after 4 weeks of 40 g/day walnut consumption prevented the TNF α stimulated IL-6 and IL-8

Table 2
LDL fatty acids and lipid mediator before and after 40 g/day walnut treatment. Only affected clusters ($P < .1$) are presented, with unaffected clusters shown in Table S3b

Cluster	Unit	Metabolite	Baseline (Mean $\pm$ SD)	4 weeks Mean $\pm$ SD	t-test P value	MANOVA P value
LDL 1	μ M	C18:3n3	1.28 $\pm$ 0.498	2.56 $\pm$ 0.895	.0001	.0002
aLA and its Epoxides	nM	15(16)-EpODE	1.04 $\pm$ 0.307	1.88 $\pm$ 1.04	.0002	
	nM	12(13)-EpODE	0.267 $\pm$ 0.13	0.508 $\pm$ 0.3	.0004	
	nM	9(10)-EpODE	1.05 $\pm$ 0.582	1.5 $\pm$ 0.845	.05	
LDL 2	μ M	C16:1n7	6.08 $\pm$ 2.71	4.88 $\pm$ 2.18	.0002	.0002
Fatty acids	μ M	C18:1n9	47.9 $\pm$ 14.5	41.1 $\pm$ 10.1	.002	
	μ M	C20:3n6	3.78 $\pm$ 1.42	3.11 $\pm$ 1.38	.0091	
	nM	15-HETE	11 $\pm$ 9.26	7.31 $\pm$ 6.19	.0032	
LDL 3	nM	5-HETE	11 $\pm$ 11.1	7.37 $\pm$ 8.35	.0073	.014
AA and DGLA mid-chain alcohols	nM	9-HETE	9.53 $\pm$ 10.9	6.14 $\pm$ 6.54	.023	
	nM	11-HETE	9.32 $\pm$ 9.61	6.05 $\pm$ 6.24	.013	
	nM	8-HETE	9.41 $\pm$ 8.73	6.44 $\pm$ 5.93	.013	
	nM	12-HETE	10.2 $\pm$ 9.31	7.31 $\pm$ 6.28	.034	
LDL 4	nM	15-HETE	1.56 $\pm$ 1.31	1.17 $\pm$ 0.91	.033	.07
	nM	16(17)-EpDPE	1.85 $\pm$ 1.27	1.12 $\pm$ 0.676	.0093	
	nM	19(20)-EpDPE	2.31 $\pm$ 1.66	1.84 $\pm$ 1.14	–	
	μ M	C22:6n3	2.77 $\pm$ 1.27	2.46 $\pm$ 1.19	–	
LDL 5	μ M	C18:2n6	108 $\pm$ 32.7	129 $\pm$ 34.1	.0037	.07
PUFAs	μ M	C22:5n3	0.781 $\pm$ 0.281	0.968 $\pm$ 0.315	.0043	
	μ M	C20:4n6	20.9 $\pm$ 6.63	20 $\pm$ 5.83	–	

Table 3
VLDL fatty acids and lipid mediator before and after 40 g/day walnut treatment. Only affected clusters ($P < 1$) are presented here, with unaffected clusters presented in Table S3c

Cluster	Unit	Metabolite	Baseline Mean $\pm$ SD	4 weeks Mean $\pm$ SD	t-test P value	MANOVA P value
VLDL 1	nM	15-HETE	4.11 $\pm$ 2.51	2.53 $\pm$ 1.53	.0001	.0001
AA and DGLA	nM	9-HETE	2.23 $\pm$ 1.47	1.51 $\pm$ 1.11	.0001	
mid-chain alcohols	nM	11-HETE	2.65 $\pm$ 1.65	1.73 $\pm$ 1.33	.0004	
	nM	15-HETE	1.02 $\pm$ 0.613	0.681 $\pm$ 0.418	.0009	
	nM	5-HETE	2.8 $\pm$ 1.58	2 $\pm$ 1.52	.0012	
	nM	8-HETE	3.78 $\pm$ 2.11	2.6 $\pm$ 1.8	.0021	
VLDL 2	nM	12-HETE	3.01 $\pm$ 1.81	2.18 $\pm$ 1.89	.0024	.0005
PUFAs and saturated fatty acids	μ M	C18:3n3	1.71 $\pm$ 1.13	3.6 $\pm$ 2.43	.0001	
	μ M	C18:2n6	51.4 $\pm$ 34.2	67.3 $\pm$ 42	.0019	
	μ M	C17:0	0.59 $\pm$ 0.504	0.797 $\pm$ 0.477	.013	
	μ M	C18:3n6	1.27 $\pm$ 1.01	1.64 $\pm$ 1.27	.022	
	μ M	C22:5n3	0.705 $\pm$ 0.629	0.941 $\pm$ 0.676	.024	
VLDL 3	nM	C16:0	64.3 $\pm$ 49.9	73.3 $\pm$ 44.4	.029	.0009
aLA Epoxides	nM	12(13)-EpODE	0.437 $\pm$ 0.301	0.794 $\pm$ 0.699	.0001	
	nM	15(16)-EpODE	1.59 $\pm$ 1.16	2.66 $\pm$ 2.32	.0004	
VLDL 4	nM	9(10)-EpODE	1.49 $\pm$ 1.11	2.14 $\pm$ 2.07	.017	.0012
LA and DHA mid-chain alcohols	nM	13-HODE	78 $\pm$ 36.4	59.4 $\pm$ 29.8	.0014	
	nM	4-HDoHE	2.5 $\pm$ 1.74	1.42 $\pm$ 1.13	.0046	
VLDL 5	nM	9-HODE	30.6 $\pm$ 14.8	24.8 $\pm$ 11.9	.012	.019
	nM	F2-IsoProstanoids	0.167 $\pm$ 0.113	0.269 $\pm$ 0.19	.019	
VLDL 6	μ M	C18:0	12.7 $\pm$ 8.17	16.8 $\pm$ 9.48	.032	
VLDL 7	nM	9-HEPE	0.642 $\pm$ 0.553	0.402 $\pm$ 0.216	.013	.036
EPA and DHA mid-chain alcohols	nM	15-HEPE	1.35 $\pm$ 1.03	0.965 $\pm$ 0.58	.038	
	nM	12-HEPE	0.943 $\pm$ 1.02	0.497 $\pm$ 0.315	.05	
	nM	17-HDoHE	5.9 $\pm$ 7.12	3.29 $\pm$ 3.18	.06	
	nM	5-HEPE	0.681 $\pm$ 0.638	0.459 $\pm$ 0.28	.048	
VLDL 8	nM	14-HDoHE	2.31 $\pm$ 2.5	1.17 $\pm$ 1	.033	.048
	nM	5-KETE	0.597 $\pm$ 0.361	0.428 $\pm$ 0.21	.048	

production in human primary diabetic adipocytes (HPDA) (Fig. 4A). Notably, the non-diabetic primary adipocytes did not show a TNF α -stimulated change in IL-6 and IL-8 (data not shown). HPDA cells treated with TNF α in the presence of LDL from the baseline (week 0), increased the levels of IL-6 and IL-8 in the media (48% and 30% respectively). LDL isolated after walnut treatment counteracted this effect, lowering IL-6 and IL-8 levels to that of uninfamed cells. Walnut treatment did not affect other measured adipokines (i.e., adiponectin, resistin, leptin, IL-1b, MCP-1 and PAI-1), and neither walnut treatment nor TNF α effected LDL uptake or the adipocyte lipid accumulation (data not shown).

3.3. LDL oxylipins, but not their parent fatty acids, are strongly correlated with the anti-inflammatory effects

To investigate associations between anti-inflammatory effects and altered fatty acids and their metabolites in LDL, correlations between pro-inflammatory adipokines, parent fatty acids and the sum of the

metabolite classes derived from those lipids were performed. This analysis allowed the visualization of changes in the correlation structure between these variables in cells with or without TNF α stimulation (Fig. 4B).

In non-inflamed adipocytes, there was no correlation between the levels of IL-6, IL-8 and aLA, LA, AA and the sum of their corresponding epoxides, diols and alcohols. On the other hand, in the inflamed adipocytes ($n = 14$), aLA-derived epoxides (EpODEs) showed negative correlations with IL-6 ($r = -0.66$; $P = .0097$) and IL-8 ($r = -0.62$; $P = .018$), which were stronger than with aLA at $r = 0.5$; $P = .07$ and $r = -0.46$; $P = .1$, respectively. Moreover, no correlations with the aLA derived alcohols (HOTEs) were observed. Arachidonic acid-derived alcohols (HETEs) were positively correlated with the levels of IL-6 ($r = 0.54$; $P = .045$) and IL-8 ($r = 0.54$; $P = .045$), while AA-derived epoxides (EpETErs) and AA itself showed no correlation with IL-6 or IL-8. Similarly to AA metabolites, LA alcohols positively correlated with IL-6 ($r = 0.52$; $P = .055$) and IL-8 ($r = 0.54$; $P = .053$) but other LA derivatives showed no relationships.

To investigate the associations of LDL lipids and oxylipins with adipokine production in more detail, we generated a Spearman ρ correlation network between all measured adipokines from the diabetic adipocytes and all measured lipids and oxylipins, in the presence or absence of TNF α (Fig. S3).

4. Discussion

The daily consumption of walnuts has been reported to have impacts on select metabolic systems, and has been associated with diverse beneficial health outcomes including improvements in inflammatory markers and select cardiovascular outcomes (reviewed in [46]). The causal links between walnut consumption and health benefits are still poorly understood, but they have generally been associated with their high levels of the omega 3 fatty acid aLA [46]. Speculation regarding the impact of phenolic bioactives have also been made [47,48], however the implications of in vivo metabolism on these compounds are poorly understood.

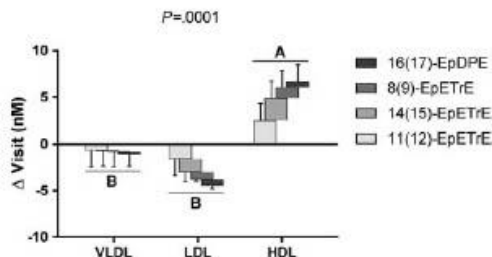


Fig. 3. Lipoprotein specific changes in long chain epoxy fatty acids induced by 40 g/day walnut treatment. A multivariate ANOVA was performed on the walnut induced change from baseline (i.e., Δ Visit = value at baseline – value after 4 weeks of walnut treatment) in long chain epoxy fatty acids. EpETE, epoxyicosatrienoic acid, EpDPE, epoxydocosapentaenoic acid. Lipoproteins with different annotations are different at $P < .05$ by a Tukey post-test. Error bars represent standard deviation.

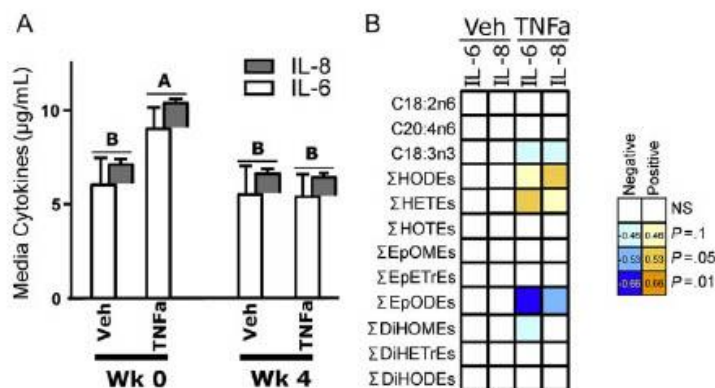


Fig. 4. The effect of LDL from subjects treated with 40 g/day of walnut on TNF α -stimulated IL-6 and IL-8 production by primary human diabetic adipocytes. A) Adipocyte culture media IL-6 and IL-8 concentrations after 6 h incubation with LDL (n=5) and either 3 ng/mL TNF α or vehicle control (Veh). B) Correlation of IL-6 and IL-8 levels with the levels of LDL oxylipins and their corresponding parent fatty acids. HODEs, hydroxyoctadecadienoic acids, HETEes, hydroxyeicosatetraenoic acids, HOTEes, hydroxyoctadecatrienoic acids, EpOMEes, epoxyoctadecanoic acids, EpETRees, epoxyeicosatrienoic acids; EpODEes, epoxyoctadecadienoic acids; DIHOMEes, dihydroxyoctadecanoic acids; DIHETEes, dihydroxyeicosatrienoic acids; DIHODEes, dihydroxyoctadecadienoic acids. Wk 0 – cells treated with LDL isolated at baseline, Wk 4 – cells treated with LDL isolated after 4 weeks of walnut supplementation. Error bars represent standard error. Letters represent significant difference according to MANOVA analysis with the $P=0.05$.

Low grade systemic inflammation is related to cardio-metabolic disorders and is characterized by increased levels of circulating pro-inflammatory cytokines like IL-6 and IL-8 (reviewed in [49,50]). In obesity, adipocytes significantly contribute to increased pools of pro-inflammatory cytokines [50]. Moreover, lipoprotein clearance is reduced in obese individuals, increasing LDL and ox-LDL levels, which have been positively associated with the levels of adipose-derived pro-inflammatory cytokines, called adipokines [51,52]. LDL is associated with adipocyte inflammation [53–55], and the level of ox-LDL has been shown to negatively correlate with plasma concentrations of the anti-inflammatory adipokine adiponectin in humans. Moreover, the direct treatment of 3 T3-L1 adipocytes with ox-LDL induces production of adipokines including resistin and visfatin [52]. Negative effect of LDL has long been associated with the LDL cholesterol load and its ability to promote foam cell formation [56], however in the current study, a modest intake of walnuts induced positive changes in LDL function without altering cholesterol loads. Here we show that LDL isolated from subjects after 4 weeks of 40 g/day (i.e., 1.6 servings/day) walnut consumption reduced the TNF α -stimulated production of the pro-inflammatory cytokines IL-6 and IL-8 by human primary diabetic adipocytes in culture. Notably, the levels of IL-6 and IL-8 in the inflamed adipocytes showed strong negative associations with the LDL composition of aLA-derived epoxy fatty acids and much less strongly with their parent fatty acid, suggesting the oxylipin load to be one of the mediators of the LDL effect. The levels of IL-6 are reported to increase with hypertriglyceridemia [57] and long-term (90 days) consumption of 15 mL/day of walnut oil has been reported to reduce triglycerides and cholesterol levels in patients with Type 2 Diabetes Mellitus [58]. Our findings suggest that walnut consumption may correct the inflammatory status without correction of hyperlipidemia. Of note, in a mouse model, IL-6 levels were reported to fluctuate between fasting and postprandial state and muscle tissue was shown to be responsible for this fluctuation [59], future studies should also focus on VLDL-mediated oxylipin delivery to the skeletal muscle and their impact on cytokine production.

We have previously reported that PUFA-rich diets as well as pathological states like metabolic syndrome or statin-resistant hypertriglyceridemia change lipoprotein oxylipins and fatty acids composition [31,32]. Walnuts are rich in LA as well as aLA. In addition, walnuts provide a substantial amount of phenolics, vitamins, minerals,

and amino acids that on their own may be of benefit, however, their specific role, let alone potential interactive effects with walnut-derived fatty acids has yet to be defined [60]. Fish oil supplementation, abundant in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), causes increases in all LOX and CYP EPA- and DHA-derived oxylipins, at the expense of oxylipins derived from other PUFAs [31–33]. On the contrary, in the current study, short term walnut treatment increased the CYP-derived epoxy-fatty acids of aLA in lipoproteins, but not aLA derived mid-chain alcohols generated by LOX metabolism. Moreover, walnut treatment reduced all PUFAs mid-chain alcohols in lipoprotein which is not accompanied by decrease in corresponding epoxy-fatty acids. Importantly, walnut treatment decreased 9-HETE, a mid-chain alcohol auto-oxidation marker [20], suggesting that the changes in lipoprotein oxylipins caused by walnut are more than just an effect of parent PUFA enrichment. The presence of antioxidant or lipoxygenase inhibitors within the food matrix along with the lipids are therefore likely important contributors to the observed effects [61]. Consistent with this idea, phenolic compounds that are present in walnuts, like caffeic acid, have been shown to inhibit 5-lipoxygenase [62].

Walnut-induced changes in oxylipins seem to be lipoprotein specific. AA derived epoxides are uniquely enriched in HDL. HDL is involved in reverse cholesterol transport but also redistribute phospholipids and cholesterol esters [63,64], lipid classes that are rich in oxylipins. This lipoprotein specific action of walnuts should not be surprising, considering the distinct physiological function of lipoproteins, as well as the distinct origin of their lipid content. While VLDL is secreted by the liver, HDL obtain their lipids from tissues in the periphery [63,64]. The enrichment of AA, aLA and LA epoxides within HDL is noteworthy, as we have previously reported that the non-esterified forms were positively associated with microvascular function [34]. Moreover, in isolation, AA derived epoxides can act as vasodilators [65]. HDL has been reported to induce nitric oxide production, and promote apoptosis and anti-inflammatory effects, while limiting LDL oxidation within the vasculature to lower cardiovascular risk, with dysfunctional HDL increasing disease risk [66]. Due in part to technological limitations, there has been limited research describing the origin of oxylipins in lipoproteins, despite their apparent physiological function. The observed specific increase in the HDL AA epoxide pool may reflect changes in peripheral tissue oxylipin metabolism. While the functional arm of this study used LDL

to assess walnut-mediated impacts on peripheral tissue inflammation, an exploration of the anti-inflammatory effect of HDL on periphery is also warranted.

4.1. Limitations

This study was originally designed as “placebo”-controlled; however, due to the apparent placebo effect, low *n*, and high variability of the placebo group, this study was analyzed as change from the baseline. The properties of the placebo group also prevented us from analyzing the walnut treatment as a dose response, with the baseline as 0 g/day, placebo as 5 g/day and the treatment as 40 g/day. This study was performed in a group of hypercholesterolemic woman and therefore extrapolation of the findings to other subject should be done with caution. Also, cell culture experiments were performed on a single batch of primary cell line and are an approximation of the in vivo condition and therefore these results should be treated as a proof of principal, demonstrating a shift in potential interactions, and not as a demonstrated physiological response.

As epoxy PUFAs were not directly added to cells, the LDL aLA-derived epoxide levels and the interference with TNF α -stimulated cytokine production is only an association and not a demonstrated physiological action. The current findings justify future studies to evaluate the direct action of aLA-derived metabolites on adipocyte inflammatory responses. Additionally, lipoproteins consist of the complex matrix of lipids, lipid mediators and additional factors like mRNA. Therefore, focusing on one component of LDL rather than the whole composition could be misleading.

4.2. Conclusion

This study demonstrates that the consumption of a modest amount of walnuts for 4 weeks differentially changed lipoprotein fatty acid and oxylipin composition. This treatment affected LDL functionality in the context of adipocyte inflammation. Specifically, LDL oxylipin concentrations, but not those of their parent fatty acids, were strongly correlated with the inhibition of a pro-inflammatory cytokines secretion by adipocytes. These findings provide a functional demonstration of a food-lipoprotein-inflammatory physiology axis positively influenced by the daily consumption of walnuts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jnutbio.2019.03.004>.

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Chapter 4:

Effects of a Flavanol-rich Lychee Fruit Extract on Vascular Function in Healthy Men and Postmenopausal Women.

Title

Effects of a Flavanol-rich Lychee Fruit Extract on Vascular Function in Healthy Men and Postmenopausal Women.

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Introduction

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide, accounting for over 17 million deaths annually²⁰⁶. While traditional risk factors such as hypertension, obesity, and dyslipidemia contribute significantly to cardiovascular events, gender-specific risks also play a critical role²⁰⁷. In women, the risk of CVD sharply increases after menopause, mainly due to a decline in estrogen levels that negatively impacts vascular health, lipid profiles, and endothelial function^{29,208-211}. The loss of estrogen, which has vasodilatory, anti-inflammatory, and lipid-regulating properties, contributes to endothelial dysfunction, increased arterial stiffness, and unfavorable shifts in cholesterol levels²¹²⁻²¹⁴. Consequently, postmenopausal women experience an accelerated risk of hypertension, atherosclerosis, and coronary artery disease⁴⁶. Given the growing interest in lifestyle-based interventions, there is a need to explore safe, evidence-based strategies to improve cardiovascular health in this population.

Flavanols, a bioactive compound in cocoa, tea, fruits, and vegetables, have emerged as promising agents in managing cardiovascular risk²¹⁵. Clinical trials have demonstrated that flavanol consumption can lower blood pressure, improve arterial stiffness, and enhance vascular health, with these effects particularly beneficial in populations with compromised vascular function^{216,217}. Oligonol (OLG), a low-molecular-weight polyphenol derived from lychee fruit and green tea (Amino Up Chemical Co., Ltd., Sapporo, Japan), is standardized to contain more monomeric and oligomeric forms of catechins and proanthocyanidins^{218,219}. Its polyphenol fraction comprises approximately 15 to 20 % monomeric catechins, 8 to 12 % dimers, and 5 to 10 % trimers of proanthocyanidins²¹⁹. Evidence suggests that OLG provides metabolic benefits, including reductions in abdominal fat and improved lipid profiles, particularly among overweight

individuals^{220,221}. Additionally, *in vitro* studies have demonstrated its anti-inflammatory and antioxidant properties and its potential to enhance insulin sensitivity^{222,223}. While these findings are promising, more clinical studies are needed to confirm the health benefits of OLG across populations.

The objective of this study was to examine the effects of two-weeks of daily OLG intake on vascular and platelet function and cholesterol metabolism in postmenopausal women. The initial study utilized three different levels of OLG (i.e. 100, 200 and 600 mg) intake to determine if sustained daily consumption could improve the measured outcomes in a dose-dependent fashion. However, upon finding no significant change in vascular function at these levels of OLG intake, a follow-up study was conducted to test whether the acute intake of 2 g of OLG can improve vascular function in a healthy group of men.

Methods

Study population

Postmenopausal women between the ages of 52 and 65 and adult males between the ages of 18 and 35 were recruited from the greater Sacramento area through public advertisements. Participants were nonsmokers and were not prescribed medication except for those to treat thyroid conditions. They also self-reported no history of disease, including CVD or diabetes mellitus. In addition, only multivitamin/mineral formulas providing no greater than 100 percent of the recommended daily value were allowed for supplementation. Women were postmenopausal, defined as having no menstrual period during the past year, and a follicle-stimulating hormone (FSH) level between 23 and 116.3 mIU/mL. Both men and women were

required to have a body mass index (BMI) below 35 kg/m². Exclusion criteria included a platelet function analyzer (PFA)-100 closure time (CT) greater than 10 percent beyond the reference range for either the adenosine diphosphate (ADP)-collagen cartridge (71–118 seconds) or the epinephrine (EPI)-collagen cartridge (94–193 seconds). This criterion was used to exclude participants with impaired platelet function or other hemostatic abnormalities. Other reasons for exclusion were routine use of aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), regular participation in high-intensity exercise. All participants provided written informed consent, which was approved by the Institutional Review Board of the University of California, Davis.

Initial eligibility was assessed through a telephone interview. Those who met the preliminary criteria were invited to the Ragle Human Nutrition Research Center for a clinical screening. The screening process involved measuring PAT and PFA-100, and a blood draw for comprehensive metabolic and lipid panels. Once enrolled, participants were asked to refrain from taking NSAIDs, aspirin, or aspirin-like medications for one week prior to each study visit, avoid their usual exercise routine for 24 hours before each visit, and fast for 12 hours before each study day visit.

Study Design:

Primary Study: Effects of Two-weeks Daily Oligonol Intake On Vascular Function and Platelet Reactivity.

Eligible study participants were enrolled into a five-week, randomized, double-blind, crossover dietary intervention trial that evaluated the effects of two-weeks of daily OLG intake providing 100, 200, and 600 mg of OLG (OLG100, OLG200 and OLG600, respectively) (Figure 4.1). These amounts were selected to test if lower flavanol levels could replicate the acute and

sustained improvements reported in previous studies that delivered 371-963 mg of cocoa flavanols²²⁴. The intervention included a one-week run-in period followed by two two-week intervention phases separated by a one-week washout period. Beginning in the run-in period, participants were instructed to consume an instant black tea beverage containing 100 mg of total polyphenols (Naturex, South Hackensack, NJ) twice a day, once in the morning and again at least six hours later, and continuing tea intake until the end of the study. Study participants were randomized and allocated to their assignment by a web-based random number generator. The participants were instructed to consume one OLG or placebo capsule twice daily, with the black tea. Both OLG and placebo were provided in identical hard-shell capsules. Depending on their intervention assignment, the participants were provided with OLG capsules that contained half of the daily dose (either 50, 100, or 300 mg), while the placebo contained dextrin and maltodextrin.

Clinical assessments occurred at the initiation and conclusion of each two-week intervention phase. For each visit, study participants were asked to arrive at the facility in the morning after an overnight fast. Baseline PAT and blood samples were collected prior to the administration of the test product, consisting of the morning portion only of OLG intake, and consumed with the morning portion of black tea. This allowed for the evaluation of both sustained effects of consistent twice-daily intake and the possible acute effects observed after the morning intake. Two hours after the morning intake all outcome measures were repeated.

Participants were allowed to maintain their usual diet throughout the study. They were required to complete two 3-day food records, one during the week after the first study visit and a second the week after the third study visit. Each 3-day food record consisted of one weekend day and two consecutive weekdays. The collected food intake data were analyzed using the Food

Processor SQL software (version 10.1.0, ESHA Research, Salem, OR), and the average intake for each time point was calculated from these records.

Follow-Up Trial: Acute Effects of High-Flavanol Intake from Oligonol

The follow-up study was conducted using a randomized, crossover, double-blind design to examine the acute effects of 2 g of OLG compared to a placebo. The 2 g amount of OLG was estimated to provide 150 mg of epicatechin, which is approximately 2 mg of epicatechin per kg of BW for an average 70 kg person. Acute improvements in PAT response have been previously observed with the provision of epicatechin at 1-2 mg/kg body weight (BW) from a high-flavanol cocoa beverage⁶³. Two study-day visits were separated by a minimum three-day washout period. Following an overnight fast and a 30-minute rest, baseline PAT and blood samples were collected. Participants then consumed the assigned intervention. The PAT measurement and blood sample collection were repeated at two and four hours post OLG or placebo intake.

Assessment of vascular reactivity

Vascular reactivity was assessed by PAT using the Endo-PAT2000 (Itamar Medical Ltd., Caesarea, Israel) as previously described in detail¹⁰⁶. Prior to each PAT measurement, participants relaxed in a supine position for a minimum of 30 minutes in a temperature-controlled room. A thimble-shaped finger probe was mounted on the middle fingers of both hands, and a blood pressure cuff was placed on the forearm of the right arm. All PAT measurements were performed supine with both arms resting at heart level. The PAT test involves a 5 to 10-minute baseline measurement, followed by 5 minutes of arterial occlusion induced by inflating a blood pressure cuff on the forearm above systolic pressure. After this

occlusion period, the cuff is deflated, initiating a 5-minute reactive hyperemia phase in the same arm. The system software automatically calculates the reactive hyperemia index (RHI) as the ratio of the average pulse wave amplitude (PWA) during the 1-minute period following the first minute of hyperemia (after cuff release) to the average PWA during the 3.5-minute baseline period. This ratio is further adjusted for any changes observed in the control arm to provide an accurate measure of vascular reactivity. The logarithmic scaled RHI, Framingham reactive hyperemia index (fRHI), was also calculated as the ratio between the pulse wave during reactive hyperemia and at baseline⁸⁵.

Assessment of arterial stiffness

PAT also calculates an augmentation index (AI) as a measure of arterial stiffness, which calculates the percentage of pulse waveform that is reflected back from the downstream vasculature. AI is calculated as the difference between the first (P_1) and second (P_2) systolic peaks (representing the forward and reflected waves) divided by the height of the first systolic peak (P_1), expressed as a percentage (i.e., $[P_2 - P_1]/P_1 \times 100\%$)⁹⁵. The AI value is adjusted to a heart rate of 75 beats per minute (AI@75) to account for the influence of heart rate variability⁹⁴. Higher AI values indicate increased arterial stiffness, which is associated with an increased CVD risk^{95,225,226}.

Assessment of platelet reactivity

Platelet reactivity was assessed using the PFA-100 (Siemens, Deerfield, IL), as previously described²²⁷. Briefly, 800 μ l of citrated whole blood (3.8% sodium citrate) was loaded into a disposable PFA-100 test cartridge containing a collagen-coated membrane with

either ADP or EPI as platelet agonists. The PFA-100 measures platelet function by determining the time required for platelet aggregation to occlude the aperture of the membrane under shear stress, simulating in vivo conditions. CT is recorded in seconds, with a maximum value of 300 seconds. Longer CT indicate reduced platelet reactivity, reflecting impaired platelet aggregation, while shorter times suggest enhanced reactivity.

Clinical blood parameters

Blood samples were analyzed for lipids and chemistry panels at the UCD Medical Center Pathology Department (Sacramento, CA).

Plasma flavanol assessment

Flavanols were extracted from plasma and analyzed using high-pressure liquid chromatography (HPLC) analysis as described elsewhere, with some modifications. Briefly, plasma samples stored at -80 °C were thawed on ice^{224,228}. Fifty µL of 1.2 M acetic acid was added to each 14 mL centrifuge tube plasma, followed by 1 mL of plasma and 50 µL of recovery standard solution (40 µM 3'-O-Ethylepicatechin in 25% methanol). Each sample was subjected to enzyme treatment to cleave glucuronidated and/or sulfated metabolites. One-hundred µL glucuronidase solution (with sulfatase activity) was added to each sample, and the samples were vortexed gently, flushed with argon, capped securely, and incubated in a 37 °C water bath for 40 min. At the end of 40 min. The samples were immediately cooled on ice, and 2 mL of 100% acidified methanol (0.5% acetic acid in methanol) was added to each sample. The samples were briefly vortexed and left on ice in the dark for 30 min. The samples were centrifuged at ~12,000 x g at 4 °C for 15 min, and the supernatant fraction was transferred to a new 14 mL centrifuge

tube. The remaining pellets were resuspended in 2 mL 100% acidified methanol, centrifuged as described above, and the supernatant fraction was transferred to the tubes containing the first supernatant. The pellets were resuspended in 1 mL 50% acidified methanol, centrifuged, and the resulting supernatant fraction was transferred to the tubes containing the previous supernatants. The combined supernatant was stored at -80 °C overnight to allow further protein precipitation and then centrifuged at ~16,000 x g at 4 °C for 15 min. The resulting supernatant fraction was transferred to 13x100 mm test tubes and concentrated to a volume of 1 – 1.2 mL in a SpeedVac concentrator (ThermoSavant, Holbrook, NY). The sample concentrates were transferred into 1.5 mL siliconized (low retention) Eppendorf microfuge tubes and further concentrated to a final volume of ~50 µL. The resulting concentrate and pellet were suspended in 50 µL 100% acidified methanol, flushed with argon, and stored at -80 °C until HPLC analysis.

Statistical analysis

Primary Study: Effects of Two-weeks Daily Oligonol Intake On Vascular Function and Platelet Reactivity.

Data were tested for normality and homogeneity of variance, and the scaled range was transformed as necessary. Results were represented as mean $\pm$ standard deviation (SD). Paired t-tests assessed the potential between group differences at baseline. The sample size was calculated from the previous study that observed dietary supplementation-induced changes in RHI in healthy adults using the expected mean difference of 0.5, a standard deviation of 0.17, and the alpha level at 0.05⁶³. A total sample size of 24 was determined to provide sufficient power at 0.8 to detect a significant difference within-between interactions with analysis of variance with repeated measures.

A mixed-effects model was used to examine the effects of treatment and time on the measure of vascular outcomes and lipid panels while controlling for cholesterol levels and accounting for individual variation. The model included the group and time as fixed factors, their interaction, cholesterol as a covariate, and the individuals as a random intercept. P-values for the fixed factors were obtained by using Satterthwaite's method. P-values of < 0.05 were considered statistically significant. All analyses were performed using the R statistical software (R studio version 2024.12).

Follow-Up Trial: Acute Effects of High-Flavanol Intake from Oligonol

Data from the follow-up study with 2 g of OLG were tested for normality, sphericity, and homogeneity of variance. Data are expressed as mean $\pm$ SD. Paired t-tests assessed the potential between group differences at baseline. Statistical analyses were conducted by repeated measures analysis of variance (ANOVA) with a Tukey's post hoc test for multiple comparisons when necessary. Since the number of subjects in the follow-up study was insufficient to meet a power level above 80%, the results need to be interpreted with extra caution due to the possibility of a false positive or negative rejection of the hypothesis. P-values of < 0.05 were considered statistically significant. All analyses were performed using the R statistical software (R studio version 2024.12).

Results

Primary Study: Effects of Two-weeks Daily Oligonol Intake On Vascular Function and Platelet Reactivity.

Population characteristics

Thirty-eight participants were enrolled in the study (Figure 4.2). Four individuals withdrew during the study, and two participants were excluded from the final analysis due to medication use that could affect the study outcome measures. Therefore, a total of 32 participants (n=9 in OLG100, n=12 in OLG200, and n=11 in OLG600) were used in the statistical analysis. Baseline characteristics between groups were similar (Table 4.1). Based on self-reports and the investigator's package tally, compliance was greater than 80%. Dietary intake of energy, macronutrients, fats, minerals, and vitamins did not change significantly between the two intervention phases or between OLG groups. Folate intake in the OLG200 group was significantly higher than in the OLG100 group at visit 1 (Table 4.2).

Microvascular function

At baseline, fasting RHI for the participants was at a level associated with low cardiovascular risk and was not significantly different among groups (Table 4.3)⁸⁷. Statistical analysis showed significant main effects for time regarding AI, AI@75, and fRHI, indicating that these vascular markers changed between the fasting (Hour 0) and two hours post-intake regardless of the treatment. AI increased from 31.60 ± 15.89 at wk0_0h to 38.32 ± 18.66 at wk0_2h and 39.38 ± 19.40 at wk2_2h, while AI@75 increased from 21.59 ± 17.08 at wk0_0h to 27.21 ± 19.32 at wk0_2h and 28.26 ± 19.35 at wk2_2h. fRHI also showed an increase from 0.80 ± 0.35 at wk0_0h to 0.96 ± 0.33 at wk0_2h, followed by 0.78 ± 0.39 at wk2_0h and 0.89 ± 0.39 at 2_2h. Additionally, overall differences in treatment effect between the OLG and placebo were observed for RHI and fRHI. Pairwise comparisons showed significant differences between OLG100 and placebo200 (2.89 ± 0.51 vs 2.17 ± 0.59) and between OLG200 and placebo200

(2.42 ± 0.52 vs 2.17 ± 0.59) for RHI. For fRHI, OLG100 differed from placebo 200 (1.10 ± 0.29 vs 0.68 ± 0.40), as did OLG200 from placebo200 (0.87 ± 0.34 vs 0.68 ± 0.40). However, there was no significant time x treatment interaction for any measures. While vascular markers changed during the study, the daily consumption of OLG for two weeks did not lead to a statistically significant improvement in microvascular function at any of the OLG levels (Table 4.3).

Platelet function

All three groups showed no significant differences after consuming OLG for 2 hours and 2 weeks (Table 4.3).

Blood pressure (BP), Fasting glucose, and Lipid panels

No significant changes were observed in systolic (SBP) or diastolic blood pressure (DBP) in any group before and after two-weeks of OLG consumption (Table 4.4). Neither fasting glucose nor HbA1c changed significantly at any level. No significant changes were shown after 2 weeks of OLG intake for fasting levels of total cholesterol, LDL, HDL, and TG.

Follow-Up Trial: Acute Effects of High Flavanol-Intake from Oligonol

Subject characteristics

Eight participants (three males and five postmenopausal females) were enrolled and completed the study. Data from all subjects were used in the analysis. No self-reported adverse events were noted, although two participants mentioned feeling nausea within minutes after consuming the OLG capsules, which dissipated within 30 minutes. Except for age, the baseline

characteristics were similar between the sexes and treatment groups (Table 4.5). Based on baseline RHI, subjects were considered low in cardiovascular risk. One subject had a Total Cholesterol:LDL ratio above four with elevated triglycerides and a low level of HDL. Despite these lipid profiles, the subject's other clinical parameters remained within healthy ranges. One female subject had a BMI between 30 and 35 kg/m².

Vascular reactivity

Both OLG and placebo consumption did not significantly change any measure of RHI, AI, AI@75 over time (Table 4.6).

Platelet function analysis

No significant changes in either ADP- or EPI-collagen closure time after OLG intake were observed (Table 4.6).

Plasma flavanol concentration

Total plasma epicatechin metabolites (i.e., sum of epicatechin and methylated epicatechin metabolites after enzyme treatment) ranged from 244 to 971 nM with a mean of 597 ± 87 nM two hours after 2 gm of OLG intake (Figure 4.3).

Flavanol content in Oligonol

Based on initial manufacturer specifications, OLG was expected to contain 7.5% epicatechin. This leads to a predicted supplemental intake of 1.7-2.4 mg/kg BW. However, analysis of four sets of OLG capsules used in the study revealed that the actual epicatechin

content was approximately 4.14 % (Table 4.7). Thus, the actual amount of epicatechin delivered in the follow-up study was significantly lower than anticipated, ranging from 0.92 to 1.33 mg/kg BW.

Discussion

We investigated the effects of 100, 200, and 600 mg of daily OLG intake over two weeks and observed no significant effects on vascular health or platelet reactivity in postmenopausal women. Vascular tone was assessed via PAT, which provided measurements for the RHI, AI, AI@75, and fRHI. No significant changes were observed in RHI or fRHI between supplemental levels. While RHI serves as a standard measure of the ability of blood vessels to dilate in response to increased blood flow, fRHI provides a transformed value that Hamburg et al. found to strongly correlate with cardiovascular risk factors such as age, obesity, and hypercholesterolemia⁸⁵.

The endothelium maintains vascular homeostasis by releasing vasoactive substances, including nitric oxide (NO), which regulate vascular tone²²⁹. Risk factors associated with menopause typically impair this regulation, leading to structural alterations and increased arterial stiffness^{46,50,51}. However, our assessment of AI and AI@75 showed no significant changes following OLG consumption. The absence of improvements in vascular response observed may be attributed to the baseline health status of our participants. In the presence of cardiovascular risk factors, RHI values typically decline linearly^{85,230}. While several studies propose RHI cutoff values between 1.5 and 1.7 to detect endothelial dysfunction, our participants exhibited a healthy baseline average RHI of 2.46²³¹. Given that individuals with an RHI above 2.08 are at

significantly lower risk for adverse cardiovascular events, the potential for measurable improvement was likely constrained by the already optimal baseline vascular reactivity^{231,232}.

Beneficial effects of OLG consumption have been reported previously, including reductions in triglycerides, body weight, abdominal circumference, and visceral fat^{220,221}. However, the current study observed no significant changes in these measures. This discrepancy may be due to the shorter intervention period of two weeks used in the present study, compared to the 10–12-week durations in previous trials^{220,221}. In addition, a minimum of four-week period is typically required for dietary interventions to observe significant shifts in lipid parameters²³³.

The results in the present studies are in contrast with earlier findings demonstrating improved PAT and platelet function after the consumption of high-flavanol and procyanidin cocoa beverages^{63,234}. One discrepancy may be the total amount of bioactive epicatechin provided in the current trial. While cocoa studies utilized amounts ranging from 70 to 203 mg of epicatechin, the supplemental levels in the initial phase of this study provided significantly lower amounts, ranging from 3.75 to 22.5 mg^{63,224,234}.

Because previous studies suggests that vascular improvements with flavanol-rich food intake are related to the total amount of (-)-epicatechin intake and its subsequent plasma metabolites, a follow-up study was conducted where healthy study participants were given 2 g of OLG^{63,82,224,227}. This was intended to provide an equivalent intake of epicatechin to 1 to 2 mg/kg BW a levels of epicatechin intake observed to produce an acute vascular response after flavanol-rich cocoa intake^{63,224,234}. Despite this increased flavanol intake, no significant changes in PAT or platelet function were observed two- or four-hour after OLG consumption.

The lack of responses in the follow-up study may be explained by an analytical discrepancy regarding the extract's composition. While initial calculations were based on an

expected epicatechin content of 7.5%, subsequent analysis revealed the actual content was 4.14%. Consequently, the actual amount of epicatechin delivered in the 2 g follow-up study was 0.92 to 1.33 mg/kg BW, which was much lower than the target concentrations in previous interventions. The analysis of plasma metabolites further explains the discrepancy in the follow-up study. Two hours after the 2 g OLG intake, total plasma epicatechin metabolites ranged from 244 to 971 nM. In contrast, previous interventions using purified (-)-epicatechin at similar levels (1-2 mg/kg BW) resulted in average plasma concentrations of approximately 2.0 μ M⁶³.

A limitation of the current trials is that we cannot account for the specific epimer of flavanols consumed. While this study measured total epicatechin within OLG, research has demonstrated that the vascular response is highly dependent on the specific epimer epicatechin, showing that (-)-epicatechin has significantly greater bioactivity in improving endothelial function compared to its counterparts (+)-epicatechin or catechin epimers¹⁶⁰. The OLG for the current trials was characterized using reverse-phase HPLC, which does not distinguish between these epimer forms. Furthermore, the reliance on enzymatic hydrolysis using glucuronidase and sulfatase for subsequent metabolite analysis likely led to an underestimation of the circulating metabolites, as Ottaviani et al. suggests that commercial enzyme treatments are incomplete and inefficient²³⁵. Recent standard methodologies utilize LC-MS/MS provides a more precise metabolic assessment¹⁶³.

While the metabolism, metabolites, and bioactivity of (-)-epicatechin following consumption have been well documented^{63,160}, the unique mixture of flavanols and procyanidins in OLG has not yet been thoroughly analyzed. Additionally, inter-individual variability in flavonoid metabolism should be considered. The metabolism and bioavailability of flavonoids can vary widely between individuals due to factors such as gut microbiome composition and

genetic variations^{236,237}. These differences affect absorption rates, biotransformation into active metabolites, and clearance times, leading to variability in physiological responses¹⁶⁵. As a result, some individuals may experience noticeable benefits from flavonoid consumption, while others may show minimal or no effects²³⁸.

Additional confounders within our short-term (two-week) trial include the daily provision of black tea (at 200 mg of total polyphenol per day) beginning one week before and throughout the entire study period, to include the washout period. Black tea has been known to be a great source of flavonoids. Studies report of a benefit of black tea intake on endothelial function in healthy individuals and patients with CAD^{83,239}. In addition to tea flavonoids, black tea contains caffeine, known to affect vascular tone²⁴⁰. Hyperemia-induced endothelial function improved after consuming caffeine acutely and chronically²⁴¹. In the present study, little information is provided on the contents of the black tea provided. Since assessments of PAT and PFA were not taken on the first day of drinking black tea, we cannot rule out that black tea provided seven days prior to the first study visit affected our measures of vascular function.

The mode of flavanol delivery (e.g., capsules vs. beverages) could possibly explain a discrepancy between studies. Liquid formulations used in the cocoa beverage studies are readily absorbed, whereas capsules require breakdown in the digestive system, leading to a slower absorption rate. However, there is limited data comparing different nutrient delivery methods. The efficacy of various supplement formulations remains largely untested. A study on vitamin C reported that the liquid form resulted in higher plasma concentrations than other forms²⁴². While this suggests enhanced absorption, further research is needed to confirm these findings across different nutrients. In the previous study, epicatechin metabolites peaked at 2 hours post-consumption and gradually declined. However, absorption and metabolism rates may vary

among different combinations of nutrients. Our analysis measured epicatechin metabolites 2 hours after OLG consumption but lacked additional time points, limiting conclusions about its metabolic profile.

Lastly, the power level to observed vascular effects after two weeks of OLG intake was under powered. Based on previous data, the sample size needed to meet 80% power was 32 participants. Based on the results of the current study, the power level needed for three levels of OLG with black tea was 15 %, considerably underpowered. Therefore, a minimum of forty-four subjects per group will be needed to avoid a Type II error or false negative.

Conclusion

We observed no impact on vascular health and platelet reactivity with either short-term or acute OLG intake in healthy men and postmenopausal women. The absence of benefits from OLG intake could be attributed to several factors. Firstly, the participants exhibited healthy vascular function at baseline, which can limit the potential for further improvement. Secondly, the bioactive epicatechin content in OLG was significantly lower than studies with reported positive effects. Study limitations include a small sample size and limited characterization of OLG, particularly for (-)-epicatechin content. In conclusion, while this study did not reveal significant cardiovascular benefits from OLG intake, it highlighted the importance of dosage, baseline health status, and individual variability in the effectiveness of dietary supplements.

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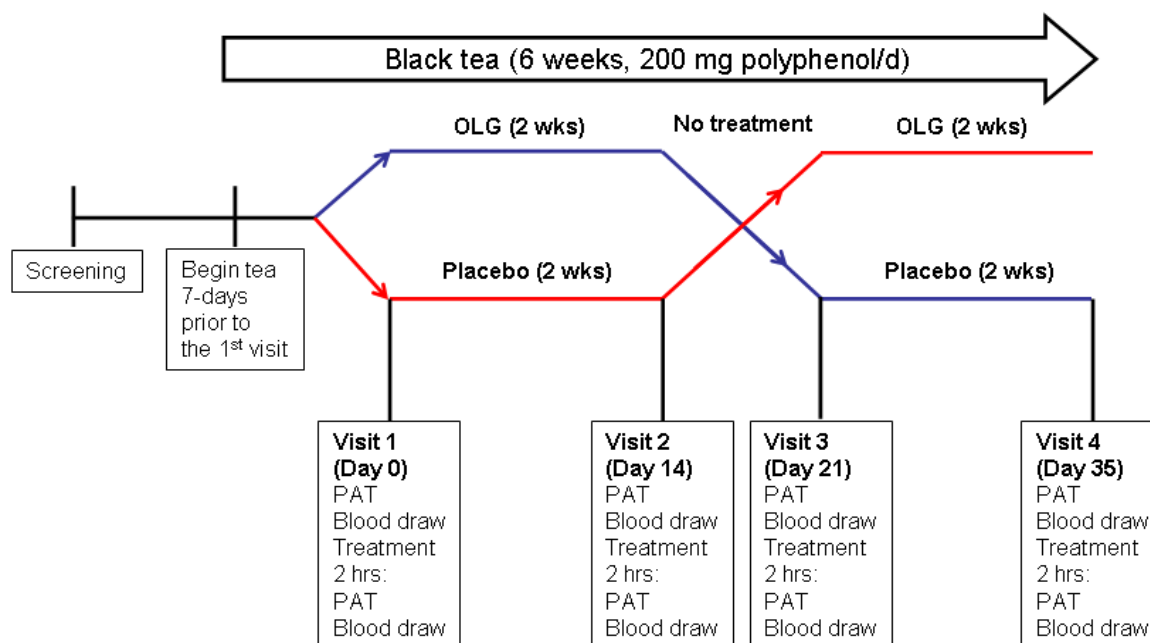


Figure 4.1. Study design of the primary study

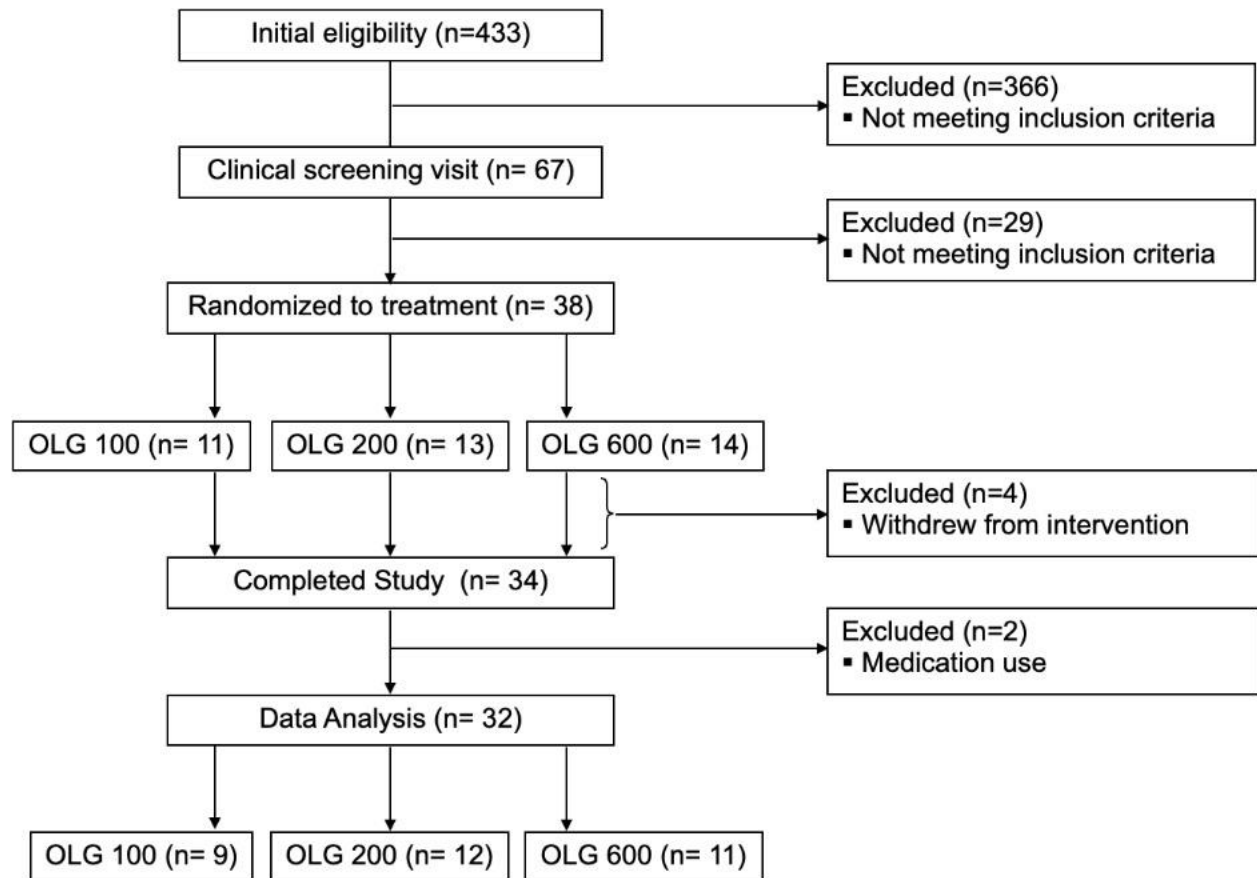


Figure 4.2. Participant flow chart

Table 4.1. Baseline characteristics of participants in Oligonol with black tea

	OLG100 (n= 9)	OLG200 (n=11)	OLG600 (n= 12)
Age	55.3 ± 2.7	54.9 ± 3.4	56.3 ± 2.7
BMI (kg/m ²)	25.4 ± 4.5	27.2 ± 4.8	26.0 ± 3.6
WC (cm)	82.8 ± 11.8	89.5 ± 10.9	86.5 ± 10.0
ST SBP (mmHg)	122.7 ± 13.8	124.5 ± 15.8	116.3 ± 10.7
ST DBP (mmHg)	74.9 ± 9.4	77.4 ± 10.8	74.9 ± 9.1
ST HR (bpm)	66.1 ± 6.4	66.5 ± 10.4	67.0 ± 6.7
SP SBP (mmHg)	113.2 ± 13.0	112.2 ± 10.2	112.0 ± 13.0
SP DBP (mmHg)	67.2 ± 7.0	68.9 ± 6.8	69.8 ± 8.4
SP HR (bpm)	59.1 ± 5.7	58.0 ± 5.7	58.7 ± 4.4
Glc (mg/dL)	95.8 ± 7.4	96.7 ± 4.0	96.1 ± 5.5
Chol (mg/dL)	234.1 ± 38.3	218.7 ± 38.8	207.6 ± 28.7
HDL (mg/dL)	59.2 ± 19.0	52.6 ± 13.1	46.9 ± 11.6
LDL (mg/dL)	156.1 ± 27.5	145.8 ± 33.2	140.8 ± 16.7
Chol:HDL	4.2 ± 1.2	4.3 ± 0.9	4.6 ± 0.9
TG (mg/dL)	93.5 ± 48.2	101.2 ± 52.3	100.3 ± 35.6
RHI	2.8 ± 0.4	2.3 ± 0.6	2.7 ± 0.7
AI	34.6 ± 16.8	32.5 ± 18.1	28.8 ± 15.1
AI75	25.1 ± 16.0	20.9 ± 19.5	18.8 ± 17.2
fRHI	1.0 ± 0.2	0.7 ± 0.4	0.8 ± 0.3
PFA-ADP closure time (s)	94.6 ± 11.2	86.7 ± 14.0	94.1 ± 17.8
PFA-EPI closure time (s)	130.7 ± 20.2	138.6 ± 46.9	147.4 ± 62.2

No significant differences were observed between groups by ANOVA. BMI, body mass index; WC, waist circumference; ST, sitting; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; SP, supine; Glc, fasting glucose; Chol, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Chol:HDL, the ratio of total cholesterol to HDL; TG, triglyceride; RHI, reactive hyperemia index; AI, augmentation index; AI75, augmentation index at heart rate of 75 beats per minute; fRHI, Framingham reactive hyperemia index; PFA, platelet function analyzer; ADP, adenosine diphosphate; and EPI, epinephrine. Values are presented as mean ± SD.

Table 4.2. Dietary nutrient intake for participants in each treatment group

	OLG100 (n=9)		OLG200 (n=12)		OLG600 (n=11)	
	Week0	Week2	Week0	Week2	Week0	Week2
Total Calories (kcal)	1401.8 ± 59.2	1640.5 ± 58.2	1630.5 ± 32.0	1544.2 ± 47.0	1530.3 ± 61.2	1555.1 ± 75.9
Protein (g)	57.6 ± 2.7	73.8 ± 3.2	70.5 ± 1.8	59.1 ± 2.2	69.2 ± 4.9	70.2 ± 5.7
Carbohydrates (g)	74.3 ± 4.3	181.6 ± 9.1	85.6 ± 2.5	218.3 ± 4.9	68.3 ± 2.1	168.4 ± 5.6
Total fat (g)	60.2 ± 3.0	66.9 ± 2.8	51.7 ± 1.4	47.0 ± 2.1	54.8 ± 3.2	59.6 ± 3.8
Saturated (g)	19.6 ± 1.2	20.4 ± 1.0	13.8 ± 0.6	12.4 ± 0.6	15.4 ± 1.1	17.1 ± 1.2
Mono (g)	17.1 ± 1.2	17.9 ± 0.9	12.5 ± 0.3	11.6 ± 0.6	17.1 ± 1.4	17.4 ± 1.5
Poly (g)	8.2 ± 0.5	9.3 ± 0.4	8.6 ± 0.4	7.4 ± 0.5	9.2 ± 0.6	10.9 ± 0.5
Cholesterol (mg)	200.6 ± 12.8	279.3 ± 21.9	139.7 ± 7.1	168.8 ± 11.2	198.2 ± 16.0	195.6 ± 18.7
Vitamin C (mg)	76.9 ± 5.6	89.3 ± 6.8	87.0 ± 5.6	87.2 ± 6.5	96.6 ± 6.5	87.7 ± 7.3
Vitamin E (mg)	5.5 ± 0.4	7.7 ± 0.9	5.1 ± 0.2	4.1 ± 0.1	5.4 ± 0.3	5.8 ± 0.4
Folate (mcg)	183.7 ± 10.1	224.0 ± 9.1	341.8 ± 12.0 [§]	191.8 ± 7.8	243.8 ± 15.8	230.4 ± 18.0
Mg (mg)	187.5 ± 9.5	194.2 ± 5.9	192.6 ± 7.3	151.9 ± 3.7	183.2 ± 7.6	172.3 ± 7.1
Ca (mg)	609.8 ± 23.9	708.7 ± 30.8	804.1 ± 24.9	696.5 ± 30.0	595.9 ± 27.9	659.2 ± 30.8
K (mg)	1820.0 ± 80.5	1740.2 ± 63.9	2516.3 ± 123.1	1734.1 ± 55.9	1874.5 ± 83.1	1780.2 ± 77.5
Na (mg)	1861.2 ± 86.1	3618.9 ± 460.6	2156.5 ± 55.7	2517.6 ± 113.7	3139.7 ± 234.9	2928.1 ± 254.4

No significant differences for any variables were found either between visits or among groups by ANOVA. §, $p < 0.05$ vs. OLG100 week 1. Values are presented as mean $\pm$ SD

Table 4.3. Two-week and 2-hour post-prandial changes in vascular and platelet function

Group	Treatment	Time	RHI	AI	AI75	fRHI	PFA-ADP (s)	PFA-EPI (s)		
OLG100	OLG	Week0	Hour0	2.8 ± 0.4	34.6 ± 12.5	26.2 ± 17.5	1.1 ± 0.4	93.8 ± 13.6	127.8 ± 17.7	
			Hour2	3.1 ± 0.5	38.8 ± 22.7	29.3 ± 22.0	1.1 ± 0.3	89.7 ± 17.1	127.9 ± 17.4	
		Week2	Hour0	2.9 ± 0.6	33.8 ± 16.1	24.1 ± 15.4	1.1 ± 0.3	92.3 ± 13.0	123.9 ± 17.0	
			Hour2	2.8 ± 0.5	48.0 ± 16.9	37.2 ± 17.4	1.1 ± 0.2	94.4 ± 16.9	134.1 ± 36.9	
		Placebo	Week0	Hour0	2.5 ± 0.6	33.3 ± 13.3	24.1 ± 13.8	0.8 ± 0.4	95.0 ± 12.0	133.2 ± 18.5
				Hour2	2.8 ± 0.7	37.1 ± 12.1	26.0 ± 12.6	1.0 ± 0.4	95.3 ± 12.1	127.3 ± 12.2
	Week2		Hour0	2.6 ± 0.3	29.2 ± 14.0	19.7 ± 15.3	1.0 ± 0.3	98.6 ± 23.9	122.8 ± 19.1	
			Hour2	2.6 ± 0.6	42.5 ± 18.4	32.4 ± 17.3	1.0 ± 0.4	90.3 ± 12.5	125.5 ± 21.2	
	OLG200	OLG	Week0	Hour0	2.3 ± 0.6	34.0 ± 19.3	22.4 ± 20.2	0.8 ± 0.3	86.8 ± 9.9	141.8 ± 46.2
				Hour2	2.5 ± 0.4	44.6 ± 21.5	32.8 ± 21.0	1.0 ± 0.3	88.0 ± 16.9	157.4 ± 74.2
			Week2	Hour0	2.5 ± 0.5	28.1 ± 12.9	17.7 ± 14.1	0.8 ± 0.3	85.6 ± 16.2	128.6 ± 29.5
				Hour2	2.4 ± 0.7	37.9 ± 16.3	26.5 ± 16.3	0.9 ± 0.4	93.4 ± 17.7	136.4 ± 58.4
Placebo			Week0	Hour0	2.3 ± 0.6	32.1 ± 18.5	21.4 ± 19.7	0.7 ± 0.3	92.2 ± 18.0	145.5 ± 58.7
				Hour2	2.3 ± 0.6	37.7 ± 17.1	25.9 ± 19.3	0.8 ± 0.4	92.4 ± 17.7	129.2 ± 31.9
		Week2	Hour0	2.0 ± 0.6	29.2 ± 15.0	19.0 ± 16.0	0.5 ± 0.4	97.2 ± 22.6	135.2 ± 33.1	
			Hour2	2.1 ± 0.6	34.2 ± 17.8	22.0 ± 18.2	0.7 ± 0.5	95.2 ± 17.2	148.7 ± 62.1	
OLG600		OLG	Week0	Hour0	2.8 ± 0.6	28.4 ± 12.1	18.6 ± 16.0	0.9 ± 0.3	92.3 ± 14.1	137.1 ± 39.9
				Hour2	2.6 ± 0.7	35.6 ± 21.1	24.6 ± 22.4	0.9 ± 0.2	92.7 ± 18.2	313.4 ± 32.5
			Week2	Hour0	2.4 ± 0.6	31.6 ± 21.5	21.3 ± 22.2	0.7 ± 0.4	88.8 ± 18.9	139.4 ± 50.2

Placebo	Week0	Hour2	2.4 ± 0.6	38.0 ± 24.0	26.2 ± 23.9	0.8 ± 0.4	95.7 ± 17.0	126.8 ± 32.5
		Hour0	2.4 ± 0.7	27.9 ± 14.3	17.9 ± 16.0	0.6 ± 0.4	98.3 ± 19.0	143.2 ± 60.6
	Week2	Hour2	2.7 ± 0.7	35.2 ± 17.6	24.1 ± 19.4	0.9 ± 0.2	96.8 ± 23.4	145.7 ± 61.6
		Hour0	2.4 ± 0.8	27.4 ± 17.1	17.7 ± 17.8	0.7 ± 0.4	99.0 ± 19.0	153.7 ± 36.4
		Hour2	2.6 ± 0.5	37.9 ± 23.5	27.8 ± 22.7	0.9 ± 0.2	97.0 ± 12.8	144.2 ± 39.1
	P-value (Time)		0.244	0.000	0.003	0.001	0.427	0.757
	P-value (Treatment)		0.004	0.574	0.680	0.003	0.161	0.267
	P-value (Time x Treatment)		0.973	0.594	0.605	0.921	0.538	0.455

No significant differences were found in the time × treatment interaction effects for any variables. OLG, oligonol, SBP, systolic blood pressure; DBP, diastolic blood pressure; Glc, fasting glucose; Chol, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Chol:HDL, ratio of Chol to HDL; TG, triglyceride; RHI, reactive hyperemia index; AI, augmentation index; AI75, augmentation index at heart rate of 75 beats per minute; fRHI, Framingham reactive hyperemia index; PFA, platelet function analyzer; ADP, adenosine diphosphate; and EPI, epinephrine. Values are presented as mean ± SD.

Table 4.4. Two-week postprandial changes in cardiometabolic outcomes

Treatment Time	OLG100				OLG200				OLG600			
	OLG100		Placebo		OLG200		Placebo		OLG600		Placebo	
	wk0	wk2	wk0	wk2	wk0	wk2	wk0	wk2	wk0	wk2	wk0	wk2
ST SBP (mm Hg)	124. 1 ± 15.1	122. 7 ± 10.4	119. 3 ± 14.2	122. 9 ± 11.5	122. 2 ± 17.5	119. 9 ± 12.9	124. 5 ± 13.7	121. 6 ± 12.3	118. 7 ± 9.6	116. 3 ± 9.6	117. 2 ± 10.1	121. 7 ± 8.2
ST DBP (mm Hg)	74.8 ± 7.7	72.6 ± 4.6	72.7 ± 9.0	74.2 ± 7.2	75.5 ± 11.3	72.4 ± 7.8	77.0 ± 8.6	77.8 ± 8.9	76.1 ± 8.6	75.5 ± 7.7	76.5 ± 8.0	76.9 ± 6.6
ST HR (bp m)	68.1 ± 9.2	64.9 ± 5.9	68.5 ± 4.5	67.5 ± 7.3	66.6 ± 8.8	64.1 ± 8.8	66.8 ± 10.4	66.5 ± 8.4	68.9 ± 8.4	65.4 ± 7.7	67.6 ± 7.7	67.7 ± 6.3
ST MAP (mm Hg)	91.2 ± 9.8	89.3 ± 6.0	88.2 ± 10.5	90.5 ± 8.3	91.1 v 12.7	88.3 ± 9.0	92.8 ± 9.5	92.4 ± 9.5	90.3 ± 8.7	89.1 ± 8.1	90.1 ± 8.0	91.8 ± 5.9
SP SBP (mm Hg)	117. 5 ± 16.2	112. 8 ± 11.1	111. 0 ± 12.7	112. 6 ± 15.4	112. 6 ± 10.7	113. 7 ± 9.0	112. 7 ± 11.3	114. 7 ± 13.2	109. 7 ± 12.3	113. 7 ± 10.0	111. 0 ± 10.0	115. 2 ± 7.9
SP DBP (mm Hg)	69.7 ± 9.7	66.3 ± 5.6	66.3 ± 6.6	66.9 ± 9.4	68.3 ± 7.2	68.2 ± 7.0	69.1 ± 7.5	70.3 ± 7.9	68.0 ± 7.5	69.8 ± 8.0	70.2 ± 7.1	72.0 ± 5.7
SP HR (bp m)	63.4 ± 12.1	60.1 ± 6.5	59.6 ± 5.0	61.1 ± 4.9	57.2 ± 4.6	58.4 ± 5.7	58.0 ± 6.8	59.1 ± 6.0	59.1 ± 7.7	58.1 ± 4.8	59.4 ± 5.9	61.1 ± 5.8

SP MAP (mm Hg)	85.6 ± 11.6	81.8 ± 7.2	81.2 ± 8.5	82.2 ± 11.2	83.1 ± 7.9	83.4 ± 7.3	83.6 ± 8.3	85.1 ± 9.3	81.9 ± 8.7	84.4 ± 8.1	83.8 ± 7.7	86.4 ± 5.8
Glc (mg/ dL)	98.1 ± 6.4	95.1 ± 4.5	94.4 ± 5.4	96.0 ± 6.0	96.5 ± 4.6	98.3 ± 8.4	96.2 ± 4.6	97.4 ± 6.1	94.9 ± 4.7	96.3 ± 3.1	98.2 ± 3.7	96.4 ± 4.6
Chol (mg/ dL)	237. 0 ± 39.9	232. 8 ± 44.3	226. 8 ± 37.0	226. 7 ± 40.4	204. 1 ± 25.7	208. 9 ± 34.6	226. 0 ± 45.8	221. 8 ± 28.1	205. 9 ± 30.0	195. 9 ± 21.2	202. 0 ± 26.2	215. 5 ± 24.0
HDL (mg/ dL)	59.8 ± 19.3	60.2 ± 16.2	58.3 ± 15.5	58.9 ± 13.4	49.7 ± 10.5	49.7 ± 9.1	54.2 ± 10.3	51.3 ± 10.1	50.1 ± 15.7	46.4 ± 11.3	44.4 ± 12.4	49.6 ± 16.8
LDL (mg/ dL)	158. 9 ± 29.7	156. 0 ± 37.9	151. 1 ± 29.8	151. 7 ± 35.7	134. 2 ± 23.3	138. 4 ± 28.4	150. 7 ± 40.2	142. 4 ± 23.0	135. 7 ± 17.6	130. 3 ± 17.7	139. 2 ± 18.9	144. 8 ± 20.7
TG (mg/ dL)	91.9 ± 41.8	82.4 ± 38.9	87 ± 39.6	79.9 ± 33.8	100. 7 ± 49.2	104. 0 ± 44.4	100. 8 ± 43.9	90.7 ± 40.9	101. 3 ± 62.1	95.5 ± 45.3	92.3 ± 32.7	105. 1 ± 55.1
HbA 1C (mm ol/L)	5.5 ± 0.2	5.6 ± 0.1	5.7 ± 0.2	5.6 ± 0.2	5.6 ± 0.3	5.6 ± 0.2	5.6 ± 0.3	5.5 ± 0.3	5.7 ± 0.3	5.7 ± 0.3	5.8 ± 0.4	5.8 ± 0.4

No significant differences for any variables were found either between visits or among groups. Wk, week; ST, sitting; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; SP, supine; Glc, fasting glucose; Chol, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and TG, triglyceride. Values are presented as mean $\pm$ SD.

Table 4.5. Baseline characteristics of participants in follow-up trial

	Participants (n=8)
Age	46.9 ± 14.2
Sex (male, female)	male=3, Female=5
BMI (kg/m ²)	25.0 ± 3.5
WC (cm)	77.6 ± 14.4
ST SBP (mmHg)	114.3 ± 6.4
ST DBP (mmHg)	72.5 ± 5.1
ST HR (bpm)	61.9 ± 8.6
SP SBP (mmHg)	107.0 ± 7.2
SP DBP (mmHg)	63.6 ± 5.7
SP HR (bpm)	53.0 ± 7.6
Glc (mg/dL)	88.5 ± 7.0
Chol (mg/dL)	183.1 ± 58.4
HDL (mg/dL)	54.9 ± 16.7
LDL (mg/dL)	103.4 ± 54.2
Chol:HDL	3.4 ± 0.9
TG (mg/dL)	66.0 ± 47.4
RHI	2.5 ± 0.7
AI	6.3 ± 27.6
AI75	-4.6 ± 25.5
PFA-ADP (s)	96.4 ± 15.3
PFA-EPI (s)	153.6 ± 18.6

BMI, body mass index; WC, waist circumference; ST, sitting; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; SP, supine; Glc, fasting glucose; Chol, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Chol:HDL, the ratio of total cholesterol to HDL; TG, triglyceride; RHI, reactive hyperemia index; AI, augmentation index; AI75, augmentation index at heart rate of 75 beats per minute; PFA, platelet function

analyzer; ADP, adenosine diphosphate; and EPI, epinephrine. Values are presented as mean $\pm$ SD.

Table 4.6. Post-prandial changes in follow-up trial

Time	OLG			Placebo		
	Hour 0	Hour 2	Hour 4	Hour 0	Hour 2	Hour 4
RHI	2.5 ± 0.7	2.7 ± 0.8	2.6 ± 0.9	2.4 ± 0.6	2.3 ± 0.7	2.5 ± 0.8
AI	6.3 ± 27.6	4.1 ± 25.3	5.2 ± 26.8	7.0 ± 28.9	5.8 ± 27.0	6.5 ± 26.4
AI75	-4.6 ± 25.5	-3.8 ± 24.7	-4.2 ± 25.0	-5.1 ± 26.1	-4.0 ± 25.3	-4.8 ± 25.8
PFA-ADP (s)	96.4 ± 15.3	98.2 ± 14.7	97.0 ± 15.0	95.6 ± 15.8	96.9 ± 15.2	97.8 ± 15.5
PFA-EPI (s)	153.6 ± 18.6	155.2 ± 17.9	154.0 ± 18.2	152.8 ± 19.1	153.9 ± 18.4	154.7 ± 18.7

No significant post-prandial changes for any measures were found over time both OLG and placebo. OLG, Oligonol; RHI, reactive hyperemia index; AI, augmentation index; AI75, augmentation index at heart rate of 75 beats per minute; PFA, platelet function analyzer; ADP, adenosine diphosphate; and EPI, epinephrine. Values are presented as mean ± SD.

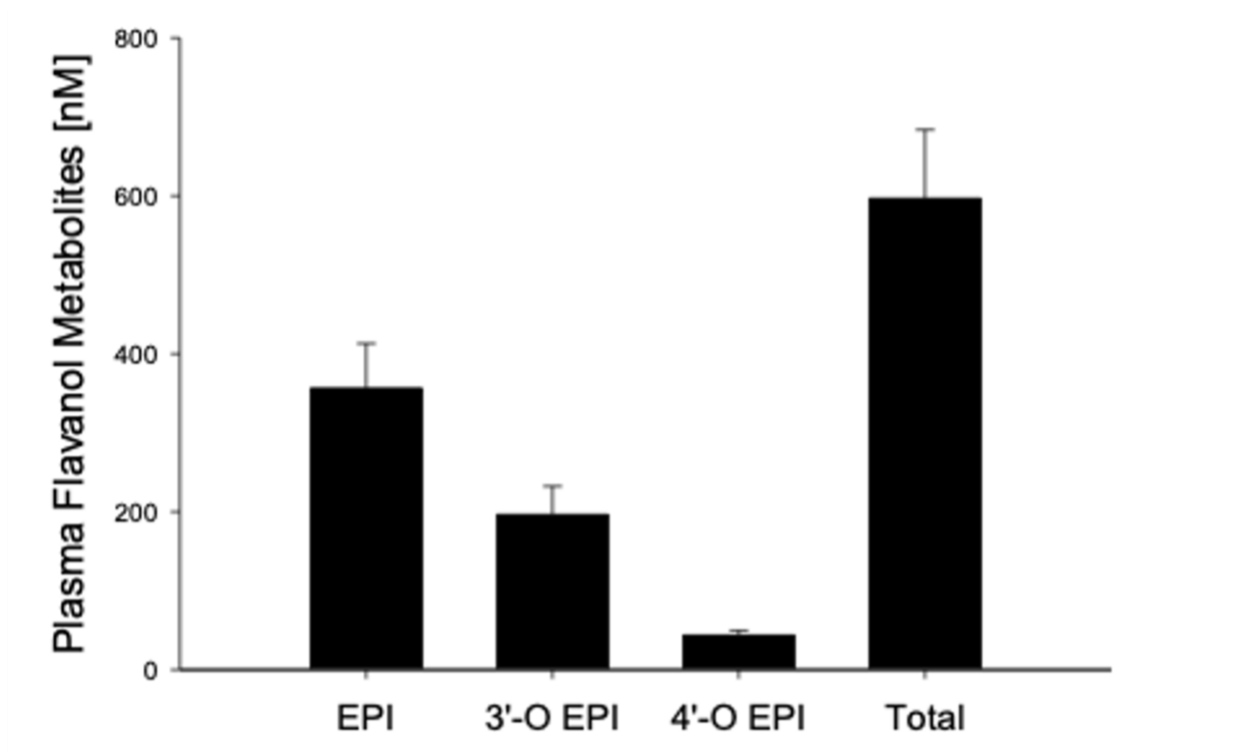


Figure 4.3. Plasma epicatechin metabolites 2 hours after high flavanol intake. Plasma epicatechin metabolites after enzyme treatment with glucuronidase and sulfatase. EPI, epicatechin; 3'-O EPI, 3'-O-methyl epicatechin; and 4'-O EPI, 4'-O-methyl epicatechin

Table 4.7. Composition of Oligonol

	% Flavanol (Actual)
Gallocatechin	0.37
Epigallocatechin	0.61
Catechin	0.41
Epicatechin	4.14
Epigallocatechin gallate	4.79
Gallocatechin gallate	0.61
Epicatechin gallate	0.73
Catechin gallate	trace

Oligonol flavanol composition was determined by high-performance liquid chromatography (HPLC) analysis performed at UC Davis. Values represent the percentage of each flavanol in the total amount of Oligonol.

Chapter 5:
Potential Impact of Dietary Components on Cardiometabolic Health
in Postmenopausal Women.

Title

Potential Impact of Dietary Components on Cardiometabolic Health in Postmenopausal Women.

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Introduction

The transition to menopause is a transformative phase in which a female's reproductive years gradually ends, but its impact extends far beyond the cessation of the menstrual cycle. As a critical window for future health, the timing and onset of menopause serve as significant indicators of a women's long-term cardiovascular and metabolic courses. While changes like vasomotor symptoms and irregular cycles are the most visible sign of perimenopause, the decline in estrogen initiates a series of silent cardiometabolic shifts^{22,23,37}. Because estrogen plays a critical role in maintaining vascular health and energy balance, its loss likely leads to adverse cardiovascular and metabolic outcomes^{27,243}.

During this transition, many women experience adverse changes that can be attributed to chronological aging²⁴⁴. However, cohort studies suggest that reproductive aging is a significant contributor to weight gain and unfavorable shifts in body composition. Specifically, menopause is often associated with a redistribution of adipose tissue toward the visceral regions. This shift is accompanied by adverse changes in lipid profiles and vascular function, which substantially increase the risk for cardiovascular disease (CVD) in women later in life^{245,246}.

Dietary intervention, particularly the Mediterranean diet (MedDiet), has emerged as an important strategy for cardiometabolic risk reduction. Rooted in the eating patterns of populations near the Mediterranean Sea, this dietary pattern is characterized by high intakes of plant-based foods and healthy fats, primarily extra-virgin olive oil (EVOO) and nuts²⁴⁷. In addition to effects on traditional lipids, the MedDiet appears to influence health through systemic inflammation and glucose metabolism, and gut microbiome²⁴⁸.

Despite the documented benefits of the MedDiet, there is limited dietary guidance specifically addressing the postmenopausal population. In order to clarify how this dietary

pattern may reduce cardiovascular risk in postmenopausal women, this review aims to integrate menopausal stage and cardiometabolic changes with mechanistic pathways modulated by key components of the MedDiet. We focus on how menopausal staging is linked to diet responsive pathways related to endothelial function, inflammation, lipid and glucose metabolism, and gut microbiome. We will consider oxylipin formation from unsaturated fatty acid metabolism, urolithin production from ellagitannin rich foods, short-chain fatty acid (SCFA) production, and estrobolome-mediated estrogen metabolism. We aim to inform nutritional strategies for cardiovascular health in postmenopausal women.

Section 1. Cardiometabolic Risk Factors in Postmenopausal Women

1.1. Hormonal and Metabolic Shifts Post-Menopause

Menopause introduces substantial physiological changes to the body, and the timing of its onset is increasingly recognized as a significant indicator of future health outcomes²². This transition marks the end of a female's reproductive years, driven by a reduction in ovarian production of reproductive hormones, estrogen and progesterone, ultimately leading to the cessation of menstrual cycles. The process is gradual, typically spanning several years (often 2-7 years), and can be divided into three phases: perimenopause, menopause, and postmenopause²³. Perimenopause, also known as the menopausal transition (MT), represents a significant physiological shift where reproductive aging, independent of chronological aging, drives adverse cardiometabolic changes³⁷. During this phase, estrogen levels fluctuate, often resulting in irregular menstrual cycles and vasomotor symptoms such as hot flushes and night sweats. Menopause is clinically diagnosed after 12 consecutive months without a menstrual period following the final menstrual period (FMP). Thereafter, the postmenopausal stage is

characterized by estrogen levels remaining consistently low for the remainder of a woman's life^{23,26}.

The dramatic decline in estrogen is the primary driver of these physiological shifts, as estrogen plays a critical protective role in the cardiovascular system and metabolic processes^{27,28}. Estrogen acts through nuclear receptors such as estrogen receptor (ER)- α and ER- β and membrane-associated G protein-coupled receptors to promote the production of nitric oxide (NO), a molecule critical for vasodilation and vascular health⁴⁸. Furthermore, estrogen maintains the regulatory balance of the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system (SNS) by activating vasodilatory components while suppressing vasoconstrictive agents like Angiotensin II ^{52,249}. Consequently, the postmenopausal decline in estrogen disrupts this balance, upregulating vasoconstrictive pathways and enhancing vascular responsiveness to sympathetic stimulation²⁵⁰.

Beyond vascular tone, this hormonal shift significantly impacts metabolism. The decline in estrogen and progesterone is coupled with an increase in follicle-stimulating hormone (FSH), which evidence suggests may affect cholesterol regulation independently of estrogen²⁴⁴. Moreover, an increase in the circulating androgen-to-estrogen ratio contributes to adverse metabolic changes, including insulin resistance and visceral fat accumulation²⁵¹. Since estrogen helps maintain metabolic homeostasis by managing energy expenditure, fat distribution, and glucose metabolism, its deficit triggers significant metabolic changes that increase the risk of disorders such as type 2 diabetes (T2D) and CVD²⁴.

Regarding cardiovascular risk, it is well established that traditional factors such as diabetes and smoking increase CVD risk in both males and females. However, for females, emerging nontraditional risk factors, such as preterm delivery, hypertensive disorders of

pregnancy, gestational diabetes, breast cancer treatment, and depression, should also be addressed²⁵². Furthermore, the specific physiological changes that occur during the MT period, including vasomotor symptoms, sleep and cognitive disturbances, depression, and anxiety, have been directly linked to an increased risk of CVD^{245,253}.

1.2. Cardiometabolic Health Markers in Postmenopausal Women

The MT and the subsequent postmenopausal period represent a significant shift in a woman's health. Many adverse cardiometabolic changes, such as weight gain and unfavorable body composition, were once generally attributed to chronological aging²⁴⁴. But major cohort studies, including the Study of Women's Health Across the Nation (SWAN) and the Melbourne Women's Midlife Health Project, suggest that reproductive aging is largely the driving factor. Specifically, these longitudinal cohorts demonstrated that adverse shifts in lipid profiles and vascular remodeling occur during the transition to the FMP. These physiological changes are significantly independent of chronological age, which suggests that reproductive aging is the primary driver of increased cardiovascular risk in midlife women^{26,254}.

Adiposity and Insulin Resistance

This hormonal shift accelerates the increase in central adiposity and abdominal visceral fat, a key change shown to dramatically raise cardiometabolic risk and mortality in postmenopausal women. While weight gain is common, the most specific change is the redistribution of adipose tissue toward visceral regions, occurring independently of total weight gain or chronological age²⁵⁵⁻²⁵⁹.

Crucially, visceral adipose tissue is not merely an inert energy storage depot but a highly metabolically active endocrine organ. This tissue secretes substances including adipokines and pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6)²⁶⁰. These cytokines create a state of chronic, low-grade inflammation that directly impairs insulin signaling pathways, decreases insulin sensitivity, and consequently disrupts glucose uptake by skeletal muscle^{261,262}. Simultaneously, the decline in estrogen disrupts the regulation of hepatic glucose production, leading to elevated blood glucose levels²⁶³. A decline in physical activity and muscle mass, commonly observed during the MT and menopause, contributes to reduced glucose utilization, further promoting the risk of insulin resistance and T2D²⁶⁴⁻²⁶⁶.

Lipid Metabolism

Estrogen typically influences lipid metabolism favorably by decreasing low-density lipoprotein cholesterol (LDL-C) and increasing high-density lipoprotein cholesterol (HDL-C) levels^{267,268}. However, the loss of estrogen during the MT triggers significant deterioration in lipid metabolism^{269,270}. Specifically, the decline in estrogen decreases hepatic LDL receptor activity, impairing LDL-C clearance and leading to consistent increases in LDL-C and apolipoprotein B (ApoB)²⁷⁰⁻²⁷². Data from the SWAN study indicate a mean LDL-C increase of 6-7 mg/dL during the MT²⁶⁸. Furthermore, while HDL-C levels may remain variable, the functional quality of HDL—specifically its capacity for reverse cholesterol transport—is often compromised^{40,273}. Collectively, these shifts contribute to a distinct postmenopausal metabolic profile driven by a cluster of inflammation, insulin resistance, and dyslipidemia.

Vascular Consequences

In parallel with metabolic dysregulation, the loss of estrogen precipitates structural and functional changes in the vasculature by dismantling its primary protective signaling pathways²⁷⁴. As previously detailed, vascular homeostasis relies on estrogen acting through nuclear receptors (e.g., ER- α and ER- β) and membrane-associated G protein-coupled receptors to drive the production of NO²⁰⁹. However, the menopausal decline in estrogen effectively uncouples these pathways. Consequently, NO production is diminished, a deficit that is further compounded by increased oxidative stress, where excessive reactive oxygen species rapidly degrade any remaining NO^{48,208}.

Studies consistently report a decline in flow-mediated dilation (FMD), a non-invasive measurement of blood vessel dilation in response to blood flow, across the menopause stages. The decline appears progressive from pre- to peri- to postmenopause^{208,275}. This resulting endothelial dysfunction directly contributes to arterial stiffness, defined as the loss of elasticity in large arteries like the aorta^{50,51}. Independent of aging and traditional risk factors, arterial stiffness, measured by pulse wave velocity (PWV), increases significantly around the time of menopause, with higher PWV indicating greater stiffness^{276,277}. Sustained low estrogen levels can promote adverse structural remodeling of the arterial wall, leading to an increase in intima-media thickness, a decrease in elasticity, and, consequently, increased systolic blood pressure by reducing the compliance of large arteries^{214,278}. In addition, the disruption in the regulatory balance of RAAS and SNS due to low levels of estrogen elevates systemic vascular resistance and blood pressure, contributing to vasoconstriction and increased blood pressure in postmenopausal women²⁵⁰.

Section 2. The Mediterranean Diet and Its Key Nutrients

2.1. Principles of the Mediterranean Diet

The MedDiet is rooted in the traditional eating patterns of populations in countries near the Mediterranean Sea and is characterized by high intakes of vegetables, fruits, whole grains, legumes, nuts, and seeds, with olive oil as the primary fats and relatively low consumption of red and processed meats and sweets²⁷⁹. This pattern provides a high density of dietary fiber, unsaturated fatty acids, and diverse polyphenols, distinguishing it from typical Western dietary patterns that are rich in saturated fat, refined carbohydrates, and ultra-processed foods^{247,280}.

EVOO and nuts are key fat sources and supply monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) together with bioactive components such as phenolic compounds and phytosterols^{281,282}. Fish and other marine foods contribute long-chain omega-3 fatty acids, while legumes, whole grains, and vegetables provide substantial amounts of dietary fiber and micronutrients²⁸³. Moderate alcohol intake in the form of red wine consumed with meals is a traditional component of the MedDiet and provide stilbenes such as resveratrol and other phenolic compounds. However, these potential cardiovascular benefits must be viewed with caution due to the known risks of high alcohol consumption²⁸⁴⁻²⁸⁶.

Beyond food choices, the MedDiet is often described as a broader lifestyle pattern that includes regular physical activity and communal eating²⁸⁷. For this review, we focus on dietary components and their mechanistic link to cardiometabolic health in postmenopausal women.

2.2. Specific Nutrients Linked to Cardiometabolic Health and Potential Mechanisms of Action

In this section, we synthesize MedDiet components that mechanistically relate to cardiometabolic outcomes during the MT and after menopause, including visceral adiposity,

endothelial dysfunction, dyslipidemia, insulin resistance, and inflammation. We focus on pathways such as oxylipin formation, urolithin production, SCFA generation, and estrobolome, which distinguish evidence in postmenopausal women from extrapolations from mixed populations for nutrition strategies in this population.

Unsaturated Fatty Acids: MUFA from Olive Oil and PUFA from Walnuts and Fish

EVOO provides a rich supply of MUFA, predominantly oleic acid consisting of up to 70-80% of EVOO²⁸⁸. Prospective cohorts from the U.S. and Europe consistently show that higher olive oil intake is associated with lower risks of total CVD, coronary heart disease (CHD) and cardiovascular mortality, particularly when replacing butter and other saturated fats²⁸⁹⁻²⁹². Large, randomized trials of MedDiet patterns supplemented with EVOO provide strong support for these observational findings^{54,293}. In the PREDIMED trial, a MedDiet enriched with EVOO reduced the incidence of major cardiovascular events compared with a low-fat control diet, alongside improvements in blood pressure and lipid profile in high-risk adults⁵⁴. PREDIMED sub-studies and related interventions confirm EVOO-rich patterns reduce 24-h ambulatory blood pressure, improve LDL-C/HDL-C/non-HDL-C, and attenuate thrombogenic/oxidative/inflammatory markers such as IL-6 and TNF- α in high-risk adults²⁹⁴⁻³⁰¹. Meta-analyses conclude that EVOO-rich diets are consistently associated with better cardiometabolic outcomes and lower cardiovascular risk in populations³⁰²⁻³⁰⁵.

The mechanisms underlying these benefits likely reflect the combined actions of oleic acid and other bioactive components of EVOO rather than oleic acid alone^{302,306}. Overall, epidemiological and clinical evidence strongly supports cardioprotective effects of EVOO-rich dietary patterns, while the mechanistic links between oleic acid and cardiovascular health remain

incompletely established^{293,307,308}. In comparison, the cardioprotective role of EVOO phenolics is well recognized; the European Food Safety Authority (EFSA) has authorized a health claim stating that olive oil polyphenols contribute to the protection of blood lipids from oxidative damage³⁰⁹. Within the MedDiet, EVOO is one part of a broader unsaturated fat matrix where nuts (particularly walnuts) and marine foods contribute complementary PUFAs and their lipid mediators that may further enhance cardiovascular protection.

Walnuts, a key MedDiet nut, provide the essential fatty acid alpha-linolenic acid (ALA). Beyond ALA conversion to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)^{310,311}, ALA serves as a parent molecule for cardioprotective, bioactive mediators³¹¹. Data from the Nurses' Health Study (NHS) showed an inverse association between ALA intake and sudden cardiac death after adjusting for the intake of EPA and DHA³¹². A comprehensive meta-analysis of prospective cohorts reported that high dietary intake of ALA lowers the risk of all-cause and CVD and CHD mortality. This protective effect appears to follow a dose-response relationship³¹³. However, findings regarding general CHD events vary by population^{314,315}.

The cardioprotective role of ALA includes its enzymatic conversion into bioactive oxylipins³¹¹. In humans, ALA is metabolized into bioactive oxylipins through three major enzymatic pathways: Lipoxygenase (LOX), Cyclooxygenase (COX), and Cytochrome P450 (CYP). Following release from membrane phospholipids by phospholipase A2 (PLA₂), ALA enters these competing pathways¹⁹⁸. The LOX pathway is the primary route, where enzymes such as 15-LOX-2 metabolize ALA into hydroxy-octadecatrienoic acids (HOTrEs), such as 13-HOTrE and 9-HOTrE³¹¹. These metabolites have demonstrated anti-inflammatory and peroxisome proliferator-activated receptor gamma (PPAR γ)-mediated effects in in vitro and animal models^{311,316}. The COX pathway, primarily mediated by COX-2 during inflammation,

converts ALA into 12-HOTrE³¹¹. Lastly, the CYP450 pathway generate epoxy-octadecadienoic acids (EpODEs). These epoxides are rapidly hydrolyzed by soluble epoxide hydrolase (sEH) into dihydroxy-derivates (DiHODEs) such as 15,16-DiHODE³¹⁷. While human data show that ALA supplementation modulates plasma oxylipin profiles, especially in populations with elevated cardiovascular risk or inflammation, direct evidence linking ALA-derived metabolites to cardiovascular outcomes in humans remains a key area for future research^{199,204,318-321}.

Although replacing saturated fats with unsaturated fatty acids such as oleic acids from EVOO and ALA from walnuts is beneficial for both sexes, women have distinct metabolic pathways for fatty acid storage and utilization that are shaped by menopausal status compared to stable male patterns^{290,313,322,323}. Premenopausal women demonstrate higher fatty acid oxidation during energy expenditure along with preferential subcutaneous storage and more postprandial triglyceride clearance, leading to a more favorable lipid profile than men before estrogen loss^{324,325}. These female-specific differences arise primarily from estrogen signaling, which upregulates mitochondrial beta (β)-oxidation enzymes and modulate lipoprotein lipase (LPL) activity to deposit subcutaneous rather than visceral fat^{325,326}. However, the postmenopausal estrogen decline, coupled with a rising androgen-estrogen ratio, uncouples these protective pathways, shifting women toward male-like risks: β -oxidation diminishes, storage shifts towards metabolically active visceral adipose tissue, and pro-inflammatory cytokines like TNF- α and IL-6 surge^{257,327,328}.

These postmenopausal shifts in fat distribution, inflammation, and fatty acid oxidation increase the relevance of long-chain omega-3 PUFAs, particularly EPA and DHA from oily fish, within the MedDiet for cardiovascular protection in women^{329,330}. Prospective cohort studies in diverse populations consistently associate frequent fish intake, most apparent at moderate levels,

with lower risks of CHD, cardiovascular mortality, and fatal or sudden cardiac events³³¹⁻³³⁴.

These findings support current dietary guidelines recommending the replacement of red and processed meats with fish as a cardioprotective protein and fat source³³⁵⁻³³⁷.

Randomized controlled trials (RCTs) of marine omega-3 supplementation and fish-based interventions provide heterogeneous evidence of reduced major adverse cardiovascular events, myocardial infarction, and cardiovascular death³³⁸⁻³⁴⁰. Interestingly, studies comparing fresh fish meals with encapsulated omega-3 fatty acids suggest that whole fish may be more beneficial for lipid profiles and cardiometabolic risk factors, due to synergistic effects of food matrix of the fish^{341,342}. Emerging dose-response analyses indicate a dose-response relationship between marine omega-3 fatty acid intake and reduced cardiovascular risk^{343,344}. Furthermore, some meta-analyses indicate that higher-dose or EPA only intake is associated with lower event rates compared with lower doses or EPA+DHA formulations^{338,345}.

The cardioprotective effects of omega-3 fatty acids likely involve improvements in lipid profiles and inflammation, modest reduction in blood pressure, and enhanced endothelial function as is well-documented in human intervention studies³⁴⁶⁻³⁴⁹. At a mechanistic level, experimental and emerging clinical data indicate that vascular inflammation and endothelial function may be improved through several pathways such as EPA and DHA incorporation into cell membranes, competition with arachidonic acid for COX and LOX in eicosanoid pathways, and production of specialized pro-resolving mediators (SPMs)^{339,348,350,351}. However, detailed mechanistic pathways remain less firmly established than the epidemiological and clinical outcome evidence^{339,348}.

Polyphenols and Other Phytochemicals

The MedDiet may protect against CVD partly through polyphenols and phytochemicals derived from EVOO, fruits, vegetables, nuts, legumes, whole grains, and moderate red wine consumption³⁵². The main classes-flavonoids (most abundant), phenolic acids, stilbenes, lignans, and ellagitannins-likely work together to affect oxidative stress, inflammation, and vascular function³⁵³. Key sources include anthocyanins (berries, red wine), flavonols (onions, apples, greens), flavan-3-ols (tea, cocoa), phenolic acids (coffee, grains, fruits/vegetables), stilbenes/resveratrol (grapes, red wine), lignans (flaxseeds, grains, nuts), ellagitannins (walnuts, pomegranates, berries), and EVOO phenolics (hydroxytyrosol, oleuropein)³⁵⁴. These components contribute to CVD risk reductions observed in women³⁵⁵.

Epidemiological studies consistently report higher polyphenol and flavonoid intakes in Mediterranean-style dietary patterns lower CVD risk in women^{283,355 138,356}. A female-focus meta-analysis found approximately 25% lower risk of cardiovascular events with high MedDiet adherence. This supports the relevance of this dietary pattern for primary prevention in female populations³⁵⁵. Postmenopausal cohorts indicate that higher flavonoid intake is associated with reduced CHD mortality, although findings are heterogeneous across specific flavonoid subclasses and outcomes³⁵⁷⁻³⁵⁹. Moreover, studies that estimated total polyphenol intake or use urinary polyphenol excretion as a biomarker further show that higher polyphenol exposure within Mediterranean-style diets is linked to lower all-cause and cardiovascular mortality and more favorable outcomes for risk factors in older adults^{138,360}.

RCTs of polyphenol-rich foods characteristic of the MedDiet provide mechanistic evidence through improvements in endothelial function and blood pressure^{361,362}. Interventions with berries, cocoa, tea, and high-phenolic EVOO consistently improve FMD, with increases of 1% to 5% that are sustained during chronic intake^{82,363}. PREDIMED sub-studies demonstrate

that higher polyphenol intake associates with reduced blood pressure, improved lipid profiles, and decreased inflammatory markers^{138,364}. In peri- and postmenopausal women, trials with berry anthocyanins, grape seed extract, and other polyphenol-rich interventions have reported improvements in FMD, reductions in blood pressure, and favorable lipid changes in some studies³⁶⁵⁻³⁶⁷. Findings from RCTs provide mechanistic support for the observed benefits and align with the endothelial, anti-inflammatory, and metabolic actions of polyphenols discussed below.

Polyphenol-rich Mediterranean foods appear to enhance endothelial health and reduce vascular inflammation, consistent with FMD improvements and lower C-reactive protein (CRP), IL-6, and oxidized LDL reported in intervention studies^{363,368}. Experimental and translational work suggests that flavonoids such as flavanols, anthocyanins, quercetin, and epigallocatechin gallate (EGCG) support NO-mediated vasodilation and attenuate activation of pro-inflammatory pathways³⁶⁹⁻³⁷³. Effects on lipid metabolism and blood pressure appear modest but consistent with known cellular actions of polyphenols, including influences on hepatic lipid synthesis, LDL receptor activity, and LDL oxidation^{367,374,375}. These mechanisms align with reductions in LDL-C and blood pressure seen in trials of quercetin-rich foods, high-phenolic EVOO, and other polyphenol-dense foods (berries, red wine, and nuts) in Mediterranean-style dietary patterns³⁷⁶⁻³⁸⁰.

Sex differences in how polyphenols are processed and act in the body likely stem more from interactions with sex hormones, their receptors, and the gut microbiome than from differences in intake and circulating levels^{381,382}. For flavonoids, sex differences in phase II conjugation such as glucuronidation and sulfation are suggested by higher hepatic UDP-glucuronosyltransferase (UGT)/sulfotransferase (SULT) expression in women and estrogen-

responsive regulation of these enzymes, but direct human data on sex-specific metabolism of flavonoids are still scarce³⁸³⁻³⁸⁵. Beyond host-level processes, most dietary polyphenols remain unabsorbed in the small intestine and reach the colon, where they undergo extensive microbial metabolism into low-molecule weight phenolic metabolites¹⁷⁰. The gut microbiota likely modulate beneficial effects by converting dietary polyphenols into bioactive phenolic metabolites and by responding to polyphenols in a prebiotic-like manner, although metabotype-specific cardiovascular outcomes in postmenopausal women remain an emerging area of research³⁸⁶. In this context, walnuts serve as a useful example, as their ellagitannins are converted by gut microbes into urolithins that are thought to mediate many of the vascular effects³⁸⁷.

Walnuts are distinguished within the tree nuts by their high content of polyphenols, particularly ellagitannins³⁸⁸. However, potential benefits of ellagitannins are limited by their complex absorption and metabolism. Ellagitannins are too large to be absorbed in the upper gastrointestinal tract. Because these cannot be absorbed intact, the colon functions as a site of extensive metabolism where the gut microbiota hydrolyzes ellagitannins into ellagic acid. Subsequently, specific bacterial communities metabolize ellagic acid into bioactive, lipophilic metabolites known as urolithins. These microbial metabolites, rather than the parent molecules, are responsible for systemic physiological effects³⁸⁹⁻³⁹¹.

The conversion of ellagitannins into urolithins relies on the composition of the host microbiome and has been characterized as individual “Urolithin Metabotypes” (UMs)³⁹². Metabotype A (UM-A) is characterized by the production of urolithin A, the metabolite strongly associated with anti-inflammatory and antioxidant activities. In contrast, Metabotype B (UM-B) produces urolithin B and iso-urolithin A in addition to urolithin A, the metabolites linked to less

favorable microbiota and metabolic profiles compared with UM-A. Lastly, Metabotype 0 (UM-0) refers to non-producers who lack the enzymatic pathways required to convert ellagic acid into bioactive urolithins^{393,394}.

The cardiovascular research on UM-A has focused on its ability to improve cardiac function by enhancing mitochondrial function in aging and heart-failure models, to lower lipid biomarkers that predict cardiovascular risk in older adults, and to modestly improve FMD in individuals with impaired vascular health³⁹⁵⁻³⁹⁷. UMs also appear to modulate cardiometabolic responses to ellagitannin-rich foods including walnuts, suggesting that microbiome-dependent urolithin production may shape individual cardiovascular benefits³⁹⁸.

Dietary Fiber

The MedDiet is well defined by its high content of dietary fiber from a wide variety of plant-based sources, including legumes such as chickpeas, lentils, and fava beans, whole grains such as barley, oats, and farro, vegetables, and fruits²⁴⁷. This plant food matrix provides soluble and insoluble fibers that modulate nutrient absorption, lipid metabolism, and gut microbial environment³⁹⁹.

Emerging evidence supports the inverse relationship between dietary fiber intake and cardiovascular risk^{400,401}. In the NHS, higher intakes of total and cereal fiber, mainly from whole grains, reduced CHD and type 2 diabetes risk in women^{402,403}. Among 7,708 postmenopausal U.S. women in National Health and Nutrition Examination Survey (NHANES), the highest tertile of fiber intake showed 15% lower all-cause mortality and 31% lower cardiovascular mortality versus in the lowest⁴⁰⁴. These cohort findings align with a recent meta-analysis of 64 prospective studies. High versus low total fiber intake is associated with 23% lower all-cause

mortality and 26% lower cardiovascular mortality, with particularly strong effects for insoluble fiber and cereal/whole-grain sources⁴⁰⁵.

The biological effects of fiber extend beyond simple cholesterol binding. Recent evidence indicates that cardiometabolic benefits of fiber originate in the colon, where non-digestible carbohydrates serve as a prebiotic substrate for microbial fermentation⁴⁰⁶. Gut microbiota convert non-digestible carbohydrates into SCFAs, including acetate, propionate, and butyrate⁴⁰⁷. These metabolites act as potent signaling molecules influencing host metabolism, inflammation, and vascular function⁴⁰⁸. These SCFAs bind to G-protein-coupled receptors (GPRs) such as GPR41 and GPR43, stimulating the secretion of glucagon-like peptide-1 (GLP-1), which, in turn, enhances insulin sensitivity and promotes satiety⁴⁰⁹. Through this pathway, fiber metabolites counteract the insulin resistance and visceral adiposity commonly observed in postmenopausal women^{410,411}.

The “estrobolome” is defined as the collection of gut microbiota involved in estrogen metabolism and exists in both men and women⁴¹². Its functional relevance likely varies by sex and life stage. Circulating estrogen and receptor signaling differ between sexes. While men maintain consistently lower levels of estrogen, women experience fluctuations during the menstrual cycle^{413,414}. In postmenopausal women, the decline in estrogen occurs alongside an age-related reduction in gut microbial diversity, raising interest in whether microbiome composition can affect residual estrogen exposure^{413,415}. Cross-sectional studies have reported that higher fecal microbial diversity and greater abundance of estrogen-metabolizing taxa are associated with higher urinary estrogen and estrogen metabolites. This suggests that microbial deconjugation and enterohepatic recirculation may partially compensate for estrogen loss, although causal evidence is still limited^{412,415-417}. Fiber-rich MedDiet patterns consistently increase microbial diversity and

SCFA production, which are linked to insulin sensitivity, lower inflammation, and more favorable cardiometabolic outcomes in midlife and older adults, including postmenopausal women^{406,408,410,411,418,419}. While direct human data on fiber-induced changes in the estrobolome are sparse, these gut-mediated pathways suggest a plausible mechanism for cardiovascular protection in this population⁴¹⁸⁻⁴²⁰.

Section 3. Evidence for Adherence to the Mediterranean Diet in Postmenopausal Women

3.1. Dietary Adherence and Cardiometabolic Outcomes in Postmenopausal Women

Large-scale prospective cohort studies indicate that higher adherence to the MedDiet is associated with substantial reductions in incident CVD and total mortality among women, particularly in the postmenopausal period⁴²¹. Findings from the Women's Health Initiative (WHI) study strongly reflect these associations. In the WHI cohort of 63,805 postmenopausal women followed for a median of 12.9 years, women with higher alternate Mediterranean Diet (aMED) score showed lower mortality outcomes. Women in the highest quintile had a 26% lower risk of all-cause mortality and a 21% lower risk of CVD mortality compared with the lowest quintile⁴²¹. The authors highlighted a potential interaction with body composition. The inverse association between diet quality and mortality was stronger among women with a waist circumference ≤ 88 cm but was attenuated and non-significant in those in central adiposity (waist circumference > 88 cm). Similarly, in WHS (n=25,994; up to 12 years), high MedDiet adherence was associated with 28% lower risk of CVD incidents compared to low adherence⁴²². Extended 25-year follow-up of WHS confirmed that high adherence was associated with a 23% and 17% reduction in all-cause and CVD mortality, respectively⁴²³. Moreover, biomarker analysis showed that inflammation was the largest contributor, explaining 29.2% of CVD

reduction and 13% of the lowering mortality^{422,423}. Metabolic contributions varied, with glucose metabolism and insulin resistance explaining 27.9% of the CVD risk reduction while small molecule metabolites contributed most to reduced mortality by 14.8%. Interestingly, both studies showed that the MedDiet contributes to health benefits largely independent of traditional risk factors like LDL-C. While the traditional lipid measures mediated 25% of the CVD incidents over 12 years, contributions from traditional lipids diminished in the 25-year analysis^{422,423}. The authors suggest that the cardioprotective benefits of the MedDiet can be explained through multiple and complex, pathways involving inflammation and metabolism, rather than solely by modifying traditional lipid profiles⁴²³. Findings from the NHS extend benefits for the MedDiet to specific cardiovascular endpoints. The NHS where 74,886 women were followed for 20 years demonstrated a 29% lower risk of CHD, a 13% lower risk of stroke, and a 39% lower risk of CVD mortality among women with the greatest MedDiet adherence¹¹⁵. These cohort findings are supported by a recent meta-analysis focusing on women. The meta-analysis pooling 16 prospective studies reported that high adherence to the MedDiet was associated with a 24% lower risk of CVD incidents and a 23% lower risk of total mortality in women across different regions and the MedDiet measures³⁵⁵.

While these prospective cohorts provide strong evidence for association, RCTs support a causal role of the MedDiet in cardiovascular risk reduction. The PREDIMED trial, the largest multicenter randomized study, enrolled individuals at high risk. Participants assigned to an energy-unrestricted MedDiet, supplemented with either EVOO or mixed nuts, experienced a 30% lower risk of the composite endpoint of myocardial infarction (MI), stroke, or cardiovascular death compared with those assigned to a low-fat control diet⁵⁴. Although the PREDIMED trial did not report sex-specific data, a substantial proportion of participants were

older adults, many of them women aged 60-80 years, and the observed primary prevention of CVD is consistent with the benefits seen in female prospective cohorts^{54,115,421-423}.

Secondary prevention trials likewise support the cardioprotective potential of the MedDiet in patients with established CVD⁴²⁴. In the Lyon Diet Heart Study, post- MI patients randomized to a Mediterranean-style diet experienced significantly fewer recurrent cardiac events and death than those assigned to a prudent Western-type diet after approximately 4 years of follow-up⁴²⁴. More recently, the CORonary Diet Intervention With Olive Oil and Cardiovascular PREvention (CORDIOPREV) compared a MedDiet with a low-fat diet in patients with CHD followed for 7 years⁴²⁵. The MedDiet group showed a significant lower occurrence of major cardiovascular events, including MI, revascularization, ischemic stroke, peripheral artery disease, and cardiovascular death, compared with the low-fat control group, supporting the benefits of the MedDiet for long-term secondary prevention. Although the study was underpowered to detect sex-specific effects, particularly in women, the findings of the study suggest the benefits of the MedDiet still remain after the onset of clinical CVD⁴²⁵.

Taken together, these clinical data demonstrated that Mediterranean dietary patterns can lower cardiovascular events in high-risk and secondary prevention populations, aligning with the women-specific cohort data to support the MedDiet as a dietary strategy for cardiovascular prevention in postmenopausal women.

3.2. Comparison with Other Dietary Patterns

Since foods and nutrients are rarely consumed in isolation, examining the health benefits of dietary patterns is essential, which gives us a holistic perspective on diet. The dietary guidelines to prevent CVD emphasize intakes of whole grains, fruits, vegetables, legumes, nuts,

and lean protein sources, with avoidance of processed foods, trans-fats, and sugar-sweetened beverages⁴²⁶⁻⁴²⁸. The MedDiet, the Dietary Approaches to Stop Hypertension (DASH), and plant-based diets have proven cardioprotective effects in varying degrees, yet each offers unique characteristics and challenges^{428,429}. The Med diet is rich in whole grains, leafy green vegetables, fruits, legumes, nuts, herbs, spices, and EVOO, with a moderate intake of lean proteins, including fish and poultry, moderate alcohol consumption, and limited red/processed meat and sweets. It naturally limits or excludes many components of the Western diet that contribute to cardiovascular disease, such as saturated fats, highly refined grains, sugar, and red meat⁴³⁰. The MedDiet distinguishes itself from other dietary patterns, such as DASH and plant-based diets, through its emphasis on EVOO and nuts as primary sources of healthy fats and a reduced intake of dairy⁴³¹. In contrast, the DASH diet is low in overall fats and does not emphasize specific oils as a fat source⁴³². Nuts and EVOO, rich in mono- and polyunsaturated fats and polyphenols, have been consistently linked to reductions in cardiovascular risk factors, particularly LDL-C, which is a main contributor to the development of CVD^{433,434}.

The DASH diet was designed specifically to lower blood pressure. It achieves this by choosing foods rich in potassium and low in sodium, while simultaneously reducing overall sodium intake. Notably, DASH diet, which increases calcium-rich low-fat dairy, has been shown to reduce blood pressure even when sodium intake is kept constant. The diet focuses on limiting processed foods, sweets, and fatty meats^{435,436}. Daily sodium intake is restricted to less than 2,300 mg, or 1,500 mg in its reduced-sodium version⁴³⁷. Significant reduction in cardiovascular risk factors such as blood pressure, total cholesterol, and LDL-C has been reported in several interventional studies with the DASH diet⁴³⁸⁻⁴⁴⁰.

Other plant-based diet share similarities with the MedDiet and DASH diet by focusing on fruits, vegetables, legumes, and whole grains but may not control sugar, fat, and sodium intakes. However, the plant-based diet, predominantly made up of plants, can be diverse in content, depending on the subsets. Depending on the subset these diets diverge based on the type of protein consumed and include the vegetarian diet, which excludes meat, poultry, seafood, and fish, with lacto-vegetarian, who include dairy products; lacto-ovo-vegetarian, who include dairy and eggs; and pesco-vegetarian, who include fish, dairy, and eggs. Finally, the vegan diet excludes all animal products. With non or low consumption of animal foods and their products, the plant-based diet contains low saturated fats and high fiber content, which helps to delay gastric emptying with moderate insulinemic and glycemic responses⁴⁴¹. Two combined observational studies, the NHS and health professional follow-up study (HPFS), showed a higher plant-based diet index, indicating it is high adherence to plant foods, was inversely associated with the incidence of CVD (hazard ratio (HR) 0.92; 95% confident interval (CI) 0.83-1.01; p-trend=0.003)⁴⁴². Similar results were reported in the PREDIMED trial in Spain that individuals with vegetarian diets showed 53 % lower cardiovascular mortality than the lowest quintile of vegetarian diet score (HR=0.47; 95% CI 0.21-1.04; p-trend=0.039 across quintiles)⁴⁴³. Plant-based diets have low processed meat or none, meaning they minimize food preservatives and additives such as nitrates and sodium commonly found in processed meats⁴⁴⁴. Consistent with meta-analyses of observational studies, a recent large cohort study reported an inverse association between processed meat consumption and risks of total mortality and major CVD⁴⁴⁵⁻⁴⁴⁸. It suggests that high food preservatives and additives consumption partly explains adverse health outcomes⁴⁴⁵. Despite its potential to prevent CVD, plant-based diets may lack certain nutrients, such as vitamin B12, vitamin D, calcium, zinc, protein, and retinol, commonly found

in animal foods and their products. Individuals on plant-based diets, especially a vegan diet, may require additional supplementation of those nutrients to avoid deficiencies⁴⁴⁹.

The MedDiet, DASH, and plant-based diets have similarities, emphasizing diets rich in fruits, vegetables, legumes, and whole grains with minimized processed foods⁴⁵⁰. Their beneficial impact on cardiovascular health is supported by compelling evidence from observational studies to large randomized controlled trials⁴⁵¹⁻⁴⁵⁵. Both the DASH and plant-based diets offer considerable benefits for cardiovascular health^{454,455}. In contrast, the MedDiet is unique in that it combines high-quality fats from EVOO and nuts, moderate animal protein intake, and polyphenol-rich foods⁴³¹. This provides a moderate and flexible dietary pattern, which may contribute to better adherence in the long term, offering a sustainable option for CVD prevention.

Section 4. Future Directions and Research Gap

Although Mediterranean-style dietary patterns are consistently associated with lower cardiovascular events and mortality in women across midlife and older adulthood, important questions remain regarding how we approach these patterns in a long term and which biological pathways mediate their effects^{115,421-423}. The MedDiet has been framed as a fixed pattern rooted in Southern European traditions, yet emerging evidence supports approaching it as flexible and culturally adaptable food combinations that preserve core cardioprotective elements, such as olive oil as primary fat, regular nuts and fish, and fiber-rich plants⁴⁵⁶. Management of women's cardiovascular risk should incorporate life-course events such as pregnancy complications and vasomotor symptoms into dietary recommendations²⁰⁷. Furthermore, determining the optimal intervention timing (pre-menopause, during the transition, or post-final menstrual period) should

be considered to effectively prevent increased visceral adiposity, arterial stiffening, and endothelial dysfunction. A limited understanding of biological mechanisms questions how these patterns influence metabolic health during MT^{457,458}. Moreover, inter-individual responses vary; for example, UMs determines walnut ellagitannin benefits, while microbiome composition modulates polyphenol and fiber-derived metabolite production^{392,457}. Yet characterization of responders versus non-responders in postmenopausal women remain poorly established. Future research should prioritize human trials that integrate hormonal staging like Stages of Reproductive Aging Workshop (STRAW)+10 criteria with multi-omics profiling, including metabolomics, lipidomics, and microbiome analyses, alongside vascular phenotyping such as FMD, peripheral arterial tonometry (PAT) and PWV^{26,459,460}. Such studies would clarify how biological pathways contribute to cardiovascular protection across menopause stages from early transition through postmenopause.

A key public health challenge is translating observational and clinical trial evidence on Mediterranean-style dietary patterns into population-level cardiovascular prevention strategies for women^{355,461}. Although the MedDiet is incorporated into major national dietary guidelines for cardiovascular prevention, postmenopausal women-specific guidance remains absent^{462,463}. Long-term adherence among postmenopausal women is another challenge. Barriers that limit access to traditional Mediterranean foods and cultural dietary preferences have long been thought to hinder adoption, with studies from non-Mediterranean regions consistently demonstrate cardiovascular benefits from adapted patterns^{456,464}. This suggests that targeted dietary counseling and education can be the primary approach for postmenopausal women^{456,465}. Future research is needed to identify counseling strategies that promote sustained Mediterranean-style dietary adherence in this population.

Section 5. Conclusion

The MT and subsequent postmenopausal period represent a distinct and critical cardiometabolic turning point in women's health. The decline in estrogen drives systemic physiological shifts that extend beyond reproductive function. These changes are characterized by a redistribution of adipose tissue toward visceral regions, chronic, low-grade inflammation, and reduced estrogen-mediated vascular protection.

Adherence to the MedDiet offers an effective strategy for both primary and secondary cardiovascular prevention. This plant-centered, nutrient-dense dietary pattern provides a unique matrix of unsaturated fatty acids from EVOO and nuts, high-quality dietary fiber, and diverse polyphenols that collectively counteract the adverse metabolic shifts associated with the MT. Benefits of this food matrix in the MedDiet are driven not only by improvements in lipids but also by gut-mediated pathways.

For the practical approach, future research should develop a life-course-integrated model that incorporates hormonal staging and utilize multi-omics profiling to understand inter-individual responses. Ultimately, translating biological insights into public health success requires individual dietary counseling and education to support sustainable adherence in postmenopausal population.

For postmenopausal women, the most supported benefits of Mediterranean dietary patterns related to cardiometabolic outcomes, including reduced cardiovascular risk mortality and improvements in body weight and fat distribution, blood pressure, lipid profile, glycemic control, and systemic inflammatory markers. On the other hand, the roles of emerging mechanistic pathways such as oxylipins, urolithins, SCFAs, and estrobolome related to estrogen metabolism, are biologically plausible but are largely inferred from experimental models rather

than direct human data, particularly demonstrated in postmenopausal women. Well-designed studies that combine precise menopausal staging with longitudinal dietary intervention and integrated multi-omic and microbiome analyses will be essential to refine Mediterranean guidance for this population.

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Chapter 6:

Concluding Remarks

This dissertation examined how Mediterranean-style dietary components influence cardiometabolic health across menopause stages. Three themes emerged: the menopausal transition is a biologically distinct period of cardiometabolic shifts for women, Mediterranean-style dietary patterns offer a promising approach for cardiovascular prevention in this population, and mechanistic pathways in oxylipins, microvascular function, and lipoprotein composition provide insight beyond traditional lipid measures.

The reviews in Chapters 1 and 5 highlight that estrogen loss during the menopausal transition drives changes in vascular, metabolic, and inflammatory pathways. Estrogen decline promotes visceral adiposity, reduces estrogen-mediated vasoprotection, and shifts a woman's metabolism toward a more atherogenic lipid and inflammatory profiles. Menopause creates unique health risks and should be considered for dietary guidance tailored to menopausal stage, rather than general advice by sex or age. Specifically, the reviews suggest that whole-food based Mediterranean dietary patterns and the inclusion of nutrient-dense nuts, such as walnuts, serve as key beneficial dietary factors for reducing menopause transition-related risks.

This dissertation work included studies on the potential vascular and anti-inflammatory effects of daily walnut intake in postmenopausal women with hypercholesterolemia. Chapter 2 found that short-term (4-weeks) of daily walnut consumption (40g) improved microvascular function and altered plasma oxylipin profiles. Particularly, increased plasma 14(15)-epoxyeicosatrienoic acid was strongly associated with improved vascular response. In a follow up trial in Chapter 3, analysis of the lipoprotein profile showed that walnut intake enriched very low-lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) fractions with alpha-linolenic acid and its epoxide metabolites. Functionally significant as LDL isolated from the walnut-intervention group suppressed the production of pro-inflammatory

cytokines, including leukotriene (IL)-6 and IL-8, in TNF- α stimulated diabetic adipocytes *ex vivo*. While Chapters 2 and 3 focused on fatty acids, Chapters 1 and 5 suggest that walnut polyphenols, the ellagitannins, also can contribute to improved vascular and anti-inflammatory outcomes. Ellagitannins are metabolized by gut microbiota into bioactive metabolites urolithins. Among these metabolites, urolithin A is specifically linked to lowering lipid biomarkers and improving mitochondrial quality. The production of specific gut microbiome-mediated metabolites characterizes individual urolithin metabotypes. These metabotypes accordingly shape the efficacy of ellagitannins in lowering cardiovascular risk biomarkers. Together, these support nuts' complementary vascular and adipose mechanisms for cardiovascular protection.

In Chapter 4, Oligonol trials showed no vascular benefit from this purported flavanol-rich supplement. Study limitations included healthy baseline vascular status, short duration, and lower-than-expected epicatechin content within the test product. Findings from this study highlight the need for attention to baseline risk, bioactive characterization of the test products, and intervention duration.

In conclusion, this dissertation identifies menopause as a distinct stage in cardiovascular prevention for women and explores from isolated bioactive compounds to whole foods. Selecting appropriate metabolic markers, such as oxylipins, vascular function, and lipoprotein composition, is essential for clearly understanding how dietary components influence cardiometabolic health. These findings provide a foundation for developing targeted dietary guideline for postmenopausal women. To refine them for application, future research should prioritize longitudinal intervention trials that track women through each stage of menopause. Furthermore, future studies should utilize multi-omic analyses, such as combining plasma oxylipins, lipoprotein composition, and gut microbiome data, to account for individual variations

in nutrient metabolism. For example, individual urolithin metabotypes influence the response to whole foods. Such an approach will enable the transition from general advice to personalized nutrition strategies for women. Finally, translating these results to the public through accessible communication and education is also essential for implementing strategies to support their cardiovascular health.