The Effect of High Polyphenol Extra Virgin Olive Oil *vs.* Low Polyphenol Olive Oil on Cardiovascular Disease Risk Markers in Australian Adults. The OLIVAUS study

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Abstract

Cardiovascular disease (CVD) is the leading cause of mortality worldwide and in Australia. In 2019, CVD was the underlying cause of 25% of all deaths in Australians. Preventive strategies are needed to counteract this trajectory. Diet plays an important role in the management and prevention of CVD. Extra virgin olive oil (EVOO), a key element of the Mediterranean Diet, has shown to be cardioprotective partly due to the presence of polyphenols. Most of this evidence is limited to Mediterranean populations.

This doctorate aims to investigate the effect high polyphenol olive oil (HPOO), compared to low polyphenol olive oil (LPOO) on CVD risk markers (high density lipoprotein (HDL) cholesterol efflux, HDL, low density lipoprotein (LDL), triglycerides, oxidised LDL (ox-LDL), total antioxidant capacity (TAC), inflammatory markers, peripheral/central blood pressure (BP) and arterial stiffness) in healthy Australian adults.

In a double-blind cross-over trial, 50 participants (mean age 38.5±13.9 years, 66% females) were randomized to consume 60 mL/day of HPOO (320 mg/kg polyphenols) or LPOO (86 mg/kg polyphenols) for 3 weeks. Following a 2-week wash-out, participants crossed-over to the alternate treatment. No significant between-group differences were observed in any of the examined markers. HDL increased significantly after LPOO and HPOO intake (+0.13 mmol/L; 95%CI 0.04 to 0.22 and +0.10 mmol/L; 95%CI 0.02 to 0.19, respectively). HPOO consumption led to a small significant increase in LDL (+0.14 mmol /L; 95%CI 0.001 to 0.28), reduced plasma ox-LDL (-6.5 mU/mL; 95%CI -12.4 to -0.5), increased TAC (+0.03 mM; 95%CI 0.006 to 0.05), reduced peripheral and central systolic BP (-2.5 mmHg; 95% CI: -4.7 to -0.3 and - 2.7mmHg; 95%CI: -4.7 to -0.6, respectively).

Our results indicate the cardioprotective effect of OO polyphenols. Longer interventions and/or higher concentrations of OO phenolic compounds are required to confirm these findings and further understand their effect on additional CVD risk markers and the involved mechanistic pathways in multi-cultural populations.

Statement of Authorship

"Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution".

I also certify that the thesis has been written by me, Katerina Sarapis. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged.

Katerina Sarapis

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OLIVAUS study Investigators and Collaborators

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List of Publications within this Thesis

Chapter 2

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Chapter 3

Sarapis, K., George, E.S., Marx, W., Mayr, H.L., Willcox, J.C., Powell, K.L., Folasire, O.S., Lohning, A.E., Prendergast, L.A., Thomas, C.J., Itsiopoulos, C., Moschonis, G. Effect of Olive Oil Polyphenols on HDL–mediated Cholesterol Efflux in healthy adults. The OLIVAUS study (to be submitted at *British Journal of Nutrition*; currently under review)

Chapter 4

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Chapter 5

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Conference presentation Abstracts

Katerina Sarapis, Elena S. George, Wolfgang Marx, Hannah L. Mayr, Jane Willcox, Katie L. Powell, Oladayo S. Folasire, Anna E. Lohning, Greg Kennedy, Andrew Pipingas, Manohar Garg, Luke Prendergast, Colleen J. Thomas, Catherine Itsiopoulos, George Moschonis. The effect of high polyphenol extra virgin olive oil on cardiovascular disease risk markers in Australian adults. The OLIVAUS study. In Proceedings of the 16th Panhellenic Conference on Nutrition & Dietetics, Athens, Greece, 2021

Katerina Sarapis, Colleen J. Thomas, Johanna Hoskin, Elena S. George, Wolfgang Marx, Hannah L. Mayr, Greg Kennedy, Andrew Pipingas, Jane C. Wilcox, Luke A. Prendergast, Catherine Itsiopoulos, George Moschonis. Reduced systolic blood pressure following high polyphenol extra virgin olive oil consumption: The OLIVAUS study. In Proceedings of the Dietitians Association of Australia National Conference, Melbourne, Australia, 2021

Katerina Sarapis, Elena S. George, Wolfgang Marx, Hannah L. Mayr, Jane Willcox, Katie L. Powell, Oladayo Folasire, Anna E. Lohning, Manohar Garg, Colleen J. Thomas, Catherine Itsiopoulos, George Moschonis. Extra virgin olive oil high in polyphenols improves antioxidant status in healthy adults. The OLIVAUS study. In Proceedings of the Nutrition Society of Australia Virtual Conference, 2020.

Katerina Sarapis, Colleen J. Thomas, Siddharth Shivantha, Elena S. George, Wolfgang Marx, Hannah L. Mayr et al. The effect of high polyphenol extra virgin olive oil on markers of cardiovascular disease risk in healthy Australian adults (OLIVAUS): Results from a double blind, randomized, controlled, cross-over feasibility pilot study. In Proceedings of the Dietitians Association of Australia National Conference, Gold Coast, Australia, 2019

Other presentations

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Title: "Effect of High Polyphenol Extra Virgin Olive Oil versus Low Polyphenol Olive Oil on Cardiovascular Disease Risk Markers in Australian Adults. The OLIVAUS study

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Title: "Effect of High Polyphenol Extra Virgin Olive Oil versus Low Polyphenol Olive Oil on Cardiovascular Disease Risk Markers in Australian Adults. The OLIVAUS study

Joint Symposium - La Trobe University and RMIT University: "Nutrition and Food Technology", November 2018. Researcher profile pitch sessions

Cobram Estate Research Briefing, Boundary Bend, Lara, June 2018

Title: "The effect of high-biophenol Extra Virgin Olive Oil on markers of cardiovascular disease risk.

Achievements

Media Release of results as part of Katerina's PhD research project investigating the effect of two different kinds of olive oil on cardiovascular disease markers (OLIVAUS study). The **audience reach** updates to **1,575,272** with a total of **153 media items/coverage** with mentions of La Trobe University.

The paper with title "The Effect of High Polyphenol Extra Virgin Olive Oil on Blood Pressure and Arterial Stiffness in Healthy Australian Adults: A Randomized, Controlled, Cross-Over Study" was recognized as the **most notable article** in the Nutritional Epidemiology category of the journal *Nutrients* in 2020.

Statement of Candidate Contribution

Table A. Percentage contribution (%) of authors to peer reviewed manuscripts

Chapter 2	Conceptualization/ Study Design	Data collection	Data analyses	Manuscript Preparation	Manuscript revision
George, ES	25%		N/A	50%	25%
Marx, W	25%			50%	25%
Mayr, HL	5%				5%
Thomas, CJ	5%				5%
Sarapis, K	5%	100%			5%
Moschonis, G	5%				5%
Kennedy, G	5%				5%
Pipingas, A	5%				5%
Willcox, JC	5%				5%
Prendergast, LA	5%				5%
Itsiopoulos, C	10%				10%

Chapter 3	Conceptualization/ Study Design	Data collection	Data analyses	Manuscript Preparation	Manuscript revision
Sarapis, K	5%	100%	70%	100%	60%
George, ES	25%				2%
Marx, W	25%				2%
Mayr, HL	5%				2%
Willcox, JC	5%				2%
Powell, KL					0.5%
Folasire, OS					0.5%
Lohning, AE					0.5%
Prendergast, LA	5%		10%		0.5%
Thomas, CJ	5%				10%
Itsiopoulos, C	10%				10%
Moschonis, G	5%		20%		10%

Chapter 4	Conceptualization/ Study Design	Data collection	Data analyses	Manuscript Preparation	Manuscript revision
Sarapis, K	5%	100%	70%	100%	60%
George, ES	25%				2%
Marx, W	25%				2%
Mayr, HL	5%				1.5%
Willcox, JC	5%				1.5%
Esmaili, T					0.5%
Powell, KL					0.5%
Folasire, OS					0.5%
Lohning, AE					1%
Garg, M					0.5%
Thomas, CJ	5%				10%
Itsiopoulos, C	10%				10%
Moschonis, G	5%		30%		10%

Chapter 5	Conceptualization/ Study Design	Data collection	Data analyses	Manuscript Preparation	Manuscript revision
Sarapis, K	5%	100%	60%	100%	60%
Thomas, CJ	5%				10%
Hoskin, J					0.5%
George, ES	25%				2%
Marx, W	25%				2%
Mayr, HL	5%				1.5%
Kennedy, G	5%				1%
Pipingas, A	5%				1%
Willcox, JC	5%				1.5%
Prendergast, LA	5%				0.5%
Itsiopoulos, C	10%				10%
Moschonis, G	5%		40%		10%

List of Abbreviations

4-AAP	4-Aminoantipyrine
AAS	Active Australia Survey
ABCA1	ATP-Binding Cassette Transporter A1
ABCG1	ATP-Binding Cassette Transporter G1
ACC	American College of Cardiology
ACE	Angiotensin Converting Enzyme
ACS	Acute Coronary Syndrome
ADMA	Asymmetric Dimethylarginine
ADP	Adenosine Diphosphate
AngII	Angiotensin II
AHA	American Heart Association
AIHW	Australian Institute of Health and Welfare
AIx	Augmentation Index
AMI	Acute Myocardial Infraction
ANOVA	Analysis of Variance
AP	Augmentation Pressure
ApoA-1	Apolipoprotein A-1
ApoE	Apolipoprotein E
ATP	Adenosine Triphosphate
AUD	Australian Dollar
BMI	Body Mass Index
BP	Blood Pressure
Ca2+	Calcium
CE	Cholesterol Estarase
CFPWV	Carotid-Femoral Pulse Wave Velocity
CHD	Coronary Heart Disease
CI	Confidence Interval
CM	Cardiometabolic Risk
CO	Cholesterol Oxidase
4-CP	4-Chlorophenol
CRF	Case Report Form

CRP	C-Reactive Protein
CV	Coefficient Variation
CVD	Cardiovascular Disease
DAP	Dihydroxyacetone Phosphate
DASH	Dietary Approach to Stop Hypertension
DBP	Diastolic Blood Pressure
DII	Dietary Inflammatory Index
EDHF	Endothelial-Derived Hyperpolarizing Factor
EDTA	Ethylenediaminetetraacetic
EE	Energy Expenditure
EFSA	European Food Safety Authority
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin -1
EVOO	Extra Virgin Olive Oil
FC	Free Cholesterol
FLOX	Fluoride Oxalate
FVOO	Functional Virgin Olive Oil
GCP	Good Clinical Practice
GK	Glycerol Kinase
GPO	Glycerol Phosphate Oxidase
GWAS	Genome-wide Association Studies
HBA	Hydroxybenzoic Acid
HDL	High Density Lipoprotein
HDL-M	Medium HDL
HDL-L	Large HDL
HDL-S	Small HDL
HDL-VS	Very Small HDL
H2O2	Hydrogen Peroxide
HPLC	High-Performance Liquid Chromatography
HPOO	High Polyphenol Olive Oil
hs-CRP	High Sensitivity C-Reactive Protein
HT	Hydroxytyrosol

ICAM-1	Intercellular Adhesion Molecule- 1
IDs	Identification Numbers
IL	Interleukin
IPAN	Institute for Physical Activity and Nutrition
ITT	Intention-to-treat
kg	Kilograms
LA	Left Arm
LDL	Low-density Lipoprotein
LL	Left Leg
LMEs	Linear Mixed Effects
LOX-1	Lectin-like Oxidized Low-Density Lipoprotein Receptor -1
LPOO	Low Polyphenol Olive Oil
LXR	Liver X Receptors
MCP-1	Monocyte Chemoattractant Protein -1
MDA	Malondialdehyde
MedDiet	Mediterranean Diet
MMPs	Matrix Metalloproteinases
MUFAs	Monounsaturated Fatty Acids
NFkB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHANES III	Third National Health and Nutrition Examination Survey
NO	Nitric Oxide
NZ	New Zealand
ox-LDL	Oxidized LDL
OO	Olive Oil
PA	Physical Activity
PAF-AH	Platelet-activating Factor Acetylhydrolase
PDGF	Platelet Derived Growth Factor
PICF	Patient Information and Consent Form
PI3Kγ	Phosphoinositide 3-Kinase Gamma
PON1	Paraoxonase Type 1
PP	Pulse Pressure

PP	Per Protocol
PUFAs	Polyunsaturated Fatty Acids
PWA	Pulse Wave Analysis
PWV	Pulse Wave Velocity
RA	Right Arm
RAAS	Renin-Angiotensin-Aldosterone System
RCT	Randomized Controlled Trial
RevCT	Reverse Cholesterol Transport
ROOs	Refined Olive Oils
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
sCD40L	Soluble CD40 Ligand
SD	Standard Deviation
SE	Standard Errors
SEM	Standard Error of the mean
SFAs	Saturated Fatty Acids
sICAM-1	Soluble Intercellular Adhesion Molecule -1
SOP	Standard Operating Procedure
SPIRIT	Standard Protocol Items: Recommendations for Interventional Trials
SPSS	Statistical Package for the Social Sciences
SR-BI	Scavenger Receptor Class B Type I
SST	Serum Separating Tube
SUCCAB	Swinburne Computerized Cognitive Assessment Battery
sVCAM-1	Soluble Vascular Cell Adhesion Molecule -1
TAC	Total Antioxidant Capacity
TC	Total Cholesterol
TG	Triglycerides
TLR	Toll-like Receptors
TNF-a	Tumor Necrosis Factor-alpha
UAE	Uric Acid Equivalents
VCAM-1	Vascular Cell Adhesion Molecule -1

VLDL	Very Low Density Lipoprotein
VOO	Virgin Olive Oil
VSMCs	Vascular Smooth Muscle Cells
WBCC	White Blood Cell Count
WC	Waist Circumference
WHO	World Health Organization
WISE	Women's Ischemia Syndrome Evaluation

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Thesis Structure

The introduction Chapter 1 includes the background, rationale and proposed aim(s) for this doctoral thesis. The chapter begins with an overview of cardiovascular disease (CVD) including the disease prevalence and burden, followed by the role of several risk factors in disease pathogenesis and progression. The rationale for diet as a strategy for CVD management is addressed with a focus on the cardioprotective effects of EVOO. However, most of the studies currently available in the literature have been restricted to populations from the Mediterranean areas, leading into the rationale of this study (OLIVAUS) that aimed to test the effect of two kinds of olive oil (OO) with different phenolic content (HPOO, 320 mg/kg phenolic content versus LPOO, 86 mg/kg phenolic content) on CVD risk markers in a healthy multi-ethnic population such as the Australian. Chapter 2 is based on the published protocol for the overarching randomized controlled trial by Marx *et al*. This chapter is a comprehensive methods chapter including a detailed description of the OLIVAUS study design, sampling procedures, randomization to treatment arms and blinding, study intervention, data collection, outcome measures and statistical analysis.

Chapter 3 in the form of a manuscript by Sarapis *et al*. (under review) explores the effect of the two kinds of OO on metabolic pathways that regulate cholesterol, such as the capacity of HDL to promote cholesterol efflux, and on serum lipids in healthy adults residing in Australia. In view of the potential antioxidant and anti-inflammatory benefits of OO polyphenols, Chapter 4 provides results from the effect of extra virgin HPOO compared to LPOO, on markers of oxidative status and inflammation in this population. This chapter is based on the published manuscript by Sarapis *et al*. Chapter 5 reports the findings of the dietary intervention with the two kinds of OO on peripheral /central blood pressure and measures of arterial stiffness, in the form of a published manuscript by Sarapis *et al*. The

OLIVAUS study is one of the first human trials to examine the effect of OO-derived polyphenols on the abovementioned hemodynamic indices by using non-invasive applanation tonometry. Finally, the concluding Chapter 6 discusses the implications of these findings and future recommendations. Figure 1 summarizes each chapter and the overall structure of this thesis.

PhD Thesis Structure

OLIVAUS study Protocol

Description of the study design sampling procedures, randomization, study intervention, data collection and outcome measures

CHAPTER 02

HDL Cholesterol Efflux Capacity

Results from the OLIVAUS study investigating the effect of olive oil polyphenols on HDL mediated cholesterol and lipid profile

CHAPTER 03

Oxidative Status & Inflammation

Results from the OLIVAUS study investigating the antioxidant and anti-inflammatory effect of olive oil polyphenols

CHAPTER 04

CHAPTER 05

Blood Pressure & Arterial Stiffness

Results from the OLIVAUS study investigating the effect of olive oil polyphenols on peripheral and central BP and measures of arterial stiffness

Conclusions & Future Recommendations

CHAPTER 06

Figure 1 A summary of thesis structure and the key components of each chapter

Chapter 1: Introduction

1.1   Cardiovascular Disease (CVD)

1.1.1   Epidemiology

Cardiovascular disease (CVD) is an umbrella term which describes a range of disorders that affect the heart and blood vessels. Key clinical manifestations of CVD include heart failure, cerebrovascular and peripheral arterial disease, and most prevalently, coronary heart disease (CHD). According to the most recent available data, CVD was the leading cause of mortality globally that accounted for approximately 17.9 million deaths (32% of all deaths worldwide) in 2019 (WHO, 2021).   In Australia, 42,300 deaths of Australians (25% of all deaths) were attributed to CVD in 2019 (AIHW, 2021). This means that on average, 119 Australians die from CVD each day at a rate of one death every 12 minutes, while the death rate was 1.4 times as high for males as for females. Furthermore, death rates increased with age, (>52% of CVD deaths occurring in individuals aged > 85 years old), and were 4.3 times and 6.2 times as high for males and for females, respectively, aged 75–84 years old (AIHW, 2021).

CHD is the most common form of CVD in Australia. In 2017-18, an estimated 580,300 Australian adults (2.8% of the population) had CHD. The prevalence of CHD was twice as high among men (3.8%) as women (1.9%), increasing rapidly with age; around 12 times as high in adults aged >75 years (13.9%) compared to those aged 45-54 (1.1%). Despite the advances in cardiovascular care and treatments, in 2018, CHD accounted for 42% of CVD deaths (11% of all deaths) (AIHW, 2021) (Figure 1.1) . CHD has two major clinical presentations: acute myocardial infarction (AMI) (also known as heart attack) and unstable angina, which constitutes Acute Coronary Syndrome (ACS). Approximately 59,100

Australians aged >25 years of age experience an ACS event each year, around 162 events daily, and these events are twice as high in men than in women (AIHW, 2021).

CVD imposes a significant economic burden on health care systems in terms of illness, disability, and premature death. Based on the Australian Institute of Health and Welfare (AIHW)/ National Hospital Morbidity Database, there were 591,000 hospitalizations (5.2% of all hospitalizations in Australia) in 2018-19, while an estimated $10.4 billion of total disease expenditure was attributed to CVD (AIHW, 2021). Considering the significant impact that CVD has on Australians, preventive strategies are urgently needed to counteract this trajectory.
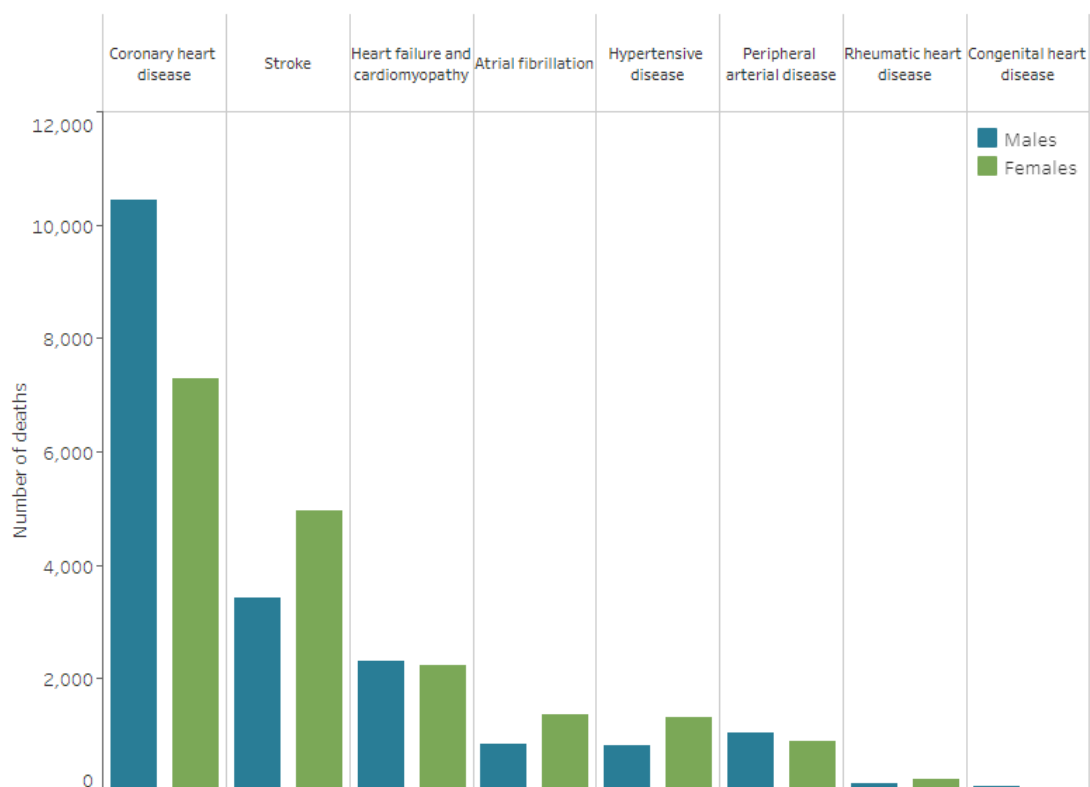
Figure 1.1   Major causes of cardiovascular disease death in Australia, 2018

Source: AIHW National Hospital Morbidity Database (AIHW, 2021)

1.1.2   Pathophysiology

Atherosclerosis-an inflammatory process

Atherosclerosis is a chronic low-grade inflammatory disorder of the blood vessel walls characterized by the accumulation of lipids, especially low-density lipoprotein (LDL), immune cells and fibrous tissue in the intima. Atherosclerosis was previously considered a cholesterol storage disease, however atherogenesis is currently understood to be a complex interaction of several risk factors (i.e., oxidative stress, dyslipidemia, hypertension, etc.) (Malakar et al., 2019).

Atherosclerosis is recognized as a sub-acute inflammatory condition, which is related to both the chronic development of plaque (atheroma) and its acute rupture (Figure 1.2). In the initial stages of atherosclerosis, risk factors (i.e., hypertension, smoking, dyslipidemia etc.) injure the arterial endothelium stimulating an inflammatory response, i.e., recruitment of immune cells from the blood stream, particularly monocytes (neutrophils, T- and B-lymphocytes), which bind to endothelial adhesion molecules (i.e., intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)) and migrate to the damaged site where they differentiate into macrophages (Jonasson et al., 1986; Tabas et al., 2015).

The endothelial cells change shape, whilst the tight junctions between endothelial cells also loosen, increasing the permeability to fluid, lipids, and leukocytes. LDL particles, enter the damaged sites of the arterial wall and undergo oxidative modification by vascular cells (i.e., endothelial cells, smooth muscle cells) and oxidizing enzymes (i.e., lipoxygenase and myeloperoxidase), leading to a loss of recognition by the LDL receptor (Borén et al., 2020). Macrophages recognize the oxidized LDL (ox-LDL) and begin to express scavenger and

toll-like receptors (TLR). Scavenger receptors bind to ox-LDL and perform phagocytosis, leading to foam cell formation, the main components of the fatty streaks. These are the initial atherosclerotic lesions which eventually evolve into a fibrous atheromatic plaque.

Activated macrophages secrete several inflammatory cytokines (i.e., interleukins IL-1, IL-6, IL-18, tumor necrosis factor-α (TNF-α)) and chemokines (Libby et al., 2009), which leads to activation and proliferation of vascular smooth muscle cells, lesion progression and finally to a vulnerable plaque by matrix degradation of its fibrous cap. Plaque rupture occurs predominately at the point where the fibrous cap is thinnest and highly infiltrated by macrophage foam cells. Increased levels of C-reactive protein (CRP), an acute phase protein synthesized in response to the other pro-inflammatory cytokines, actively contribute to the inflammatory processes and plaque rupture (Paffen & DeMaat, 2006). The latter can lead to thromboembolism and coronary artery occlusion, resulting in myocardial infarction (MI) and other acute cardiac events (Hansson et al., 2015).
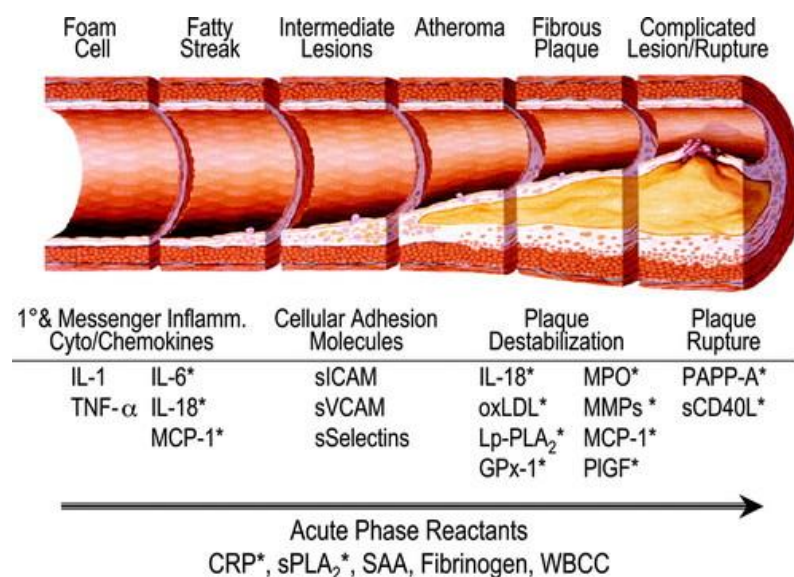
Figure 1.2   Schematic representation of the progressive development of atherosclerosis.

Injury to and inflammation of the endothelium plays a significant role in the initiation, progression and final stages of atherosclerosis. Endothelial cells produce adhesion molecules that interact with monocytes (inflammatory cells); macrophages secrete cytokines and chemokines, which lead to activation and proliferation of vascular smooth muscle cells, lesion progression and finally to plaque destabilization by matrix degradation of its fibrous cap. IL, interleukin; TFN-a, tumor necrosis factor-a; MCP-1, monocyte chemoattractant protein-1; sICAM-1; soluble intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ox-LDL, oxidized low density lipoprotein; Lp-PLA$_2$, lipoprotein associated phospholipase A$_2$; GPx-1, glutathione peroxidase; MPO, myeloperoxidase; MMPs, matrix metalloproteinases; PlGF, placental growth factor; PAPP-A, pregnancy-associated plasma protein-A; sCD40L, soluble CD40 ligand; CRP, C-reactive protein; sPLA$_2$, secretory type II phospholipase A$_2$; SAA, serum amyloid A; WBCC, white blood cell count. (*Wolfgang Koenig and Natalie Khuseyinova. Biomarkers of atherosclerotic plaque instability and rupture. Arteriosclerosis, Thrombosis, and Vascular Biology, 2007(27), 15-26. https://www.ahajournals.org/doi/10.1161/01.atv.0000251503.35795.4f; used with permission*).

1.2   Cardiovascular Disease (CVD) risk factors

Global comparative risk assessment studies have estimated that "hundreds of thousands or millions of CVD deaths" are attributed to several risk factors, some of which are non-modifiable while others are modifiable (Tzoulaki et al., 2016).

1.2.1   Non-modifiable CVD risk factors

Age, gender, ethnicity and family history are considered as the most important non-modifiable CVD risk factors (Malakar et al., 2019). According to the World Health Organization, ageing is recognized as a significant risk factor, with the prevalence of CVD increasing every decade after the age of 55 years (WHO, 2021). CVD develops later in females compared to males, with strong evidence for increased levels of endogenous oestrogen during women's reproductive age protecting against cardiovascular events

(Khamis et al., 2016). Supporting this fact, the women's Ischemia Syndrome Evaluation (WISE) study reported that young women with oestrogen deficiency have a 7-fold increase in the risk of heart disease (Bairey Merz et al., 2003). Moreover, females with declining oestrogen levels due to early menopause (< 40 years) have a lower life expectancy (~2 years) compared to those with normal or late menopause (Ossewaarde et al., 2005). In contrast to females, males have a 2-fold higher incidence of heart disease and related mortality, but the gap in morbidity declines with increasing age, as elderly women (>55 years; post-menopausal) experience a greater incidence of cardiovascular events (Gao et al., 2019).

Some ethnic groups (e.g., South-Asians and Aboriginal and Torres Strait Islander people) are showing higher rates of CVD mortality and morbidity than others (McGorrian et al., 2011). This can be partly explained by genetic differences among populations worldwide and/or changes in lifestyle behaviors (i.e., diet and physical activity), due to urbanization and 'Westernization' (Forouhi & Sattar, 2006). Family history has also been shown to play an important role in the onset of CVD at a young age (Goff et al., 2014; Malakar et al., 2019). Genome-wide association studies (GWAS), suggest that numerous genes and their variants are significantly implicated in the pathogenesis of CVD (McPherson & Tybjaerg-Hansen, 2016). For instance, disease-causing genes have been identified for familial hypercholesterolemia, mutations of which can lead to the premature onset of CVD (Malakar et al., 2019).

1.2.2   Modifiable CVD risk factors

According to the INTERHEART study, which was a large cohort study including population groups from 52 countries, nine modifiable factors were found to account for 90% of the risk of developing MI: dyslipidaemia, hypertension, type 2 diabetes, abdominal

obesity, psychosocial factors, smoking, increased alcohol consumption, physical inactivity and poor diet (Yusuf et al., 2020). Other large longitudinal studies such as the Framingham Heart Study (Fox et al., 2008) and the Third National Health and Nutrition Examination Survey (NHANES III) (Vasan et al., 2005) have demonstrated a strong correlation of at least one risk factor with CVD incidence. The major modifiable cardiometabolic and lifestyle CVD risk factors are described in further detail below.

1.2.2.1   *Cardiometabolic risk factors*

Oxidative stress and vascular inflammation play a significant role in the pathogenesis of atherosclerosis since they are closely related to endothelial dysfunction (Saggese et al., 2015). Oxidative stress is a "state of imbalance" between oxidants (also known as reactive oxygen species (ROS)) and antioxidants. Excessive formation of ROS is commonly observed in pathophysiological conditions, leading to cellular alterations and endothelial dysfunction (Sies, 1997). The latter, promotes a pro-inflammatory environment evidenced by increased expression of adhesion molecules (i.e., VCAM-1, ICAM-1, E-selectin) and chemoattractant molecules (i.e., MCP-1), subsequently resulting in a) increased susceptibility to foam cell formation (atherosclerosis) and b) impaired vascular homeostasis. Impaired ability to regulate vascular tone and increased inflammation could lead to high BP and vascular remodeling (Siti et al., 2015).

Hypertension

Blood pressure (BP) is the pressure that circulating blood exerts against the arterial wall, with pressure varying from maximum (systolic blood pressure, SBP), immediately following contraction of the heart, to minimum pressure (diastolic blood pressure, DBP), between contractions, when the heart relaxes and refills (Poulter et al., 2015). One of the

main systems involved in BP regulation is the Renin-Angiotensin-Aldosterone System (RAAS) (Ferrari, 2013). Low blood volume activates kidney cells to secrete renin, an enzyme which is responsible for converting angiotensinogen (protein of hepatic origin) to angiotensin-I. The latter is further metabolized to angiotensin-II (AngII), by angiotensin converting enzyme (ACE), which is primarily found in the vascular endothelium of the lungs and kidneys. AngII increases BP via two mechanisms: a) stimulating the release of aldosterone, which increases sodium and water reabsorption in the kidneys and b) direct vasoconstriction of arterioles. This leads to greater vascular resistance, therefore causing an increase in BP (Ferrari, 2013; Manrique et al., 2009). Figure 1.3 is a schematic representation of the RAAS system.
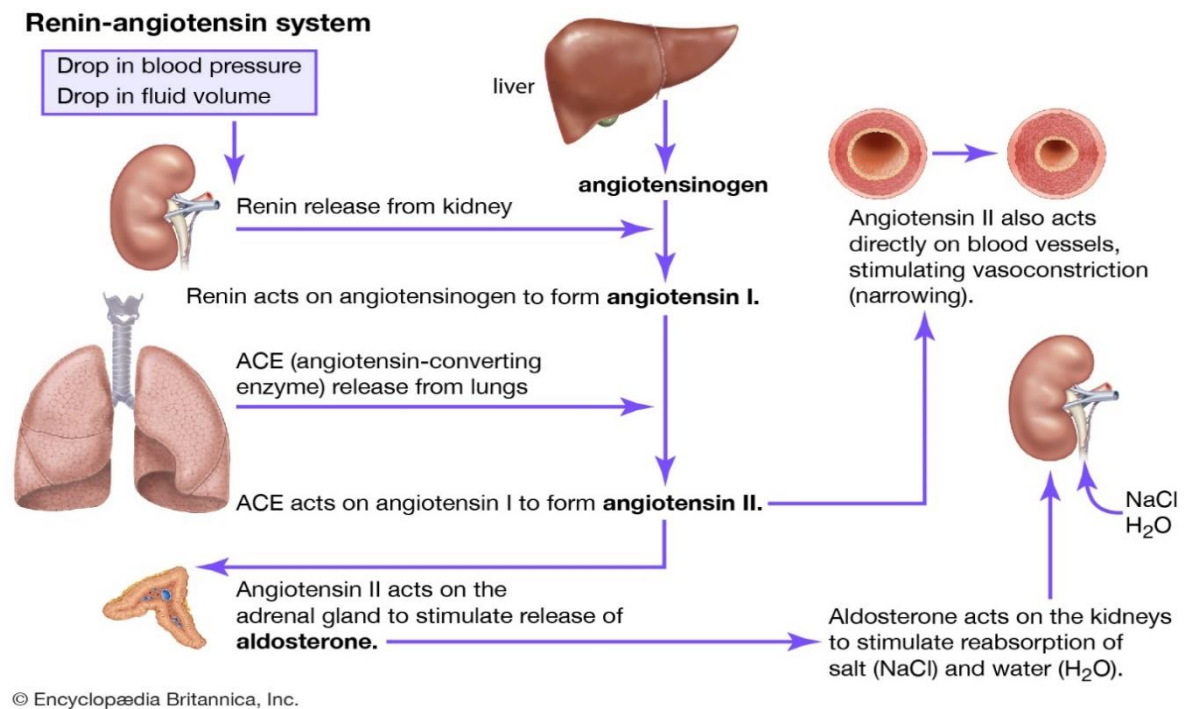
Figure 1.3   **Renin-Angiotensin-Aldosterone System (RAAS).**

> The RAAS plays an important role in regulating vascular resistance and blood volume, and in turn arterial blood pressure (*By courtesy of Encyclopædia Britannica, Inc., copyright 2015; used with permission*).

Increased levels of ROS as well as AngII have been reported to trigger intracellular pathways (via activation of AT-1 receptor) that promote vascular inflammation and subsequently hypertension (Grote et al., 2004). Furthermore, ox-LDL also appears to play a significant role in the pathogenesis of hypertension (Ryoo et al., 2011). One of the main ox-LDL receptors that has gained attention due to its proinflammatory potential, is the Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (Kattoor et al., 2019). Exposure to ROS, ox-LDL and numerous proinflammatory stimuli have been shown to

trigger LOX-1 expression. In particular, the inflammatory cytokine TNF-α has been shown to increase LOX-1 expression via activating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) inflammatory signaling pathway, which in turn stimulates the expression of NF-kB dependent adhesion molecules, such as VCAM-1 and ICAM-1 (Stein et al., 2010). Oxidized LDL has also been reported to activate Arginase II, which downregulates the synthesis of nitric oxide (NO) by competing with endothelial nitric oxide synthase (eNOS). Reduced bioavailability of NO could result in impaired vasorelaxation and therefore hypertension (Giles et al., 2012; Zhou et al., 2013). Figure 1.4 illustrates the link between endothelial dysfunction markers, inflammatory markers and oxidative stress with hypertension and atherosclerosis (Saggese et al., 2015).

Arterial stiffness

There is evidence supporting the role of vascular inflammation also in the pathogenesis of vascular remodeling. Vascular remodeling involves structural changes in the arteries that cause stiffening and reduced vascular compliance, thereby leading to increased BP (Ng et al., 2012; van Bussel et al., 2012). Increased pulsatile forces resulting from hypertension lead to high levels of stress on the arteries, causing the elastic tissue in the tunica media to fatigue (Hodis & Zamir, 2009). The latter, leads to fragmentation of the elastin fibres. The damaged fibres are then progressively replaced by collagen, which is stiffer and less compliant resulting in reduced vessel distensibility. The degradation of the elastic fibres is often accompanied by calcium salt deposition, leading to additional arterial stiffness via calcification (Lillie & Gosline, 2007).

It is also known that stiffening of the large thoracic conduit arteries may increase the velocity of aortic pulse waves and pulse pressure (PP). Increased pulse wave velocity

(PWV) results in the earlier return of pressure waves, hence leading to an augmentation of central (aortic) systolic pressure and reduction in central diastolic pressure (O'Rourke & Safar, 2005). Vascular remodeling is initially intended as an adaptation to perpetuate blood flow, however these structural modifications may ultimately lead to adverse vascular complications.
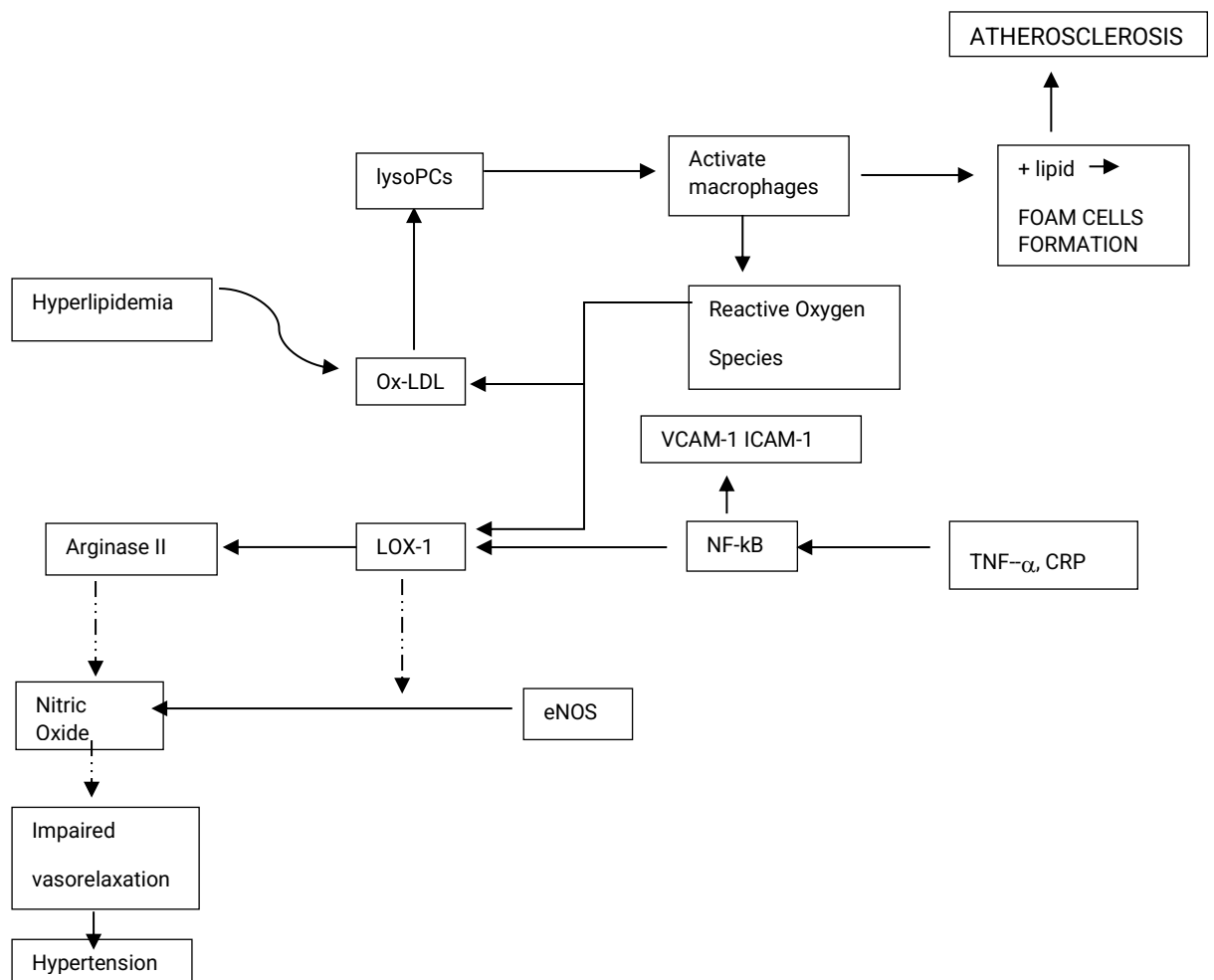
Figure 1.4   Schema of the link between endothelial dysfunction markers, inflammatory markers and oxidative stress with hypertension.

Oxidized low density lipoprotein (ox-LDL) and Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) play a significant role in the development of hypertension. Arginase II, enzyme that reduces nitric oxide formation; eNOS,

endothelial nitric oxide synthase, LysoPC, lysophosphatidylcholine is a chemoattractant for T cells and monocytes, promoting endothelial dysfunction; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; TNF-α, tumor necrosis factor-α; CRP, C-reactive protein. Solid arrows indicate induce; dashed arrows indicate inhibit. (*Hawa N. Siti, Y. Kamisah and J. Kamsiah. The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease (a review). Vascular Pharmacology, 71 (2015), 40–56.* https://pubmed.ncbi.nlm.nih.gov/25869516/; *used with permission*).

Dyslipidemia is also considered as a major modifiable risk factor for cardiovascular events. Dyslipidemia is characterized by elevated plasma levels of total cholesterol (TC), LDL cholesterol and triglycerides (TG), and reduced high density lipoprotein (HDL) cholesterol, often associated with excess abdominal adiposity and insulin resistance (Siri-Tarino & Krauss, 2016). Dyslipidemia results in altered arterial endothelial cell permeability that allows the accumulation of lipids, especially small, dense LDL in the arterial wall, where they bind to extracellular matrix and aggregate (Farràs et al., 2020). Modifications in LDL composition and particle size including oxidative modifications (i.e., ox-LDL), result in unstable LDL particles, which are no longer recognized by LDL receptors but by macrophage receptors; ox-LDL is then taken up by macrophages forming foam cells which expand and accumulate in the cell wall leading to plaque formation (Farràs et al., 2020; Kunitake et al., 1990).

On the other hand, serum HDL has been reported to be inversely correlated with CVD risk (Farràs et al., 2020). HDL apolipoprotein A-1 (ApoA-1) has been shown to exert an anti-inflammatory effect on the vascular system, by reducing proinflammatory cytokine levels, i.e., TNF-α, IL-6 and IL-8, and inhibiting the expression of cytokine-induced adhesion molecules (i.e., VCAM-1 and ICAM-1) (Papageorgiou et al., 2016). Furthermore, HDL

protects LDL against oxidation whilst reduces the biological activity of ox-LDL (Farràs et al., 2020). In addition, HDL is considered as an important vasoprotective agent since it stimulates the release of endothelial NO, which plays a pivotal role in maintaining vascular tone (Farràs et al., 2020; Loscalzo, 2001).

Role of HDL-mediated cholesterol efflux in CVD

Serum HDL has several antiatherogenic properties, including the ability to mediate macrophage cholesterol efflux. Previous studies have demonstrated an inverse association between HDL cholesterol, known as the "good cholesterol", and the risk of cardiovascular events, with elevated HDL levels to be considered as atheroprotective (Farràs et al., 2020; Rohatgi et al., 2014). Recently, however, emerging evidence suggests that impaired HDL function, rather than low HDL cholesterol concentrations, may explain HDL-associated CVD risk (Farràs et al., 2020).

The main biological function of the HDL particle appears to be its involvement in the reverse cholesterol transport (RevCT) pathway, in which excess cholesterol is removed from peripheral cells and transported to the liver for excretion in bile and faeces. Cholesterol efflux, which is the initial step of RevCT occurs via pathways that involve cell membrane transporters, plasma lipid acceptors, plasma proteins and enzymes, and hepatic cellular receptors (Berrougui et al., 2015; Helal et al., 2013). Figure 1.5 illustrates the metabolic pathways that are involved in the removal of excess cholesterol from macrophage foam cells. Briefly, these involve: 1) aqueous diffusion, 2) the ATP-binding cassette transporter A1 (ABCA1) dependent cholesterol efflux pathway, 3) the ATP-binding cassette transporter G1 (ABCGI), 4) the scavenger receptor class B type I (SR-BI), and 5) endogenous production of lipid-poor apolipoprotein E (apoE).

More specifically, 1) aqueous diffusion involves the desorption of free cholesterol (FC) from the cell membrane into the surrounding aqueous phase. Desorbed FC molecules then collate with HDL particles, leading to their uptake into the lipoprotein acceptor. Other acceptors involved in this stage are HDL subclasses, such as large HDL (HDL-L), medium HDL (HDL-M) and small HDL (HDL-S), 2) the primary acceptor for cholesterol efflux via the ABCA1 pathway is cholesterol/lipid poor apoA-1. The binding of apoA-1 with ABCA1 increases the levels of ABCA1 transporter in the plasma membrane. Both ABCA1 transporter and apoA-1 facilitate transport of cholesterol from the endocytic compartment to the cell membrane via an intracellular pathway, 3) the ABCG1 transporter promotes efflux of cholesterol from foam cells to HDL particles. Of note, increased expression of the ABCA1 and ABCG1 receptors is mediated by LXR transcription factors, 4) the SR-B1, an integral membrane protein, mediates bidirectional efflux of unesterified FCs between macrophage cells and HDL or other acceptors, and 5) apolipoprotein-E (apoE) containing HDL (E-HDL), which is synthesized in the liver and macrophages, also facilitates the efflux of cholesterol from the macrophage by binding to the ABCA1 transporter. Cholesterol is then delivered to the liver by E-HDL particles through the interaction with the SR-B1 and LDL receptors (Rosenson et al., 2012).
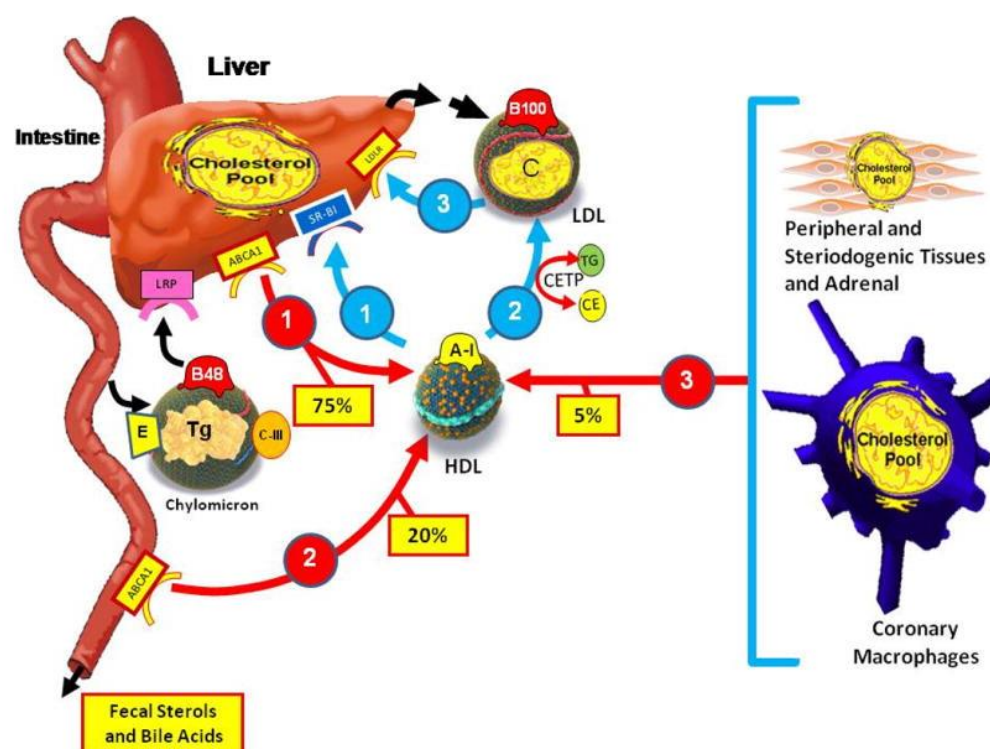
Figure 1.5   Representation of HDL metabolic pathways

One important pathway for cholesterol-mediated efflux from macrophage foam cells involves interaction between the ATP-binding cassette transporter A1 (ABCA1) with lipid-poor apoA-I (preβ migrating HDL or very-small HDL (HDL-VS)). Subsequently, the ATP-binding cassette transporter G1 (ABCGI) mediates macrophage cholesterol efflux through interactions with cholesterol-containing alpha HDL particles (small HDL, medium HDL (HDL-M), large HDL [HDL-L] and very large (HDL-VL). On the other hand, the scavenger receptor class B type I (SR-BI) receptor, mediates bidirectional efflux of unesterified FCs between macrophage cells and HDL or other acceptors. (*Robert S. Rosenson, H. Bryan Brewer, Jr, W. Sean Davidson, Zahi A. Fayad, Valentin Fuster, James Goldstein, Marc Hellerstein, XianCheng Jiang, Mic hael C. Phillips, Daniel J. Rader, Alan T. Remaley, George H. Rothblat, Alan R. Tall, and Laurent Yvan-Charvet.(2012).   Cholesterol   Efflux   and   Atheroprotection. Circulation,   125(15),1905–1919. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4159082/; used with permission*).

HDL functionality can be affected in several ways. For instance, the oxidative damage of apoA-1 observed in patients with CVD impairs its ability to promote cholesterol efflux through the ABCA1 pathway (Shao et al., 2010). Furthermore, acute phase response to inflammation and alterations in HDL enzymes and lipid transfer proteins have also been found to affect HDL metabolic pathways (Farràs et al., 2020). Studies of large populations have shown that efflux of cholesterol from macrophage cells is impaired in patients with CVD (Khera et al., 2011; Li et al., 2013). Results from a recent case control study, the EPIC-Norfolk study, which involved 1745 initially healthy people who later developed fatal/non-fatal CHD, confirmed a significant inverse association between HDL-c efflux and incident CHD (Saleheen et al., 2015). Similarly, results from the Dallas Heart study, a multiethnic population-based cohort study, demonstrated an inverse association between HDL mediated cholesterol efflux and incident atherosclerotic CVD in 2924 adults free from CVD at baseline (Rohatgi et al., 2014). Thus, the observation that cholesterol efflux capacity is correlated with cardiovascular events, further supports the use of HDL efflux as a parameter in guiding the development of new HDL-target therapies for humans.

1.2.2.2 *Lifestyle risk factors*

Factors such as tobacco smoking, excessive alcohol consumption, physical inactivity and poor diet are recognized as the most important modifiable lifestyle risk factors related to CVD (Badimon et al., 2019; Mozaffarian et al., 2008). In particular, smoking has been found to increase the risk of developing CVD, with tobacco smokers having double the risk of experiencing a cardiovascular event compared to non-smokers (Banks et al., 2019). The risk is greater at younger ages (<50 years), also increases with prolonged use of tobacco and greater intensity of smoking. The association between CVD and smoking has also been reported to be higher in females than their male counterparts, potentially due to the

downregulation of the estrogen-dependent vasodilation of the arteries caused by smoking (Gao et al., 2019).

Data from numerous epidemiological studies have demonstrated a complex relationship between alcohol intake and cardiovascular events, with the dose and pattern of alcohol consumption strongly influencing these associations (Potter et al., 1986; Rosito et al., 1999; Seppä & Sillanaukee, 1999). In particular, low-to-moderate daily alcohol consumption (i.e., <15 to 20 g/day, 1 to 2 standard drinks) is associated with a decreased incidence of heart disease and mortality compared to greater amounts of alcohol intake (Piano, 2017). Several factors seem to be involved in the alcohol's positive and/or adverse effects on cardiovascular conditions, including modifications of lipid profile, carotid intima-medial thickness and insulin sensitivity, homeostatic factors such fibrinogen levels and platelet reactivity, and inflammation. Lower doses of alcohol intake are shown to be correlated with reduced inflammation, as indicated by markers such as CRP and certain interleukins, whilst higher levels have been found to induce oxidative stress and endothelial inflammatory response (Piano, 2017).

Sedentary behavior and physical inactivity are also major modifiable risk factors associated with metabolic disorders and increased CVD risk. Long-term studies have demonstrated that regular physical activity and frequent exercise are strongly correlated with a lower risk of developing cardiovascular and respiratory diseases (Nystoriak & Bhatnagar, 2018; Paffenbarger et al., 1986). Interestingly, death rates among both genders have been found to be inversely correlated with cardiorespiratory fitness levels, even in the presence of other CVD risk factors such as smoking, high BP, and dyslipidemia (Nystoriak & Bhatnagar, 2018). Data from recent cardiovascular cohort studies have shown that sustained physical activity is correlated with an improved inflammatory marker profile, decreased heart failure

risk, and improved survival at 30 years follow-up in individuals with CHD (Moholdt et al., 2018; Vella et al., 2017). A significant dose-response relationship between daily sedentary time and both all-cause and CVD mortality has also been previously reported (Lavie et al., 2019).

1.3   Diet and CVD

There is strong evidence supporting the relationship between diet and cardiovascular health (Fung et al., 2001; Hu et al., 2000; Menotti et al., 1989). Poor dietary habits characterized by increased intake of foods high in saturated fats and sugar have been strongly associated with the development of atherosclerosis and other cardiometabolic related conditions such as metabolic syndrome, diabetes, and hypertension (Benjamin et al., 2018; Curry et al., 2018; Fox et al., 2004; Vasan et al., 2005). Previous research has demonstrated that a "Western" dietary pattern is associated with increased risk of diet-related chronic disease. This dietary pattern is characterized by a high intake of red and processed meat, high-fat dairy products, refined grains, discretionary foods (i.e., sweets, pastries, biscuits, cakes and desserts) and alcohol (Manzel et al., 2014).

On the contrary, epidemiological and clinical studies suggest that dietary patterns that are plant-based, high in dietary fibre and low in animal fats are cardioprotective (Chiavaroli et al., 2018; Keys, 1995; Lankinen et al., 2019; Tuttolomondo et al., 2019). There are several examples of such dietary patterns, i.e., the Dietary Approach to Stop Hypertension (DASH), a well-researched dietary intervention, which is designed specifically for blood pressure lowering effects and features vegetables, whole grains, low fat dairy, fish, poultry, lean meat, nuts and beans (Saneei et al., 2014); the Nordic diet, which comprises of whole grains from oats, barley and rye, berries, vegetables, fatty fish and rapeseed (Lankinen et al., 2019); the Portfolio diet, which has shown to have beneficial effects on CVD markers

as a "portfolio" of four cholesterol-lowering foods/nutrients (i.e., nuts, dietary pulses, soluble fibre, plant sterols, soy protein) (Chiavaroli et al., 2018). Finally, a healthy dietary pattern which has been widely studied over several decades for its cardioprotective properties, and for which there is the most scientific evidence, is the Mediterranean diet (MedDiet) (Keys, 1995; Tuttolomondo et al., 2019).

1.3.1 Mediterranean Diet

The MedDiet was first described in the late 1950's by Keys and colleagues (Keys et al., 1986) who identified the cardioprotective effect of traditional dietary patterns across 'Seven Countries' that surround the Mediterranean Sea; Spain, Italy, Greece, Turkey, North Africa, Lebanon and Israel. This study found that the incidence of CHD was particularly low in the Greek Island of Crete, owing to low content of saturated fat in the MedDiet and high consumption of plant foods and olive oil (OO) (Keys et al., 1986). Thus, the Cretan Diet is considered a 'traditional' Mediterranean dietary pattern which Keys himself described as a diet rich in whole grains, fruit, vegetables, low in meat and processed foods, with a considerable amount of fat deriving from OO and nuts. This dietary pattern seemed to be a possible determinant of the significant difference in CVD prevalence between Mediterranean cohorts and the Western population in the Seven Countries Study.

Since the Seven Countries Study, numerous meta-analyses of observational and clinical studies have also demonstrated that individuals who adhere to the MedDiet have a significantly lower risk in CVD incidence and mortality (Dinu et al., 2018; Liyanage et al., 2016; Sofi et al., 2014). In this context, data from the Lyon Heart Study showed that adherence to the Mediterranean dietary pattern was associated with a reduction of CVD events and death for up to four years after the first infarction, thus establishing this diet as a staple in secondary prevention (de Lorgeril et al., 1999). The traditional MedDiet

emphasizes an abundance of plant-based foods (fruit, root vegetables), grains (mainly whole), legumes, nuts/seeds, consumption of moderate amounts of fish/seafood, poultry and wine, low amounts of dairy products and red meat, whilst extra virgin olive oil (EVOO) is the main source of dietary fat (George et al., 2018) (Figure 1.6). Thus, the diet's high content in foods rich in antioxidants, fibre, polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) could potentially explain the observed health benefits (Martínez-González et al., 2019).

Although the observed protective effects of the MedDiet may be attributed to the overall dietary pattern, the significant inverse associations with cardiovascular events also suggest the potential role of the diet's single key foods and/or nutrients in determining disease risk outcomes (Grosso et al., 2017). In this sense, results from a multicentre clinical trial, the PREvencion con DIeta MEDiterannea (PREDIMED) study, where high CVD risk participants were randomly assigned to a MedDiet supplemented with EVOO or nuts or a low-fat control diet, showed that supplementation with EVOO and with nuts were both independently effective in reducing CVD risk compared to the low-fat diet (Casas et al., 2016). These findings highlight the importance of taking into consideration not only the diet as an overall but also its individual key food components in order to fully understand any observed health benefits.

Mediterranean Diet Pyramid
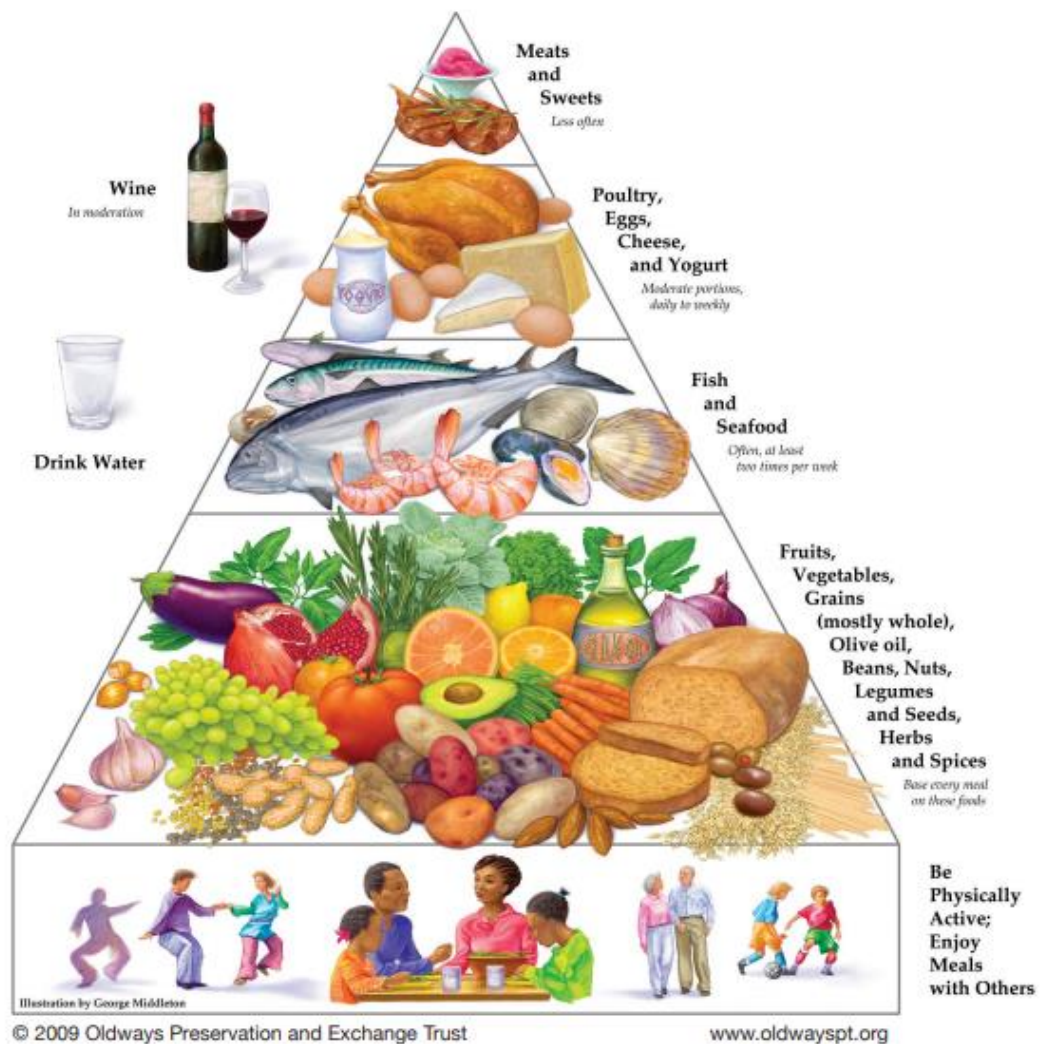
Figure 1.6   The Mediterranean Diet Food Pyramid.

The Mediterranean Diet (MedDiet) consists predominantly of vegetables, fruits, legumes, nuts and seeds with moderate intake of poultry and fish/seafood and small amount of red meat. Olive oil is the primary source of fat in the diet (*Image used with permission by Oldways, www.oldwayspt.org*).

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1.3.2   Extra virgin olive oil

Extra virgin olive oil composition

Olive oil (OO), a hallmark of the MedDiet, is obtained from the olive tree fruit (Olea europaea L, Oleacea family). It is classified into three categories according to the degree of acidity[1]: extra virgin olive oil (EVOO) (<0.8% acidity), virgin olive oil (VOO) (0.8-0.2%, acidity) and lampante OO (>2%, acidity) (Pérez et al., 2014).   OO is composed of two fractions. The saponifiable fraction represents approximately 98% of the oil's composition and consists of MUFAs (i.e., oleic acid: 55–83%), PUFAs (i.e., linoleic fatty acid: 3.5–21%) and SFAs (i.e., palmitic fatty acid: 7.5–20%, stearic fatty acid: 0.5–5%). The unsaponifiable fraction constitutes 1–2% of the total content of OO, and includes more than 230 compounds grouped into six categories: (1) sterols; (2) hydrocarbons (e.g., squalene and carotenoids); (3) volatile compounds; (4) triterpenic and aliphatic alcohols; (5) pigments and (6) phenolic compounds or polyphenols (Ghanbari et al., 2012).

EVOO phenolic compounds are secondary plant metabolites responsible for OO's sensory characteristics (aroma and flavor) and oxidative stability. They are classified in the following main groups based on their chemical structure: phenolic acids and their derivatives, lignans, flavonoids, phenolic alcohols (i.e., hydroxytyrosol, tyrosol), secoiridoids (i.e., oleuropein, oleacein, oleocanthal) and hydroxy-isocromans (Rodríguez-López et al., 2020). The synthesis of phenolic compounds occurs in the olive fruit as a response to the fruit's ripening process and interactions with microorganisms, but they are also products of chemical and enzymatic reactions that occur during the oil's extraction

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[1] Acidity is defined as a percentage, as grams of free fatty acids (i.e., oleic acid) in 100 grams of oil.

procedure (Cicerale et al., 2010; Rodríguez-López et al., 2020).

OO's phenolic content highly depends on several factors, such as the plant variety, environmental conditions (region, soil, climate), maturation of the fruit, storage of the final product and extraction techniques (Ghanbari et al., 2012). VOOs, which are obtained by mechanical extraction methods (i.e., direct-press or centrifugation) preserve high phenolic content (ranging from 200–800 mg/kg), while refined OOs (ROOs) that are subjected to both physical and/ or chemical processing, present a lower phenolic content profile (ranging from 62-198 mg/kg) (Frankel et al., 2013; Rothwell et al., 2013).

1.4   Effects of extra virgin olive oil on CVD risk markers

Growing evidence indicates that regular consumption of EVOO reduces the risk of developing chronic diseases (i.e., CVD, diabetes and cancer) (Badimon et al., 2019; Mozaffarian et al., 2008). Besides its monounsaturated fatty acid content, the antioxidant and anti-inflammatory effects of phenolic compounds present in EVOO are suggested to contribute to its beneficial effects (Sarapis et al., 2020). The mechanisms by which OO polyphenols elicit an anti-inflammatory effect, particularly in CVD, involves: (1) antioxidant activity; (2) modulation of signaling pathways and receptors (i.e., blocking the signaling and expression of chemokines and cellular adhesion molecules); (3) reduction of the adhesion of immune cells (T lymphocytes and monocytes) to the endothelium; and (4) improvement of vascular function (Banks et al., 2019; Papageorgiou et al., 2016). Figure 1.7 demonstrates the effect of dietary antioxidants on oxidative stress and inflammation on a vascular level.
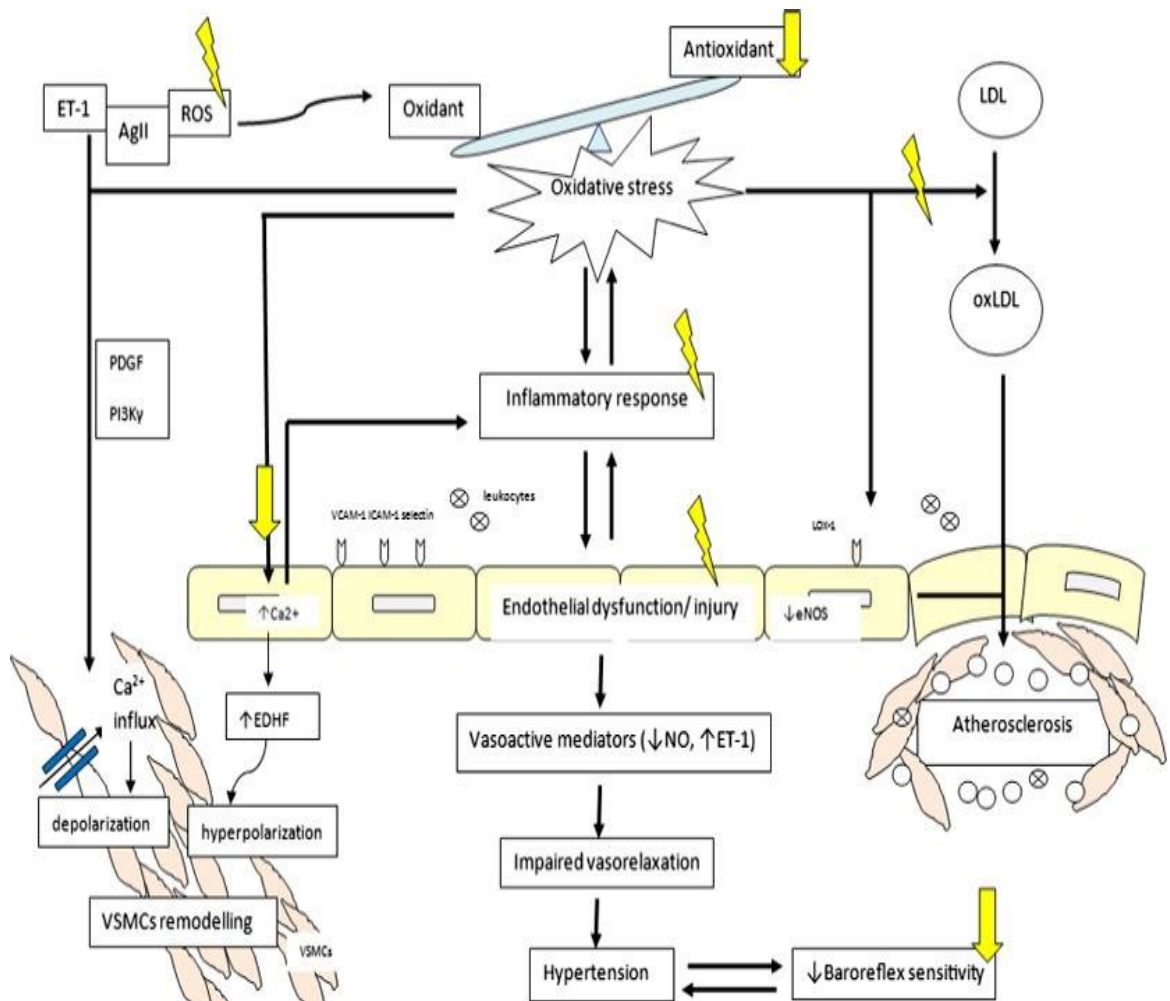
Figure 1.7   **Schema of the effect of dietary antioxidants on oxidative stress and inflammation on a vascular level.**

"Oxidative stress may occur by several stimulants, such as ROS, AgII, ET-1 and inflammatory cells. Oxidative stress, inflammation and endothelial dysfunction synergistically contribute to the damaging process. Oxidative stress may cause inflammation which further results to injury of the endothelium, hence leading to endothelial dysfunction, and consequently to impaired vascular homeostasis (reduced bioavailability of NO and increased release of the vasoconstrictor ET-1 peptide). Impaired ability to regulate vascular tone and increased inflammation could lead to increased susceptibility to formation of foam cells (atherosclerosis) and hypertension. Oxidative stress may also cause endothelial cells Ca^{2+} influx which in turn aggravates inflammatory response. Antioxidants may act as scavengers for ROS, increase

antioxidant enzymes, reduce oxidative and inflammatory process, improve EDHF[2] and Baroreflex[3] sensitivity and prevent endothelial dysfunction. **Abbreviations:** ET-1, endothelin-1; AgII, angiotensin II; ROS, reactive oxygen species; LDL, low density lipoprotein; oxLDL, oxidized LDL; eNOS, endothelium nitric oxide synthase; LOX-1, lectin-like oxidized low density lipoprotein receptor; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; Ca^{2+}, calcium; EDHF, endothelial-derived hyperpolarizing factor; PDGF, platelet derived growth factor; PI3Kγ, phosphoinositide 3-kinase gamma; NO, nitric oxide; VSMCs, vascular smooth muscle cells . Symbols indicate: ⚡antioxidant prevent; ⬇ antioxidant promote".

(Hawa N. Siti, Y. Kamisah and J. Kamsiah. The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease (a review). Vascular Pharmacology, 71 (2015), 40–56. https://pubmed.ncbi.nlm.nih.gov/25869516/*; used with permission)*

1.4.1   Effect of EVOO on oxidative stress and vascular inflammation

There is strong evidence supporting the beneficial effects of OO polyphenols on oxidative stress, inflammation and endothelial dysfunction in humans. A recent systematic review and network meta-analyses compared the effects of daily intake (20 -75 mg, daily dose) of EVOO (>200 mg/kg, phenolic content) vs. LPOO (<200 mg/kg, phenolic content) on several CVD risk markers, including markers of oxidative stress and inflammation, predominantly in healthy adults. There was a significant decrease in ox-LDL, CRP and IL-6 levels following EVOO intake compared to LPOO (Schwingshackl et al., 2019). Another recent systematic review and meta-analyses evaluating the effect of HPOO vs. LPOO (150-800 mg/kg vs. 0-132 mg/kg, phenolic content, respectively; 25-75 mL, daily dose) on CVD risk factors reported that adherence to HPOO significantly improved malondialdehyde

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[2] Endothelial-derived hyperpolarizing factors (EDHF) are other vasoactive mediators that regulate vascular tone apart from NO, ET-1 and prostaglandin

[3] The baroreflex is one of the body's homeostatic mechanisms that helps to maintain blood pressure at nearly constant levels.

(MDA) [4] and ox-LDL measurements. Subgroup analyses and individual studies also reported improvements in inflammatory markers (George et al., 2019).

Authors in another network meta-analysis explored the role of OO with different phenolic content (ranging between 2 and 607 mg/kg) in the modification of metabolic factors, such as oxidative stress and inflammation (Tsartsou et al., 2019). The data confirmed the protective effect of OO polyphenols on LDL oxidation and inflammatory markers, demonstrating that lower polyphenol concentrations (i.e., 60 mg/kg, phenolic content) may still be sufficient to exert cardioprotective effects. Of note, the anti-inflammatory effect of the OO was more pronounced in patients with CVD or with an established metabolic syndrome. Moreover, in the PREDIMED study, high CVD risk participants were randomly assigned to 1 of 3 diets: a MedDiet supplemented with EVOO or nuts or a low-fat control diet. Compared with baseline, at 3 and 5y, adherence to both MedDiet groups supplemented with EVOO (255 mg/100 g, phenolic content; 50 ml, daily dose) or nuts (30 g mixed nuts/day), respectively, demonstrated significant reductions of 16% in circulating hs-CRP, IL-6, TNF- a, and MCP-1, compared to the low-fat diet intervention arm (Casas et al., 2016).

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[4] *Malondialdehyde* (MDA) is formed during oxidative degeneration as a product of free oxygen radicals. It is an indicator of lipid peroxidation.

1.4.2 Effect of EVOO on BP and measures of endothelial dysfunction and arterial stiffness.

Endothelial dysfunction has been accepted as an early determinant in the development of hypertension since the endothelium is considered as an important regulator of vascular homeostasis (Sanchez-Rodriguez et al., 2018). Impaired balance between vasoconstricting and vasodilating molecules, such as ET-1 and NO, respectively, could lead to the pathogenesis and /or maintenance of high BP since both play a pivotal role in maintaining vascular homeostasis (Brunner et al., 2005; Dhaun et al., 2008).

Large-scale studies have shown that adherence to a MedDiet supplemented with EVOO was associated with lower peripheral SBP via the production of plasma NO, in both hypertensive and normotensive patients (Rafael Moreno-Luna et al., 2012; Patino-Alonso et al., 2015; Perona et al., 2004). Furthermore, a meta-analysis reported that an OO with at least 150 mg/kg phenolic content can exert a moderate lowering effect on SBP, but no effect on DBP (George et al., 2019). Other authors have described a decrease in peripheral DBP but no changes in SBP when consuming 25 mL/day EVOO (366 mg/kg) compared to medium (164 mg/kg) or low (2.7 mg/kg) phenolic content OO, in healthy men (Covas et al., 2006). Recently, it has been found that VOO intake (366 mg/kg, phenolic content; 25 mL daily dose) can also exert a beneficial effect on the expression of genes related to the RAAS system, demonstrating the antioxidant and ant-inflammatory potential of OO polyphenols (Martin-Pelaez et al., 2017).

As previously mentioned, stiffening in the larger central arterial system and increased central (aortic) BP significantly contribute to CVD and are positively associated with hypertension (Bulas et al., 2017). Although brachial cuff BP has been extensively used for the diagnosis of hypertension, this method does not allow for the estimation of vascular

stiffness and central BP (London, 2008). Thus, other techniques have been used to assess these hemodynamic parameters. The standard method for non-invasive estimation of arterial stiffening (and central BP) is applanation tonometry, in which pulse waves in carotid arteries are detected by a tonometer (Stergiou et al., 2016).

Of note, the effect of EVOO polyphenols on markers of arterial stiffness and central BP measured via applanation tonometry has not been previously reported (to our knowledge) in the scientific literature. However, there are some studies that have explored the effect of OO polyphenols on surrogate markers that are closely related to poor endothelial function (i.e., asymmetric dimethylarginine (ADMA))[5], NO, ET-1), hence impaired ability to regulate vascular tone. In this context, decreased levels of plasma ADMA and increased NO concentrations were reported after adhering to a MedDiet supplemented with VOO (564 mg/kg, phenolic content; 60 mL daily dose) compared to a MedDiet with a polyphenol-free OO, for 8 weeks, in women with normal-high BP and/or at stage 1 hypertension (SBP 130-139 mmHg or DBP 80-89 mmHg). The same authors examined the hyperemic response after intermittent ischemia and observed increased plasma levels of nitrite/nitrate, supporting a key role of EVOO polyphenols on vascular tone modulation (R. Moreno-Luna et al., 2012). Another study demonstrated a small but significant reduction in plasma concentrations of ET-1 after a 3-week dietary intervention with EVOO (124 mg/kg, phenolic content; 30 mL, daily dose) in healthy adults (Sanchez-Rodriguez et al., 2018). A study by Widmer et al. reported significant improvements in endothelial

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[5] Asymmetric dimethylarginine (ADMA), a methyl derivate of the amino acid arginine, is a surrogate marker of poor endothelial function. ADMA, inhibits NO synthesis, thus, impairing endothelial function and promoting atherosclerosis

function biomarkers (i.e., ICAM-1) following EVOO intake (340 mg/kg, phenolic content; 30 ml, daily dose) for 4 months, in participants with early atherosclerotic endothelial dysfunction (Widmer et al., 2013). *In vitro* studies have also shown that OO polyphenols, particularly hydroxytyrosol and its metabolites, reduce the expression of adhesion molecules (i.e., E-selectin, sVCAM-1, sICAM-1) thus positively affecting endothelial function (Dell'Agli et al., 2006).

1.4.3   Effect of EVOO on serum lipids

The health benefits of OO, primarily as a constituent of the MedDiet, are widely recognized, with the European Food Safety Authority (EFSA) approving the replacement of saturated fats with a daily intake of 20 g of EVOO in order to achieve and/or maintain normal plasma cholesterol levels ("Scientific Opinion on the substantiation of health claims related to olive oil and maintenance of normal blood LDL-cholesterol concentrations (ID 1316, 1332), maintenance of normal (fasting) blood concentrations of triglycerides (ID 1316, 1332), maintenan," 2011). There is sufficient evidence supporting the effect of OO polyphenols on serum lipids, however, contradictory findings are reported in the literature.

For instance, a recent meta-analysis of 26 randomized controlled trials (RCTs) that compared the effects of HPOO vs. LPOO on several CVD risk factors, demonstrated a beneficial effect of HPOO (150-800 mg/kg, phenolic content; 25-75 mL, daily dose) on serum total cholesterol and HDL cholesterol in healthy adults, compared to the LPOO (George et al., 2019). Furthermore, a one-year intervention with a MedDiet supplemented with VOO (255 mg/100 g, phenolic content; 50 mL, daily dose) improved several LDL characteristics related to its atherogenic profile (i.e., resistance against oxidation, size and composition) but did not alter circulating LDL-c concentrations in a subsample of subjects at high cardiovascular risk in the PREDIMED study (Hernáez et al., 2017). On the

contrary, no significant changes were observed in serum lipids, in another meta-analysis that also explored the effect of two kinds of OO differing in their phenolic content (HPOO >150 mg/kg, phenolic content vs LPOO <5 mg/kg, phenolic content; 25-76 mL, daily dose) in healthy adults and patients with CVD (Hohmann et al., 2015).

There is evidence supporting the effect of OO polyphenols on serum HDL-c, however the data are once again contradictory. In 2015, a meta-analysis reported no effect of VOO intake (150 mg/kg, phenolic content) on circulating HDL-c in both healthy and adults with CVD (Hohmann et al., 2015), while another systematic review demonstrated that EVOO intake (2.28 to 75 g daily dose) resulted in increased concentrations of serum HDL-c in dyslipidemic subjects (Rondanelli et al., 2016). Other studies have also shown that increased concentration of OO polyphenols in the lipoprotein fraction may increase HDL particle size, stability and antioxidant status, but not circulating HDL-c levels (Hernaez et al., 2014). Increased circulating HDL-c and reduced TG concentrations have also been reported in a European multicenter study, the EUROLIVE study, after consumption of three types of OO differing in their phenolic content (2.7 mg/kg, phenolic content; 164 mg/kg, phenolic content; and 366 mg/kg, phenolic content, respectively). Of note, the increase in HDL-c levels was dose-dependent with the OO phenolic content (Covas et al., 2006).

VOO consumption has also been reported to affect HDL characteristics and metabolism. In this context, consumption of VOO has been shown to improve the anti-inflammatory capacity of HDL by increasing the ability of HDL particles to inhibit the expression of cytokine-induced adhesion molecules (i.e., VCAM-1 and ICAM-1) in healthy adults (Papageorgiou et al., 2016). Furthermore, it has been reported that consumption of VOO

(793 mg/kg, phenolic content; 25 mL daily dose) and FVOO[6] (500 mg/kg, phenolic content; 25 mL daily dose) has the potential to reduce oxidized lipid levels through enhanced HDL enzyme[7] activity in healthy and hypercholesterolemic adults, respectively (Cherki et al., 2005; Farràs et al., 2015). Moreover, EVOO intake (366-793 mg/kg, phenolic content; 25 mL daily dose) has been reported to improve the HDL monolayer fluidity, but also modify HDL particle numbers and size (Farràs et al., 2015; Helal et al., 2013; Hernaez et al., 2014).

1.4.4 Effect of EVOO on HDL-mediated cholesterol efflux.

Numerous studies have investigated the effect of OO consumption on circulating HDL-c, with contradictory results (Blanco-Molina et al., 1998; Covas et al., 2006; Marx et al., 2019; Tsartsou et al., 2019). However, limited studies have examined the effect of OO phenolic compounds on the anti-atherogenic activity of HDL particles, such as their capacity to promote cholesterol efflux from macrophages. Previous studies have shown that HDL fluidity and oxidative status are principal determinants for cholesterol efflux and that dietary antioxidants can enhance the resistance to lipid peroxidation (Fernández-Castillejo et al., 2017). For example, a traditional MedDiet supplemented with VOO (366 mg/kg, phenolic content; 25mL daily dose) improved cholesterol efflux in adults with high cardiovascular risk (Hernáez et al., 2019). A beneficial effect of HPOO intake on HDL-c efflux capacity was also reported in a recent systematic review and meta-analyses, which examined the effect of HPOO vs. LPOO (150-800 mg/kg vs. 0-132 mg/kg, phenolic

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[6] FVOO: Functional Virgin Olive Oil

[7] Paraoxonase type (PON1) and platelet-activating factor acetylhydrolase (PAF-AH)

content, respectively; 25-75 mL, daily dose) (George et al., 2019). However, most of the studies included in this meta-analyses were conducted in Mediterranean populations.

Hence, additional studies, with diverse ethnicities are required to confirm the observed beneficial effect of HPOO, and determine if there are genetic differences that may predispose individuals to the cardiovascular benefits associated with polyphenol intake. In addition, previous research has primarily assessed the effect of EVOO as part of the MedDiet in populations with existing comorbidities such as CHD, type 2 diabetes, and cancer (Tsartsou et al., 2019). Therefore, additional research is warranted, to explore the cardioprotective benefits of EVOO-derived polyphenols in a healthy population.

1.5   Olive oil polyphenols - Knowledge gap and implications for future research

The traditional MedDiet, known for its cardioprotective effect, has been widely recognized to favourably affect many CVD risk factors including dyslipidemia, hypertension, oxidative stress, and inflammation. In addition, increased CVD risk has been partly attributed to low plasma levels of HDL-c. However, recent evidence suggests that impaired HDL function, rather than low HDL-c, may explain HDL-associated CVD risk. EVOO, a key component of the MedDiet, has been reported to provide a cardioprotective effect through mediating improvements in cardiovascular risk factors. Although the cardio-protective properties of EVOO have been primarily attributed to its high content in MUFAs, it contains an array of unique polyphenols that have also shown to improve measures of lipid peroxidation, inflammation and glucose metabolism due to their antioxidant and anti-inflammatory properties.

Overall, there is a strong scientific basis for attributing consumption of EVOO to cardioprotective properties. Despite this evidence, the unique cardioprotective polyphenols

present in EVOO are not currently recognized by dietary and CVD guidelines, hence demonstrating the need for additional high-level evidence. Furthermore, most of the studies currently available in the literature have been restricted to populations from the Mediterranean areas. It remains unclear whether EVOO consumption will achieve the same effects in a multi-ethnic population with different habitual food cultures, therefore highlighting the need for additional research. To the best of our knowledge there is scarce evidence on the effects of EVOO polyphenols on the metabolic pathways that regulate HDL. Hence, to add to the existing evidence in this area, the cardiovascular-related effect of OO polyphenols on HDL-c efflux needs to be further explored in multiethnic populations such as Australians. Finally, to further understand the mechanisms involved in the cardioprotective effect of EVOO-derived polyphenols, further clinical research is needed to replicate previously reported improvements in routinely measured cardiovascular markers.

1.6   Thesis Preface

The OLIVAUS study is a double blind, cross-over, randomized controlled trial (RCT) that aims to investigate the effect of extra virgin HPOO (360 mg/kg, phenolic content) compared to LPOO (86 mg/kg, phenolic content) consumption on CVD risk markers in healthy Australian adults. This 10-week RCT involves the recruitment of participants from Melbourne, Australia who were required to be within the age range of 18-75 years and body mass index (BMI) 18.5-40 kg/m^2. Eligible participants underwent randomization to receive either extra virgin HPOO or LPOO for 3-weeks each. After a 2-week wash-out period they then crossed-over to the alternate treatment arm. The study's aims and outcomes form the basis of this doctoral work and are outlined below.

1.7 Study Aims and Outcomes

The primary research aim of this doctoral thesis is:

1. To investigate the effect of 3-weeks extra virgin HPOO vs. LPOO consumption on markers of cardiovascular disease risk that are related to cholesterol metabolism and specifically to HDL mediated cholesterol efflux, in healthy Australian adults.

Secondary research aims of this doctoral thesis are:

2. To investigate the effect of high vs. low polyphenol olive oil intake on traditional cardiovascular biomarkers such as serum lipid levels (i.e., total, HDL, LDL, triglycerides), and;

3. To determine the effect of high vs. low polyphenol olive oil consumption on plasma oxidative status (i.e., total antioxidant capacity (TAC) ox-LDL) and inflammatory markers (hs-CRP), and;

4. To explore the effect of the dietary intervention on peripheral (brachial) and central (aortic) systolic and diastolic BP and measures of arterial stiffness (i.e., augmentation pressure (AP), augmentation index (AIx), Carotid-Femoral Pulse Wave Velocity (CFPWV)).

1.8   Hypotheses

The hypotheses for this doctoral thesis are:

1. Serum levels of HDL-c efflux will exhibit greater increase in participants following 3-weeks of daily consumption of HPOO compared with LPOO.

2. Individuals randomized to the extra virgin HPOO treatment arm will have greater improvements in serum cholesterol levels compared to the LPOO treatment arm.

3. High polyphenol olive oil intake will result in improved measures of oxidative status and low-grade inflammation in a multicultural Australian cohort.

4. Consumption of HPOO versus LPOO for 3 weeks will reduce peripheral and central systolic and diastolic BP and improve measures of arterial stiffness in a multicultural Australian cohort.

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Chapter 2: Methods

The following chapter presents content from a published manuscript entitled "*Effect of high polyphenol extra virgin olive oil on markers of cardiovascular disease risk in healthy Australian adults (OLIVAUS): A protocol for a double blind randomized, controlled, cross-over study*" (Marx et al., 2019) . This is the protocol paper for this doctoral work, published in 2019 in Nutrition & Dietetics (Impact Factor, 1.742). Reprinted with permission from all authors. A full copy of the publication can be found in Appendix A.

2.1   Chapter Overview

This chapter describes the methods employed to undertake the OLIVAUS study, a clinical trial investigating the effect of two kinds of olive oil (OO) of quantified higher and lower polyphenol content respectively, on markers of cardiovascular disease (CVD) risk in healthy adults. More specifically, this chapter describes the OLIVAUS study design, sampling procedures, randomization to treatment arms and blinding, study intervention, data collection, outcome measures and statistical analysis. Additional details regarding the methodological procedures of the OLIVAUS study can be found in the relevant Standard Operating Procedure (SOP) protocols, available in the Appendices of the thesis.

Investigator Involvement in Research Tasks

The involvement of the candidate and other investigators in the various research tasks related to this thesis work are outlined in Table 2.1. The candidate was significantly involved in all stages of data collection, entry and analyses.

Table 2.1 **The involvement of the candidate and other investigators in the various research tasks related to this thesis**

Research Task	Investigators	Location
Study design		
Trial Protocol	Prof. Catherine Itsiopoulos	La Trobe University
	Dr Elena S. George	
	Dr Wolfgang Marx	
	A/Prof Colleen J. Thomas	
Randomisation Tables	**Candidate**	La Trobe University
Standard Operating Procedure protocols (SOPs)	Prof George Moschonis	
Dietary Intervention (olive oil preparation)	Dr Jane Willcox	La Trobe University
Coding		
Data collection/training		
Screening/recruitment	**Candidate**	La Trobe University
	Siddarth Shivantha	
	Johanna Hoskin	
Face-to face appointments and phone reviews	**Candidate**	La Trobe University
	Siddarth Shivantha (pilot study)	
	Johanna Hoskin	
Venepuncture	**Candidate**	La Trobe University
	Tammy Esmaili	
	Dorevitch Pathology, Pty. Ltd	Bundoora
Anthropometry	**Candidate**	La Trobe University
	Siddarth Shivantha (pilot study)	
	Johanna Hoskin	
Blood pressure and arterial stiffness (SphygmoCor)	**Candidate**	La Trobe University
	Siddarth Shivantha (pilot study)	
	Johanna Hoskin	
Cognitive performance Test (SUCCAB)	**Candidate**	La Trobe University
	Siddarth Shivantha (pilot study)	
	Johanna Hoskin	

Research Task	Investigators	Location
Assessment of dietary intake (Food diary)	**Candidate** Johanna Hoskin	La Trobe University
SphygmoCor & SUCCAB training	Prof Andrew Pipingas Dr Greg Kennedy	Swinburne University
Data entry/analyses		
Data entry	**Candidate** Siddarth Shivantha (pilot study) Johanna Hoskin (food diaries)	La Trobe University
Pathology data analyses	Prof. Manohar Garg Dr Anna Lohning Dr Katie Powell Dr Oladayo Folasire PathWest Lab. Pty, Ltd	University of Newcastle, NSW Bond University, QLD Fiona Hospital, WA
Statistical analyses	**Candidate** Prof. George Moschonis Prof Luke Prendergast	La Trobe University
Project Supervision	Prof George Moschonis (primary supervisor) Prof. Catherine Itsiopoulos (co-supervisor) A/Prof Colleen J. Thomas (co-supervisor) Dr Elena S. George (external) Dr Wolfgang Marx (external)	La Trobe University Deakin University
Project administration	**Candidate** Dr Elena S. George Dr Wolfgang Marx Dr Jane Willcox	La Trobe University
Project funding acquisition	Prof Catherine Itsiopoulos Dr Elena S. George Dr Wolfgang Marx Dr Hannah L. Mayr A/Prof Colleen J. Thomas Prof George Moschonis	La Trobe University

2.2   OLIVAUS study design

The OLIVAUS study is a 10-week double-blind, cross-over, randomized controlled trial (RCT) that has been designed to evaluate the effect of high polyphenol EVOO (320 mg/kg, polyphenol content) compared to low polyphenol olive oil (LPOO) (86 mg/kg, polyphenol content) on traditional and novel CVD risk markers in healthy Australian adults. A pilot study was conducted (within the context of the OLIVAUS trial and as part of an Honours project by research student Mr. Siddarth Shivantha) before commencement of the main OLIVAUS study in order to test the feasibility of the trial's protocol and the data collection tools (Sarapis et al., 2019). Figure 2.1 provides an illustration of the study design. The trial protocol (registered 30/04/2018, updated 13/02/2019) has been registered with the Australia New Zealand Clinical Trials Registry (ACTRN12618000706279) and was created in accordance with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) statement (Chan et al., 2013).

The OLIVAUS study was conducted in accordance with the Guidelines for Good Clinical Practice (GCP) and the Declaration of Helsinki and the CONSORT reporting guidelines. All procedures involving the study participants were approved by the Human Research Ethics Committee of La Trobe University (HEC17-067) Appendix B. Written informed consent was obtained from all enrolled subjects and co-signed and dated by the doctoral candidate (KS).

2.3   Sampling procedures

2.3.1   Recruitment of OLIVAUS study participants

All OLIVAUS participants were recruited in Melbourne, Australia via La Trobe University staff email database advertising, word of mouth and study posters on display at the campus.

Individuals who were interested in receiving more information about the trial contacted the researcher[8], who provided a detailed study overview. In particular, the researcher explained to each participant the nature of the treatment, its purpose, the procedures, expected duration and the potential risks and benefits involved along with any discomfort participation in the trial and consumption of OO may entail. Ample time and opportunity was allowed for each subject to enquire about details and to decide whether to participate in the trial. If agreeable, a screening procedure was undertaken to discern eligibility. Fifty adult healthy participants out of 105 individuals that were initially screened, were recruited and enrolled in the study between July 2018 and July 2019.

2.3.2   Screening assessment and enrollment

All potential participants completed a standardized screening questionnaire via a telephone interview or face-to-face meeting to assess their eligibility to participate in the trial (Appendix C). Those who met the eligibility criteria were then provided with a Patient Information and Consent Form (PICF) and were invited to enroll in the study via written informed consent (Appendix D). Once written consent was obtained, the researcher arranged with participants the timing of their visits on campus for the baseline and follow-up measurements.

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[8] Where reference is made to a 'researcher' in this section the doctoral candidate (KS) was involved for the majority or all of this work.

2.3.3   Eligibility criteria

The inclusion and exclusion criteria used in the OLIVAUS study to identify eligible participants are listed below.

Inclusion criteria

- Age: 18-75 years;

- Body Mass Index: $18.5 \leq BMI \leq 40$ kg/ m^2.

Exclusion criteria

- Non-English-speaking;

- Pregnant or lactating women;

- History of adverse reactions to olive oil;

- Currently prescribed warfarin, anti-coagulant therapy, statin medications, all oral hypoglycaemic agents, insulin, cyclosporine, tacrolimus, immunosuppressant agents, antihypertensive agents, and nonsteroidal anti-inflammatory drugs (hypothesised interactions), hormone replacement therapy, anti-depressant medication;

- Use of antioxidant supplements or medications with antioxidant properties;

- A habitual diet with ≥ 1 tablespoons of olive oil per day;

- Dieting (i.e., special types of diets, gluten free, weight loss ≥ 5 kg etc.);

- Current smoker;

- Diagnosed with any of the following conditions: hyperlipidaemia; diabetes mellitus; hypertension; inflammatory conditions (e.g. rheumatoid arthritis), intestinal disease (e.g. inflammatory bowel disease); irritable bowel syndrome, food intolerances, blood coagulation disorders, any cognitive or mood disorder, any other physiological condition or disease that could impair adherence.

2.4   Randomization to treatment arms and blinding

Participant identification numbers (IDs) were assigned sequentially to individuals once they signed the PICF. Enrolled participants were randomly assigned, in a 1:1 ratio, to one of two treatment arms, i.e., high polyphenol EVOO (treatment 1) or LPOO (treatment 2), using the block-randomization method of a software program for sequence. Blocks of 6 participants were generated by a senior researcher, who was not directly involved in the participant recruitment or data collection phase. In addition, to ensure the double blinding of the study, the two kinds of OO were supplied in dark-colored glass containers with similar size and shape; each bottle was assigned a different code number that was concealed from study participants and research team members and was disclosed to researchers only after the completion of the statistical analyses. Treatment allocation and randomization SOP can be found in Appendix E.

2.5   Withdrawal criteria

Participants were informed of their right to withdraw from the study at any stage without prejudice to their medical or dietetic treatment. In addition, investigators could also withdraw participants from the study if they believed it was in their best interest. A relevant form was required to be completed (explaining the reason of withdrawal) and to be signed by the participant (Appendix D).

2.6   Composition of intervention olive oils

The polyphenol profile for each OO intervention was determined by Modern Olives Laboratory Services (Lara, Australia), a Commonwealth Government accredited testing agency, using high-performance liquid chromatography. All high polyphenol EVOO was sourced from Boundary Bend P/L, from the same harvest/lot and stored under the same conditions. An EVOO with a confirmed 320 mg total polyphenols per kg OO (mg/kg) was

provided to participants as the HPOO. A low polyphenol OO was obtained from a local supermarket where a bulk purchase of the same brand from the same lot number was made. This OO was confirmed to have a total polyphenol concentration of 86 mg/kg upon assessment. The bottles were stored at a constant temperature ranging from 16 to 20°C, with the exclusion of light as per the manufacturer's instructions to prevent any changes in the chemical composition (i.e., oxidative stability) of the olive oil[9]. The polyphenol composition of the two OOs used in the OLIVAUS study is summarized in Table 2.2. The full chemical analysis reports can be found in Appendix F.

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[9] Olive oil begins to deteriorate from the time of extraction and as such different methods should be adopted in order to reduce the rate of this deterioration during storage. Factors such as low storage temperatures, the exclusion of oxygen and light are shown to be crucial in maintaining a longer shelf life of olive oil. Sanmartin, C., Venturi, F., Sgherri, C., Nari, A., Macaluso, M., Flamini, G., Quartacci, M. F., Taglieri, I., Andrich, G., & Zinnai, A. (2018). The effects of packaging and storage temperature on the shelf-life of extra virgin olive oil. *Heliyon*, *4*(11), e00888. https://doi.org/10.1016/j.heliyon.2018.e00888 .

Table 2.2   The polyphenol composition of the two olive oils used in the OLIVAUS trial

Polyphenol composition	High polyphenol Extra Virgin Olive Oil (mg/kg)	Low polyphenol/ Refined Olive Oil (mg/kg)
Hydroxytyrosol	3.3	5.3
Tyrosol	2.5	5.1
Vanillic acid + Caffeic acid	3.5	0.0
Vanillin	2.9	0.6
p-Coumaric acid	13.0	1.0
Hydroxytyrosol Acetate	0.0	0.0
Ferulic acid	9.8	0.8
0-Coumaric acid	0.0	0.0
Decarb. Oleuroaglycone,Ox	6.5	2.7
Oleacein	71.7	6.3
Oleuropein	17.2	1.0
Oleuro aglycone, Al	11.0	0.8
Tyrosol acetate	2.9	0.1
Decarb. Ligstraglycone, Ox Al	11.6	3.8
Oleocanthal	29.5	11.2
Pinoresinol + 1 acetoxy pinore	26.0	5.5
Cinnamic acid	3.7	2.0
Ligstroside aglycone, Al	3.2	0.4
Oleuro aglycone, Ox Al Hy	13.8	3.0
Luteolin	13.9	1.8
Oleuro aglycone, Al Hy	44.6	3.0
Ligstro aglycone, Ox Al Hy	6.4	1.4
Apigenin	9.3	1.0
Methyl- Luteolin	5.0	3.0
Ligstroside aglycone, Al Hy	9.0	7.9
Total polyphenols-HPLC*	320.3	86.4

*HPLC, high-performance liquid chromatography. The data in this table has been transcribed from a Modern Olives-Laboratory Report on the two olive oils. The composition analysis was completed on 7th May 2018 for the high polyphenol extra virgin olive oil and 17th July 2018 for the low polyphenol/refined olive oil.

2.7   Study Intervention

Enrolled participants were required to consume daily 60 mL raw high polyphenol EVOO or LPOO for 3 weeks and then crossed-over to the alternate treatment arm for another 3-weeks. A 3-week study duration was chosen based on previous literature, where most studies were relatively short in duration with most intervention phases lasting on average, 3 weeks (George et al., 2019). Two washout periods, of 2 weeks each, preceded the first and the second intervention phases of OO administration. Figure 2.1 provides a visual representation of the study flow. The study intervention including visit time-points (T) and data collection is described in further detail below.
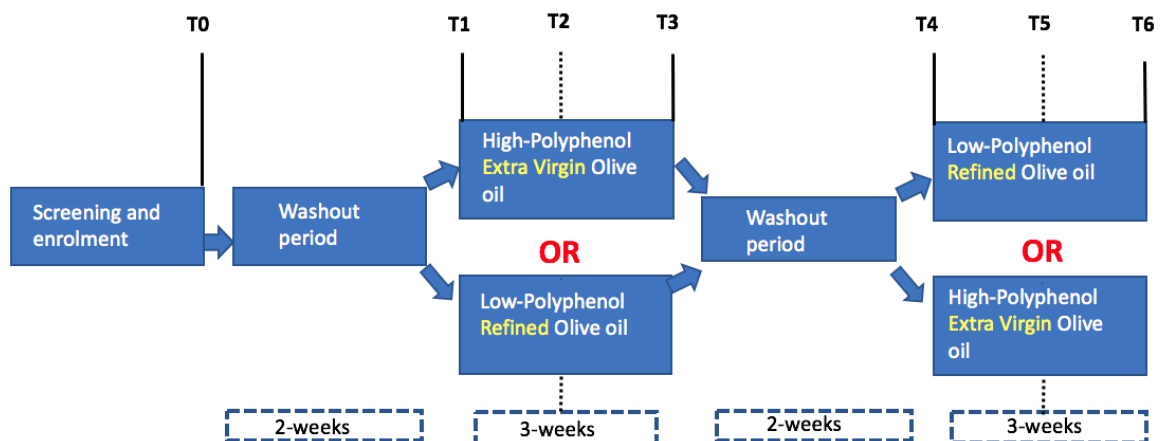
Figure 2.1.   Illustration of the cross-over study design of the OLIVAUS trial.

   T0 = screening and enrolment,
   T1&T4 = Start of intervention phase,
   T2&T5 = Mid-intervention phase (email/phone update),
   T3&T6 = End of intervention phase.
   T, time. (Reproduced from Honours thesis: Mr. Siddharth Shivantha, 2018).

2.7.1   Washout period: T0-T1 and T3-T4

Enrolled participants were asked to undergo a 2-week wash out period, where they were instructed to cease consumption of OO, olive products and antioxidant supplements (except

for iron, calcium and Vitamin-D) prior to commencing the first intervention phase (T0-T1) and between OO administration periods (T3-T4). The 2-week duration of the wash-out period was considered to be adequate to avoid any carry-over effect and is commonly used in other crossover diet interventions (Fito et al., 2005). Finally, participants were asked to attend each baseline intervention phase meeting (T1 and T4) in a fasted state (10-12h).

2.7.2 Commencement of first and second intervention phase: T1 and T4

At the commencement of the first and second baseline intervention phase (T1 and T3), participants attended a 1-hour appointment in the morning with the research staff at the nutrition clinical rooms (La Trobe University, Bundoora). A standardized questionnaire was used to collect information on participants' socio-demographic characteristics (only at T1). Physical activity (PA) levels were also assessed only at the first baseline intervention phase (T1). Data collection including anthropometry, fasting blood, blood pressure (BP) and measures of arterial stiffness, dietary intake and cognitive performance took place at each face to face appointment.

At the end of each pre-intervention appointment, participants were supplied with the total amount of 1.26 L of OO required per 3-week period. The research staff provided detailed instructions to the participants on the dosage regimen required for the trial and suggestions as to how to incorporate the OO in their meals in its raw, uncooked form. This included dressing salads or vegetables and drizzling the OO on prepared meals such as soups or casseroles, and ensuring leftover amounts were also consumed. In addition, study participants were advised to consume the OO in multiple doses during the day, (i.e., in their breakfast, lunch, dinner) while they were discouraged from drinking the OO. Measuring cups were provided to ensure the required volume (60 mL) of OO was accurately consumed. Participants were also asked to record any adverse reactions or events during

the intervention in a log sheet, and this information was also reported in the participant Case Report Form (CRF). Due to the cross over design of the trial, participants changed to the alternate dietary treatment arm at the commencement of the second intervention phase (T4).

2.7.3 Mid-intervention phase: T2 and T5

The research staff contacted participants via email or phone (each phone call lasted 10-15min) approximately 1.5 weeks into each intervention phase (T2 and T5) to discuss their progress and whether they had experienced any adverse events during the trial period. Furthermore, study participants were reminded to consume the required amount of OO in its raw form with suggestions on how to achieve this. Finally, the researcher confirmed the date of the next appointment.

2.7.4 End of first and second intervention phase: T3 and T6

Participants attended a follow-up appointment at the end of each intervention phase (T3 and T6), where study assessments were repeated as per T1 and T3 appointments. PA levels were assessed only at the end of the second intervention phase (T6). To encourage study retention, participants received a $25 AUD gift voucher ($50 AUD in total) at the end of each intervention stage (T3 and T6). Furthermore, to assess the level of adherence to the intervention, participants were instructed to return the OO bottles (containers) at the end of each intervention period (T3 and T6) so that the daily amount of unconsumed OO could be measured. Study participants were also instructed to keep a self-report record of daily OO consumed during each intervention phase using a diary/checklist provided to them which was reviewed at the end of each intervention period (T3 and T6). Finally, at the completion of the trial (T6), research staff assessed blinding by asking the participant to comment on

the organoleptic characteristics of each type of OO (i.e., taste, color, smell) as well as the order they think they received the two intervention OOs.

2.8 Data collection and outcome measures

The data related to the outcome measures that were collected as part of the current study, including the data collection tools and methods, are described in this section. An overview of study data collection at each visit timepoint is shown in Table 2.3. The doctoral candidate was responsible for the accuracy, completeness and legibility of the data, which were recorded in the CRF and in all required reports.

2.8.1 Socio-demographic data

At the first baseline intervention phase (T1), details regarding age, gender, language(s) spoken at home, level of education, ethnicity and parental country of birth was collected from participants using a standardized questionnaire (Appendix G).

2.8.2 Lifestyle Data

Lifestyle data collection included dietary intake and PA. Dietary intake data wase collected at four time-points (T1, T3, T4 and T6), while PA was assessed only during the week preceding the first intervention period (T1) and at the conclusion of the study (T6).

2.8.2.1 Dietary intake

A 3-day food diary was used to collect information on the dietary intake of study participants during two weekdays and one weekend day (preferably non-consecutive) at baseline and follow-up of each 3-week intervention phase (T1, T3, T4 and T6) (Appendix H). Specifically, study participants were instructed to record details on their intake of food and beverages, including information on the quantity (via household measures), type/brand

and cooking methods of the consumed items. Furthermore, the timing and amount of the consumed intervention OOs was also recorded. The level of detail required to be recorded in the diary, as well as additional strategies on how to incorporate raw, uncooked OO in their habitual diet was provided at a pre-baseline meeting by the doctoral candidate. The completed food diaries were then returned and checked by the research team members for potential wrong or missing entries during the scheduled interviews with the study participants. All dietary intake data was analyzed for energy, macro- and micronutrient content using FoodWorks 9™ software (Xyris Software Pty Ltd, Queensland, Australia) and relevant databases: Australia—AusFoods 2017, AusBrands 2017, AUSNUT 2011-13. In addition, a daily consumption log sheet was used to assess the level of adherence to the dietary intervention. Specifically, participants were instructed to record the volume of OO consumed at the end of each 3-week intervention period (T3 and T6).

2.8.2.2. Physical Activity

In order to identify potential confounding effects of any changes in physical activity (PA) levels, participants were required to complete the Active Australia Survey (AAS) questionnaire (AIHW, 2003) during the week preceding the interviews at the first baseline (T1) and at the last follow-up meeting (T6). The survey consists of eight questions for assessing participation in PAs of different intensity, such as walking, vigorous gardening/yardwork, other vigorous activities and moderate activities. For each activity type, there are two questions: (1) number of sessions and (2) total time spent in each activity (minutes and/or hours) during the previous week (Appendix H) In order to calculate PA-related energy expenditure (EE) per week for each type of activity, the following equation was used: *PA related EE=(MET of PA*total weekly time spent on the specific PA (in*

min)*3.5ml O₂/kg/min *Body Weight (kg)/1000)*5[10]. Finally, by summing the PA, EE for each type of PA the total weekly energy expenditure (in Kcal) was calculated.

2.8.3 Anthropometric Data

Anthropometric data collection included measurement of participant's standing height (cm), weight (kg) and waist circumference (WC) (cm). These measures were collected at baseline and follow-up of each 3-week intervention phase (T1, T3, T4 and T6) and at the same time of the day, preferably in the morning, by the doctorate candidate. Protocols for anthropometric measurements were developed based on the measurement techniques and equipment recommended within the International Standards for Anthropometric Assessment published by the International Society for the Advancement of Kinanthopometry. (Arthur Stewart, 2011) (Appendix I). Height and weight were used to calculate Body Mass Index (BMI) by dividing weight in kg by height in m^2 (kg/m^2). Cut off points for BMI were set based on the World Health Organization (WHO) guidelines (underweight, BMI < 18.5 kg/m^2; normal weight, BMI 18.5–24.9 kg/m^2; overweight, BMI 25.0–29.9 kg/m^2; obese, BMI 30 kg/m^2) (WHO, 1995). Detailed protocols for all measurements are outlined below.

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[10] **MET**= metabolic equivalent of task. One MET equates with the oxygen consumption (O_2) required at rest or sitting quietly and is assumed to be 3.5 mL/O_2/min × kg body weight. The index is used to express O_2 uptake or intensity of activities as multiples of the resting or 1 MET value and is useful for describing and prescribing exercise of different intensities. Activities range from 0.9 MET (sleeping) to 18 METs (running) Hills, A. P., Mokhtar, N., & Byrne, N. M. (2014). Assessment of physical activity and energy expenditure: an overview of objective measures. *Front Nutr*, *1*, 5. https://doi.org/10.3389/fnut.2014.00005

2.8.3.1 Body height

Standing height (cm) was measured with study participants in minimal clothing and without any hair ornaments (i.e., large hair grips, head bands, pony tail holders etc.) and barefoot, using a wall-mounted stadiometer (SE206, Seven Hills, NSW, Australia). Participants were instructed to stand straight under the stadiometer's headboard, with the head in the horizontal Frankfurt Plane[11], feet together, knees straight, and heels, buttocks, and shoulder blades in contact with the vertical surface of the stadiometer; arms were required to be hanging loosely at the sides with palms facing the thighs (Figure 2.2). Gentle traction was applied to the mastoid processes to stretch the spine and minimize effects produced by diurnal variation. Participants were asked to take a deep breath and stand tall to aid the straightening of the spine. The stadiometer's movable headboard was then lowered to the crown of the participant's head. The height measurement was taken at maximum inspiration, with the examiner's eyes level with the stadiometer's headboard to avoid parallax errors. Two measurements were taken to the nearest 0.1 cm, the mean value of which was recorded. If the two height measurements differed by more than 0.4 cm, then a third reading was taken.

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[11] The Frankfurt Plane is an imaginary line passing through the upper margin of the external ear canal and across the top of the lower bone of the eye socket, under the eye. This position is important if an accurate reading is to be obtained.
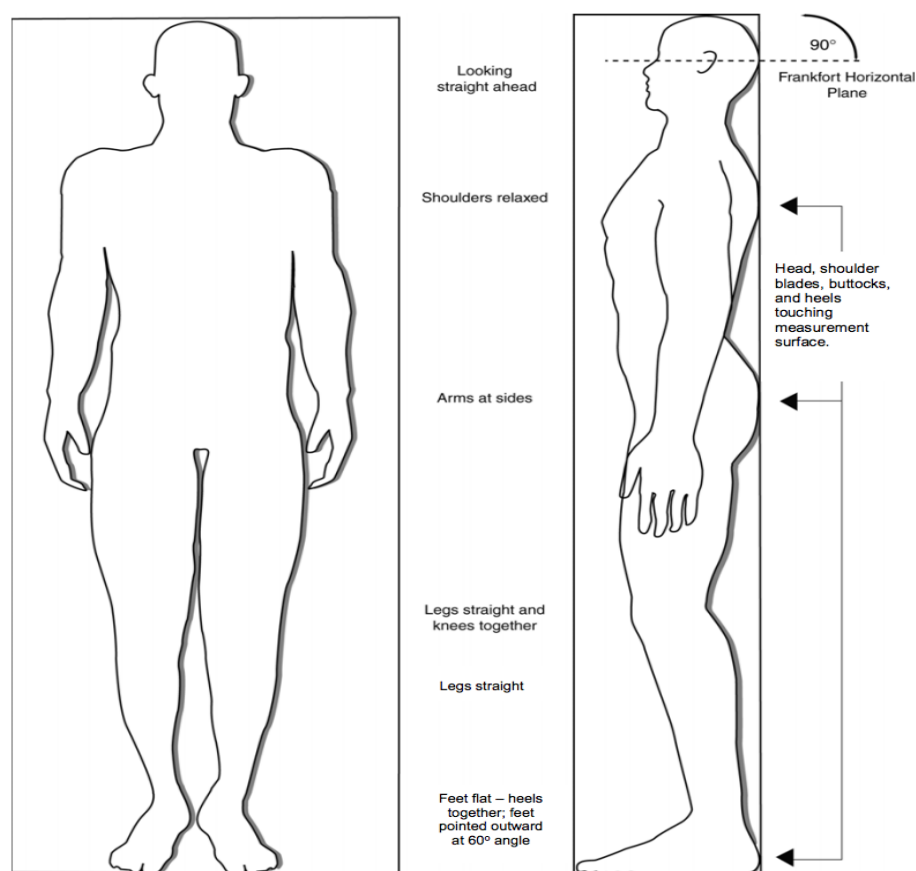
Figure 2.2 Positioning of study subject for height measurement

Horizontal line is in the Frankfurt plane, which should be in a horizontal position when height is measured. (Reproduced from Robbins GE, Trowbridge FL, in: Nutrition Assessment: A Comprehensive Guide for Planning Intervention by M.D. Simko, C. Cowell, and J.A. Gilbride (eds), p.77, with permission of Aspen Publishers, Inc., C 1984).

2.8.3.2 Body weight

Body weight was measured with study participants in light clothing and barefoot using a digital scale (WM203, Willawong QLD, Australia). The scale was placed on a hard, flat surface and adjusted to '0 kg' (zero-balance) before each measurement. Study participants were asked to stand in the center of the weight scale platform and look straight ahead, standing unassisted, and relaxed. Two consecutive measurements of weight were taken,

after a normal expiration, to the nearest 0.1 kg, and the mean value recorded.

2.8.3.3   *Waist circumference*

Waist circumference (WC) was measured using a flexible steel tape calibrated in cm with mm graduations (Luftkin W606PM, Sparks, MD, USA). Participants were asked to stand erect with the abdomen relaxed, arms at the sides, feet together, and their weight equally divided over both legs. The elastic tape was then applied horizontally around the abdomen directly over the skin at the umbilical level (Figure 2.3). Participants were asked to breath out gently at the time of the measurement to prevent them from contracting their muscles or from holding their breath. The measurement was taken without the tape compressing the skin. Two readings were taken to the nearest 0.1 cm, the mean value of which was recorded. If the two measurements differed by more than 0.4 cm, then a third reading was taken. Gender-specific WC cut-off points proposed by the WHO were used to categorize study participants for CVD risk: normal (WC < 94 cm in men and < 80 cm in women), high CVD risk (WC 94–102 cm in men and 80–88 cm in women) and very high CVD risk (WC > 102 cm in men and 88 cm in women) (WHO, 2008).
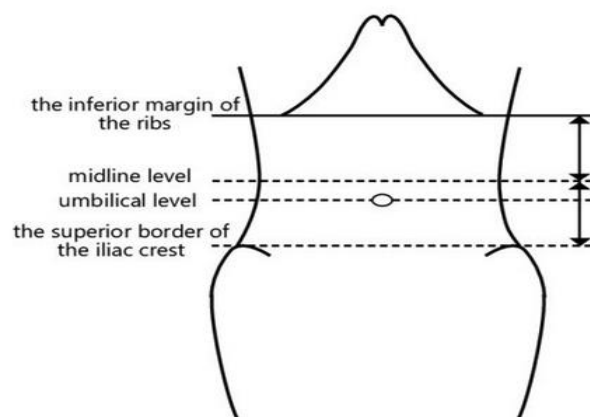

Figure 2.3   Measurement of waist circumference at umbilical level

2.8.4   Biomarkers

2.8.4.1   *Blood collection and processing*

Biomarkers were measured from early-morning venous blood samples collected from the participants during the trial by the doctoral candidate (KS). Participants attended La Trobe University following a 10-hour overnight fast. Blood collection occurred at four time points (baseline of the first (T1) and second intervention period (T4), with follow up tests at the end of the first (T3) and second intervention period (T6)). Whole blood was collected only at baseline of the first intervention period (T1). If the initial attempt to collect blood by the doctoral candidate was unsuccessful, blood was taken at a commercial pathology clinic (Dorevitch Pty. Ltd.) or by a certified phlebotomist based at La Trobe University within 48 hours of the scheduled appointment.

Five different test tubes were used per study participant to collect 28 mL of venous blood, which was then processed for plasma and serum separation. For the purpose of plasma separation, blood was collected into four tubes that contained different types of anticoagulant, i.e., one ethylenediaminetetraacetic (EDTA) containing tube of 6.0 mL for plasma extraction (appointed for ox-LDL measurement), a second EDTA containing tube of 4.0 mL for whole blood storage (future analysis), one heparin containing tube of 6.0 mL (for measurements of TAC) and one fluoride oxalate (FLOX) containing tube of 4.0 mL for plasma extraction and measurements of fasting plasma glucose concentrations. Serum separation was achieved by collecting the remaining blood into one tube (SST) of 8.0 mL containing a coagulation activator (appointed for HDL-c efflux, TC, LDL, HDL, TG, and hs-CRP measurements). Collected venous blood was centrifuged (Hettic Rotina 420r, Massachusetts, USA) at 2350 rpm for 10 min at 4 ^{0}C and the extracted plasma and/or serum was apportioned into aliquots of 500 μl each and stored at -80 ^{0}C until analysis. An

overview of the blood collection test tubes used for each of the measured biomarkers at each visit time point is presented in Table 2.3. Blood collection, processing, handling and storage SOP can be found in Appendix J.

Table 2.3   Overview of the blood collection test tubes used for each of the measured biomarkers at each visit time point.

Time-point	Test tube	Blood volume	Biomarkers
Baseline (T1, T4) & Follow-up (T3, T6)	EDTA tube (plasma extraction)	6.0 mL	ox-LDL
Baseline (T1)	EDTA tube (plasma extraction)	4.0 mL	Whole blood
Baseline (T1, T4) & Follow-up (T3, T6)	Lithium Heparin tube (plasma extraction)	6.0 mL	TAC
Baseline (T1, T4) & Follow-up (T3, T6)	FLOX tube (plasma extraction)	4.0 mL	Glucose
Baseline (T1, T4) & Follow-up (T3, T6)	SST tube (serum extraction)	8.0 mL	HDL-c efflux, TC, LDL, HDL, TG, hs-CRP
Total blood		**28.0 mL**	

Oxidized LDL, ox-LDL; total antioxidant capacity, TAC; high density lipoprotein cholesterol efflux capacity; HDL-c efflux, total cholesterol, TC; low density lipoprotein, LDL; high density lipoprotein, HDL; triglycerides, TG; ethylenediaminetetraacetic, EDTA; fluoride oxalate, FLOX; serum separating tube, SST.

2.8.5   Biochemical analyses methods

All biochemical analyses presented in this doctorate were performed by accredited laboratories based at Bond University, Queensland (for HDL-c efflux and TAC concentration determination), University of Newcastle, New South Wales (for ox-LDL

concentration determination) and Fiona Stanley Hospital, PathWest, Western Australia (for lipid profile and hs-CRP concentration determination). The biochemical methods used for the determination of the concentrations of the abovementioned biomarkers are described in the following sections.

2.8.5.1 HDL cholesterol (HDL-c) Efflux

Cholesterol efflux was measured using the Cholesterol Efflux Fluorometric Assay Kit (BioVision; Milpitas, CA, USA) following the manufacturer's instructions. Briefly, 5x104 J774A.1 cells were seeded into 96-well tissue culture plates and grown in supplemented phenol red-free DMEM for 24 hours. Cells were washed with serum-free, phenol red-free DMEM and labelled for 1 hour in 1:1 ratio labelling reagent to serum-free, phenol red-free DMEM. The labelling medium was removed before the cells were incubated in equilibration medium for 18-19 hours. Samples were pre-treated with serum treatment reagent to remove interferents (LDL/VLDL) prior to addition to the cells according to the protocol. 2 µL of each pre-treated human serum sample, assayed in duplicate, was added and incubated with the cells for 4 hours at 37 °C. Supernatant was transferred to black walled 96-well plates and fluorescence measured using a FLUOstar Omega spectrophotometer (BMG LabTech, Vic, Australia) with excitation and emission wavelengths 485 and 520 nm, respectively. Cells were lysed and the lysate was transferred to black-walled 96-well plates and fluorescence measured. Percentage cholesterol efflux was measured using the following equation.

% Cholesterol Efflux = (RFU[12] of supernatant / RFU of cell lysate + RFU of supernatant) x 100

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[12] RFU=Relative Fluorescence Units

2.8.5.2 Lipid profile

Cholesterol

The Alinity c Cholesterol Assay kit (Abbott GmbH & Co; Wiesbaden, Germany) was used for the quantitation of cholesterol in human serum (assayed in duplicate; sample volume for single test: 1.5 µL). In brief, cholesterol esters are enzymatically hydrolyzed by cholesterol esterase to cholesterol and free fatty acids with this method. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase to cholest-4-ene-3-one and hydrogen peroxide. The hydrogen peroxide combines with hydroxybenzoic acid (HBA) and 4-aminoantipyrine to form a chromophore (quinoneimine dye) which is quantitated at 500 nm. The measuring interval of the Alinity c Cholesterol assay is 7 to 705 mg/dL (0.18 to 18.26 mmol/L).

Low-Density Lipid (LDL)

The Alinity c Direct LDL Assay kit (Sekisui Diagnostics P.E.I. Inc; Charlottetown, Canada) was used for the direct, quantitative determination of LDL cholesterol in human serum (assayed in duplicate; sample volume for single test: 2 µL). Briefly, the method is in a two-reagent format and depends on the properties of a unique detergent which solubilizes only the non-LDL particles. The cholesterol released is consumed by cholesterol esterase and cholesterol oxidase in a non-color-forming reaction. A second detergent solubilizes the remaining LDL particles and a chromogenic coupler allows for color formation. The enzyme reaction with LDL in the presence of the coupler produces a color intensity that is proportional to the amount of LDL cholesterol present in the sample. The measuring interval of the Alinity c Direct LDL assay is 1 to 800 mg/dL (0.03 to 20.69 mmol/L)

High Density Lipid (HDL)

The Ultra HDL Assay (Abbott GmbH & Co; Wiesbaden, Germany) is a homogeneous method that was used for directly measuring HDL cholesterol concentrations in serum without the need for off-line pretreatment or centrifugation steps (assayed in duplicate; sample volume for single test: 1.7 µL) . The method uses a two-reagent format and depends on the properties of a unique detergent. This method is based on accelerating the reaction of cholesterol oxidase (CO) with non-HDL unesterified cholesterol and dissolving HDL cholesterol selectively using a specific detergent. In the first reagent, non-HDL unesterified cholesterol is subject to an enzyme reaction and the peroxide generated is consumed by a peroxidase reaction with DSBmT yielding a colorless product. The second reagent consists of a detergent (capable of solubilizing HDL cholesterol), cholesterol esterase (CE), and chromagenic coupler to develop color for the quantitative determination of HDL cholesterol. The measuring interval of the Alinity c Ultra HDL assay is 5 to 180 mg/dL (0.13 to 4.66 mmol/L).

Triglycerides

The Alinity c Triglyceride Assay (Abbott GmbH & Co; Wiesbaden, Germany) was used for the quantitation of triglyceride in human serum (assayed in duplicate; sample volume for single test: 1.5 µL). With this method, triglycerides are enzymatically hydrolyzed by lipase to free fatty acids and glycerol. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol- 3-phosphate and adenosine diphosphate (ADP). Glycerol-3- phosphate is oxidized to dihydroxyacetone phosphate (DAP) by glycerol phosphate oxidase (GPO) producing hydrogen peroxide (H_2O_2). In a color reaction catalyzed by peroxidase, the H_2O_2 reacts with 4-aminoantipyrine (4-AAP) and 4-chlorophenol (4- CP) to produce a red colored dye. The absorbance of this

dye is proportional to the concentration of triglyceride present in the sample. The measuring interval of the Alinity c Triglyceride assay is 7 to 1420 mg/dL (0.08 to 16.05 mmol/L).

2.8.5.3 Inflammation marker – C-Reactive Protein

The Alinity c CRP Vario assay (SENTINEL CH; Milano, Italy) was used for the quantitative immunoturbidimetric determination of high sensitivity (hs)-CRP in human serum (assayed in duplicate; sample volume for single test: 4.0 µL). In brief, when an antigen-antibody reaction occurs between CRP in a sample and anti-CRP antibody, which has been absorbed to latex particles, agglutination results. The latter is detected as an absorbance change (572 nm), with the rate of change being proportional to the quantity of CRP in the sample. Two different methods (high sensitivity (CRP16) and wide range (CRP48)) were used to cover a wide analytical measurement range. The measuring interval of the Alinity c CRP Vario assay High Sensitivity application is 0.04 to 16.00 mg/dL (0.40 to 160.00 mg/L).

2.8.5.4 Oxidative status markers

Total Antioxidant Capacity (TAC) of plasma samples was measured using the OxiSelect TAC Assay Kit (Cell Biolabs; San Diego, CA, USA) following the manufacturer's instructions. Briefly, plasma from blood samples collected in heparin treated tubes was centrifuged to remove precipitate prior to use in the assay. 20µL of each sample, assayed in duplicate, was mixed with reaction buffer in a 96-well plate and the absorbance at 490 nm measured using a FLUOstar Omega spectrophotometer. Copper Ion Reagent was added to the samples and incubated for 5 minutes before the reaction was stopped and a second absorbance measurement recorded. Antioxidant capacity of the samples was calculated by

subtracting the initial absorbance reading from the second reading and comparing the net optical density readings to a 5-parameter fit uric acid standard curve. The detection limit was 0.0039 mM.

Plasma ox-LDL concentrations were measured using the oxidized-LDL ELISA kit (Mercodia AB; Uppsala, Sweden). This ELISA assay is a solid phase two-site enzyme immunoassay in which 2 monoclonal antibodies are directed against separate antigenic determinants on the oxidized apoB molecule. The detection limit was 0.6 mU/L.

2.8.6 Haemodynamic indices

2.8.6.1 *Peripheral and central blood pressure*

Peripheral (brachial) and central (aortic) blood pressure (BP) were measured using applanation tonometry with a SphygmoCor XCEL device (Model XCEL, AtCor Medical, Sydney, Australia), at baseline and follow-up examinations at each intervention period (T1, T3, T4, T6). Following a minimum of 5 min rest in the supine position, peripheral brachial systolic BP (SBP) and diastolic BP (DBP) were measured using a blood pressure cuff affixed to the upper left arm. Three consecutive BP recordings were made and the average of the last two recordings was used for data analysis. In addition, central SBP and DBP, as well as pulse pressure (PP) measures were automatically derived via the brachial BP cuff. The BP categories recommended by the American College of Cardiology (ACC) / American Heart Association (AHA) were used to classify study participants into those with Normal BP (SBP/ DBP < 120/ 80 mmHg), Elevated BP (SBP 120–129 mmHg and DBP < 80 mmHg), Hypertension Stage I (SBP 130–139 mmHg or DBP 80–89 mmHg) and Hypertension Stage II (SBP > 140 mmHg or DBP > 90 mmHg) ("Correction to: 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA Guideline for the

Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines," 2018).

2.8.6.2 *Non-invasive arterial stiffness measurement*

Measures of peripheral and central arterial stiffness, using pulse wave analysis (PWA) and pulse wave velocity (PWV), were also obtained non-invasively with the SphygmoCor XCEL device (Model XCEL, AtCor Medical, Sydney, Australia). PWA is a non-invasive, valid and reliable technique to investigate mechanical properties of the arterial tree, using central blood pressures and analysis of systemic arterial wave reflection. Peripheral arterial stiffness indices of augmentation pressure (AP) and the augmentation index (AIx) were derived automatically by the device as part of the standard BP measurement procedure. The AP was calculated as the difference between the first and second systolic peak, while the AIx was calculated as the percentage contribution that the AP makes to the overall PP (AIx = AP/PP x 100). PWV was measured using a tonometer (pressure sensor) which was placed on the carotid artery to capture the carotid waveform, while a femoral cuff was placed high on the left thigh in order to capture the femoral waveform, as shown in Figure 2.4. The PWV was then calculated by dividing the distance between the carotid and femoral measurement sites by the transit time. This method is considered the gold standard technique for assessing central arterial stiffness.
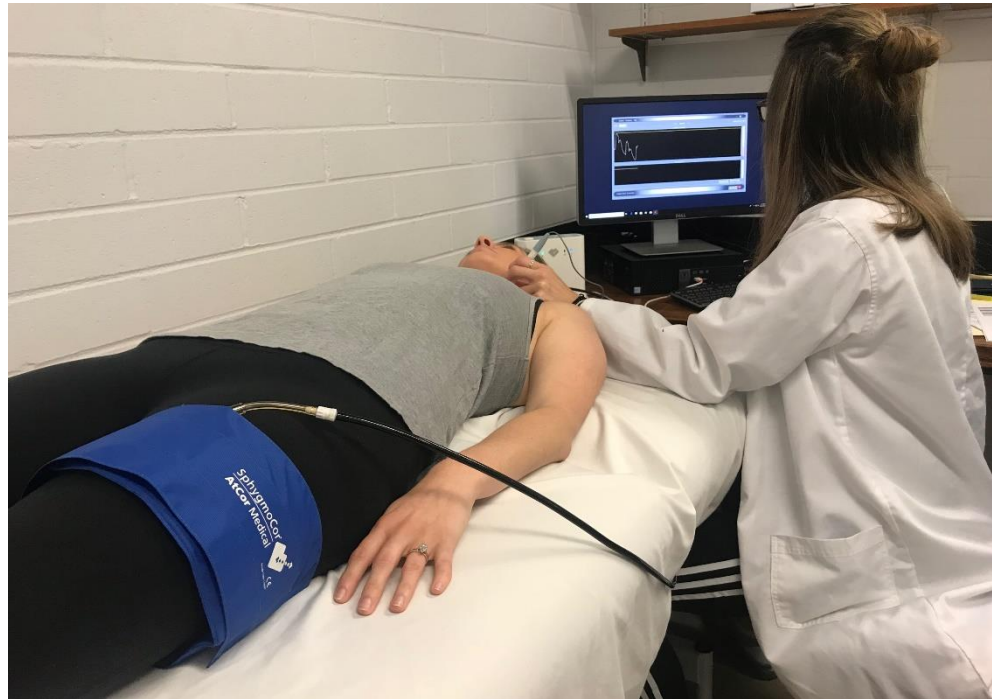
Figure 2.4   Pulse wave velocity measurement using the SphygmoCor device.

With the participant lying in a supine position, a femoral BP cuff was placed on the left upper leg (de facto measurements of femoral artery blood flow/BP). On the same correlating side (participant's left) the researcher identified the carotid artery in the neck and a tonometer was placed on the strongest pulse point to capture the PWV measurement
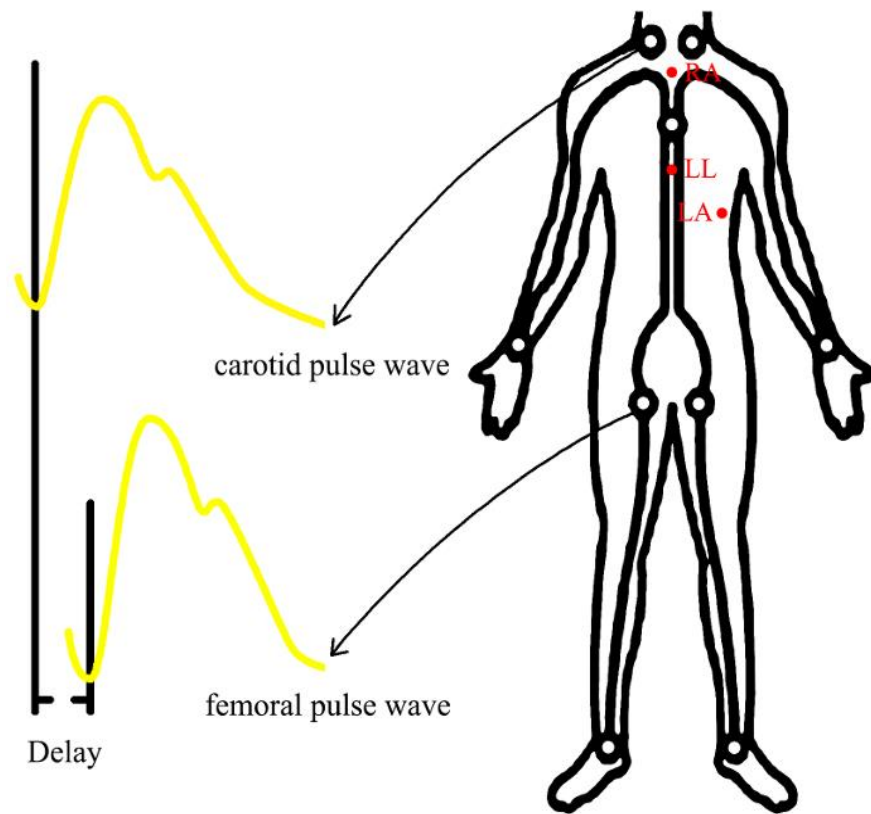
Figure 2.5   The positions of the electrodes and the segments of measured arteries.

The device measures the transit time as the delay between arrival of the pulse wave at the femoral artery and the carotid artery. PWV is calculated by dividing the traveled distance by transit time (distance/time = PWV). RA, right arm; LL, left leg; LA, left arm. (Benjamin et al., 2018).

2.8.7   Cognitive Performance test

Study subjects were invited to participate in an optional cognitive performance assessment in order to investigate the effect of dietary polyphenols on cognition. These measures were conducted at all four time points (T1, T3, T4 and T6) using a validated computer based cognitive battery consisting of eight measures, the Swinburne Computerized Cognitive Assessment Battery (SUCCAB). This battery, which has been validated in several other studies investigating the cognitive effect of dietary supplementation (Harris et al., 2012; Pipingas et al., 2008), uses a simple 5 button box and reports eight measures of cognitive functioning: (1) Simple and (2) Choice Reaction Times, (3) Immediate and (4) Delayed Recognition, (5) Congruent and (6) Incongruent Stroop color-words, (7) Spatial Working Memory and (8) Contextual Memory. At first baseline meeting, each measure/task was preceded by a brief practice task to familiarize the participant with the overall procedure. The stimulus presentation was via a laptop to ensure clear task visibility. Finally, participants were instructed to respond as quickly and as accurately as possible. All testing was undertaken onsite at La Trobe University clinical room.

The primary outcome projected to be measured includes spatial working memory, immediate, delayed and contextual recognition response time. The secondary outcome projected to be measured includes response time and accuracy on the individual SUCCAB measures, with the exception of the Congruent Stroop and Reaction Time tasks, as ceiling effects for accuracy are anticipated, response times will be the sole outcome for these tasks. The outcome measurements have not been analyzed for this thesis submission.

Table 2.4   Overview of study data collection and visit timepoints

Timepoint	Questionnaires/ Diaries	Collection	Other
Screening/ enrollment (T0)	-Screening questionnaire -Provide 3-day food diary - Provide AAS questionnaire		Consent form
Baseline-Intervention phase 1 (T1)	-Socio-demographic questionnaire -Review 3-day food diary -Review AAS questionnaire	-Anthropometry (height, weight, WC, BMI) -Fasting blood collection -BP (aortic, central) & measures of arterial stiffness -Cognitive performance test	-Supply bottles with the intervention OO -Delivery of participant booklet*
Mid-intervention (T2) Phone call/Email			-Progress and adverse events assessment -Remind participants of form completion -Confirm follow-up meeting time
End-intervention phase 1 (T3)	-Review 3-day food diary -Review daily OO consumption diary/adverse events	-Anthropometry (height, weight, WC, BMI) -Fasting blood collection -BP (aortic, central) & measures of arterial stiffness -Cognitive performance test	-Dispense gift card (voucher) -Collect returned OO bottles -Delivery of participant booklet*
Baseline-Intervention phase 2 (T4)	Review 3-day food diary	-Anthropometry (height, weight, WC, BMI) -Fasting blood collection -BP (aortic, central) & measures of arterial stiffness -Cognitive performance test	-Supply bottles with the intervention OO -Delivery of participant booklet*

Timepoint	Questionnaires/ Diaries	Collection	Other
Mid-intervention (T5) Phone call/Email			-Progress and adverse events assessment
			-Remind participants of form completion
			-Confirm follow-up meeting time
End-intervention phase 2 (T6)	-Review 3-day food diary	-Anthropometry (height, weight, WC, BMI)	-Dispense gift card (voucher)
	- Review daily OO consumption diary/adverse events	-Fasting blood collection	-Collect returned OO bottles
		-BP (aortic, central) & measures of arterial stiffness	
	-Review PA questionnaire		
		-Cognitive performance test	
	-Review blinding questionnaire		

*Participant booklet includes 3-day food diary, daily OO consumption diary/adverse events log and AAS questionnaire. AAS, Active Australia Survey; WC, waist circumference; BMI, body mass index; OO, olive oil.

2.9 Adverse events

Adverse events were monitored at all timepoints. In the occasion of experiencing a significant adverse event, participants would be withdrawn from the study. All adverse events would be reported to the trial steering committee, comprised of the Principal Investigator and trial staff. The Human Research Ethics Committee would also be notified, as appropriate. Emergency unblinding would occur for serious adverse events deemed related to the study product. All participant data was securely stored either in onsite locked cabinets or password protected documents on secured university servers with restricted access to the research team only.

2.10   Statistical Methods

2.10.1   Sample size estimation

A power calculation was completed to determine appropriate participant sample size for the OLIVAUS trial based on the intended primary outcome, HDL-c efflux (%). Based on the results of previous research (Hernaez et al., 2014), a sample size of 40 was considered adequate to provide sufficient statistical power to detect a statistically significant 5% difference in HDL-C efflux between the two intervention phases with 80% power and 5% level of significance. To account for a 20 % level of potential attrition, this sample size was expanded to 50 participants.

2.10.2   Statistical analyses

For all continuous variables, the Kolmogorov-Smirnov test was performed to examine the normality of their distribution. Repeated-measures ANOVA (analysis of variance) and linear mixed effects (LMEs) models with a random intercept to account for repeated measures were used to examine the between-group differences (treatment effect; i.e., extra virgin high *vs* low polyphenol OO) of mean values at each time point of measurement, the within-group changes (time effect) from baseline to follow-up in each intervention arm, and the differences in the changes from baseline to follow-up between the two intervention arms (treatment x time interaction effect). Both per protocol (PP) and intention–to-treat (ITT) analyses were performed. The PP analyses were conducted in study participants who had full data from baseline to follow-up in the first or the second intervention period. For the ITT analyses, multiple imputations were conducted in order to compensate for all missing values. Five imputed models derived from this process. Considering that the PP and the ITT analyses provided similar results, (i.e. mean values, mean changes and statistical significance), the results from the ITT analyses are presented in the OLIVAUS

study published articles. In all statistical analyses, adjustments were made for gender and age, as these represent the most common confounding factors in biomedical research. Data is presented either as mean ± SD, as estimated marginal means and standard errors (SE) or as mean change and 95% Confidence Interval of change (CI) for continuous variables and as frequency (n) and percentage (%) for categorical ones. All reported *P* values are two-tailed, and the level of statistical significance is set at P< 0.05.   Statistical analyses were conducted by GM, KS and LP, using either the SPSS statistical software for Windows (IBM, Version 24.0; IBM, Armonk, NY, USA) or the R statistical software Version 3.6.1, as appropriate. Details concerning the statistical analyses performed for the examination of the effect of treatment to each one of the examined outcomes are described within the respective methods sections in the relevant chapters.

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Chapter 3: Effect of Olive Oil Polyphenols on HDL-mediated Cholesterol Efflux and lipid profile

This chapter reports the findings in relation to the primary outcome of the OLIVAUS study; namely high-density lipoprotein cholesterol efflux (HDL-c efflux). This chapter also reports the findings in relation to one of the secondary outcomes of the OLIVAUS study; namely serum lipids.

After a brief introduction, the chapter content is an exact copy of the manuscript entitled: " *Dietary intervention with high polyphenol extra-virgin olive oil does not enhance HDL-mediated cholesterol efflux capacity. The OLIVAUS study*", will be submitted to the British Journal of Nutrition. The article has been reformatted for thesis presentation.

Contributions:

Conceptualization/study design, C.I., E.S.G., C.J.T., and W.M.; investigation, K.S.; data curation, K.S., and G.M.; statistical analysis, K.S., L.P., and G.M.; writing—original draft preparation, K.S.; review and editing, all authors; supervision, G.M., C.J.T., and C.I.; project administration, K.S., E.S.G., J.C.W., and W.M.; funding acquisition, C.I., E.S.G., W.M., H.L.M., and C.J.T.

3.1   Chapter overview

It is well established that serum high-density lipoprotein (HDL) is inversely correlated with cardiovascular disease (CVD). The main function of the HDL particle is its role in the reverse cholesterol transport process. Specifically, HDL facilitates the efflux from the body of excess cellular cholesterol, transporting it to the liver for excretion in bile and feces. Cholesterol efflux occurs via two major pathways that involve the ATP-binding cassette receptors ABCA1 and ABCG1 and the scavenger receptor B1 (SR-B1). Specifically, ABCA1 facilitates the efflux of phospholipids and free unesterified cholesterol from cells to lipid-poor apolipoprotein A-1 (apoA-1) through a process that involves the binding of apoA-1 to the ABCA1 transporter, while ABCG1 and SR-B1 mediate cholesterol efflux from macrophages to HDL.

The traditional Mediterranean diet (MedDiet), known for its cardioprotective effect, has been shown to improve several CVD risk factors (i.e., lipid profile, low grade inflammation, and blood pressure). Of particular relevance to the proposed study, is the large servings of extra virgin olive oil (EVOO; 60–80 mL daily) which contains an array of unique polyphenols known for their antioxidant and anti-inflammatory properties. To further understand the mechanisms involved in the cardioprotective effect of EVOO-derived polyphenols, additional research is needed to a) replicate previously reported improvements in routinely measured cardiovascular markers (e.g. serum lipids) in the Australian population and b) investigate the effect of high polyphenol EVOO on cholesterol metabolic pathways (e.g. HDL-c efflux). Increased CVD risk has been partly attributed to low plasma levels of HDL-c; however it is the functional quality, not the quantity of circulating HDL *per se*, which is the more biologically significant as recently reported.

In view of the potential benefits of OO polyphenols on HDL functionality, the following paper provides results examining the effect of two kinds of OO differing in their phenolic content on a) the capacity of HDL to promote cholesterol efflux and b) serum lipid levels in a multi-ethnic Australian population.

3.2   Publication details

Dietary intervention with high polyphenol extra-virgin olive oil does not enhance HDL-mediated cholesterol efflux capacity. The OLIVAUS study

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3.3   Abstract

Olive oil polyphenols have been shown to improve high density lipoprotein (HDL) anti-atherogenic function, thus demonstrating beneficial effects against cardiovascular risk factors. The aim of the present study was to investigate the effect of extra-virgin high polyphenol olive oil (HPOO) vs. low polyphenol olive oil (LPOO) on the capacity of HDL

to promote cholesterol efflux in healthy adults. In a double blind, randomized cross-over trial, 50 participants (aged 38.5±13.9 years, 66% females) were supplemented with a daily dose (60 mL) of HPOO (320 mg/kg polyphenols) or LPOO (86 mg/kg polyphenols) for three weeks. Following a 2-week wash-out period, participants crossed-over to the alternate treatment. Serum HDL cholesterol (HDL-c) efflux capacity, circulating lipids (i.e., total cholesterol (TC), triglycerides (TG), HDL cholesterol, low density lipoprotein (LDL) cholesterol), and anthropometrics were measured at baseline and follow-up. No significant differences between treatments were observed in terms of changes from baseline to follow-up. A non-significant increase in HDL-c efflux was observed within both the LPOO and HPOO treatment arms by 0.54% (95% CI -0.29 to 1.37) and 0.10% (95% CI -0.74 to 0.94), respectively. Serum HDL cholesterol increased significantly after LPOO and HPOO intake, by 0.13 mmol/L (95% CI 0.04 to 0.22) and 0.10 mmol/L (95% CI 0.02 to 0.19), respectively. A small but significant increase in LDL of 0.14 mmol /L (95% CI 0.001 to 0.28) was observed following the HPOO intervention. Our results indicate that the higher phenolic content in HPOO did not result in any additional benefits on HDL-c efflux capacity compared to LPOO, thus highlighting the need for further research in this field.

Keywords: olive oil; extra virgin olive oil; polyphenols; cardiovascular disease; cholesterol efflux; HDL-cholesterol; adults

3.4 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality globally and accounted for 18.6 million deaths in 2019 (Roth et al., 2020). Endothelial dysfunction is a main contributor to the progression of cardiovascular events and any alterations in endothelial function have been associated with the development of atherosclerosis (Berenji Ardestani et al., 2020). It is well established that dyslipidemia plays a major role in the initiation and progression of atheromatous plaque formation. Indeed, dyslipidemia is recognized as an independent risk factor for the development of atherosclerosis-related vascular disease (Hedayatnia et al., 2020; Nepal et al., 2018; Rondanelli et al., 2016). Whereas elevated serum levels of low-density lipoprotein (LDL) and by-products of oxidation reactions with LDL (i.e., oxidized LDL) are involved in the progression of the atherogenic process (Helkin et al., 2016), serum HDL-c is inversely correlated with CVD. Indeed, an increase in serum HDL-c levels by 0.026 mmol/L (1 mg/dL), has been demonstrated to reduce CVD risk by 2% and 3% in male and female adults, respectively (Kutkiene et al., 2019; Rondanelli et al., 2016).

One of the main anti-atherogenic functions of HDL is the regulation of cholesterol homeostasis through the reverse cholesterol transport (RevCT) pathway, in which excess cholesterol is removed from peripheral cells and transported to the liver for excretion in the bile and feces (Favari et al., 2015; Kutkiene et al., 2019). The efflux of cholesterol from cells to HDL is considered the primary step of RevCT and occurs via two major pathways: the ATP-binding cassette receptors ABCA1 and ABCG1 and the scavenger receptor B1 (SR-B1). Specifically, ABCA1 facilitates the efflux of phospholipids and free unesterified cholesterol from cells to lipid-poor apolipoprotein A-1 (apoA-1) through a process that involves the binding of apoA-1 to the ABCA1 transporter, while ABCG1 and SR-B1

mediate cholesterol efflux from macrophages to HDL (Fernández-Castillejo et al., 2017; Helal et al., 2013; Rohatgi et al., 2014).

Previous evidence indicates that certain diets have beneficial effects on cardiovascular health (Collins C, 2017). The traditional Mediterranean diet (MedDiet) has been widely reported over several decades as one of the 'healthiest' dietary patterns for the prevention and management of CVD (Dinu et al., 2018; Kelly, 2010). Various components of this dietary pattern are cardioprotective, including the high consumption of extra virgin olive oil (EVOO). Olive oil (OO) has a favourably high content of mono-unsaturated fatty acids (MUFA) (55-83% oleic acid) as well as important minor bioactive compounds, including polyphenols (i.e., tyrosol, hydroxytyrosol and oleuropein) (Donini et al., 2015; Estruch et al., 2006). Polyphenols have been demonstrated to mediate the prevention and management of CVD and associated risk factors (e.g., hypertension and dyslipidemia) through various mechanistic pathways (Hohmann et al., 2015). In particular, OO polyphenols have demonstrated cardioprotective effects not only through the scavenging of free radicals due to their antioxidant properties, but also via a plethora of anti-inflammatory cell signaling and molecular mechanisms (Berrougui et al., 2015; Farràs et al., 2013).

Increased CVD risk has in part been attributed to low plasma levels of HDL-c. However, more recently, developing evidence has emerged that impaired HDL function, rather than low HDL levels, may explain HDL associated risk in these conditions. EVOO polyphenols have shown to improve the functionality of HDL, especially the capacity to promote cholesterol efflux, by reducing HDL oxidative modifications and improving the physiochemical properties of HDL. They also activate ABCA1 expression, which is a key protein involved in cholesterol efflux (Berrougui et al., 2015). Moreover, results of the EUROLIVE Study demonstrated that the daily consumption of polyphenol-rich OO

significantly increased HDL levels and promoted cholesterol efflux from macrophages, in healthy male volunteers (Hernaez et al., 2014). A beneficial effect of HPOO intake on HDL-c efflux capacity and HDL-c was also reported in a recent systematic review and meta-analyses, which examined the effect of HPOO versus LPOO on several cardiovascular risk factors (George et al., 2019).

While numerous *in vitro* and *in vivo* studies have explored the effect of OO on serum lipid levels (Berrougui et al., 2015; Blanco-Molina et al., 1998; Carluccio et al., 2007; Covas et al., 2006), most of the intervention studies are limited to Mediterranean populations that are accustomed to high OO intake (Hohmann et al., 2015), thus highlighting the need for additional evidence in multi-ethnic populations with different habitual food cultures. Hence, the primary aim of the present study was to investigate the effect of 3-weeks daily consumption of either raw extra virgin HPOO (320 mg/kg, phenolic content; 60 mL/day) or LPOO (86 mg/kg, phenolic content; 60 mL/day), on HDL-mediated cholesterol efflux in Australian adults with no previously diagnosed medical conditions. The secondary aim was to compare the effect of the two OO treatments on serum lipids (i.e., TC, triglycerides (TG), LDL and HDL cholesterol).

3.5   Material and Methods

3.5.1   Study design and procedure

The OLIVAUS study (Marx et al., 2019) was a double-blind, cross-over, randomised controlled trial (RCT) that aimed to investigate the effect of extra virgin HPOO compared to a commercially available LPOO on several CVD risk factors in a healthy adult population. A pilot study was conducted prior to the main study, in order to test the feasibility of the study protocol and the data collection tools (Sarapis et al., 2019). The trial

protocol has been registered with the Australia New Zealand Clinical Trials Registry ACTRN12618000706279 and was created in accordance with the SPIRIT statement (Chan et al., 2013). This trial was conducted in accordance with the Guidelines for Good Clinical Practice and the Declaration of Helsinki and CONSORT reporting guidelines. The trial team has obtained written approval for the protocol and Patient Information and Consent Form from the La Trobe University Human Research Ethics Committee (HEC17-067).

Study participants were recruited in Melbourne, Australia through La Trobe University using email advertisements, mailing lists, word of mouth, and posters on campus. The inclusion and exclusion criteria used in the OLIVAUS study to identify eligible participants are presented elsewhere (Marx et al., 2019). Enrolled participants were randomly allocated in a 1:1 ratio, to one of the two treatment arms, i.e., extra virgin HPOO or LPOO. Randomization was performed in blocks of six using a computerized random number generator in excel software. The block randomization sequence was developed by an independent senior researcher not otherwise involved in the study.

Study participants received a daily dose of 60 mL of either HPOO or LPOO, over two intervention periods of 3 weeks each, added in their usual diet in its raw form. The two kinds of OO had the same nutrient composition (i.e., fat-soluble vitamins and fatty acids) but differed in their phenolic content (320 mg/kg in HPOO vs. 86 mg/kg in LPOO). Two washout periods of 2 weeks each, in which study participants were instructed to avoid consumption of olives and OO, preceded the first and the second intervention periods of test oils administration. A 2-week washout period was chosen on the basis that this was sufficient to eliminate the carry-over effect of OO polyphenols between interventions, considering the short half-life of OO's phenolic compounds (Miró-Casas et al., 2001). Furthermore, a daily dose of 60 ml OO was chosen in the current study, since this reflects

the habitual amount consumed in Mediterranean populations where the cardioprotective benefits of virgin OO (VOO) have previously been reported (George et al., 2019; Hohmann et al., 2015; Schwingshackl et al., 2019).

The intervention OOs were stored in dark, sealed, similar in appearance and colour containers, thus ensuring blinding of the participants and researchers, and were supplied to study subjects at the beginning of each intervention period. To ensure further blinding to the kind of OO, each container was assigned a different code number that was concealed from study participants and researchers. The code was disclosed only after the completion of the statistical analyses. Participant's adherence to the intervention was assessed by measuring the volume of unconsumed OO returned at the end of each intervention period. To confirm further adherence, study participants were also asked to record the daily volume of OO consumed over each 3-week intervention period using a log sheet. This information was collected by the researchers at the end of each intervention period. Detailed descriptions of the study's protocol and information on the phenolic concentrations and composition of the two intervention OOs are provided elsewhere (Marx et al., 2019).

3.5.2 Measurements

3.5.2.1 *Socio-demographics, use of medication and dietary supplements*

At the first baseline intervention period, study participants' socio-demographic data and any information related to medication and dietary supplement intake was collected during a scheduled interview by trained researchers using a standardized questionnaire. (Marx et al., 2019)

3.5.2.2 Dietary intake

A 3-day food diary was used to collect dietary intake data during two non-consecutive weekdays and one weekend day at baseline and follow-up of each intervention period. Participants were instructed to record details on the foods and beverages consumed, including the type/brand, quantity in household measures and cooking methods. Emphasis on strategies that incorporate OO into their habitual diet in a raw, uncooked form was provided by the researchers. All dietary intake data was analyzed for energy, macro- and micronutrients' content using FoodWorks®9 software (Xyris Software Pty Ltd, Queensland, Australia).

3.5.2.3 Physical Activity

Physical activity (PA) was assessed during the week preceding the interviews at the first baseline and at the last follow-up meeting using the Active Australia Survey (AAS) questionnaire (AIHW, 2003), a tool that has been validated in the Australian population. This questionnaire is designed to assess participation in a range of leisure-time physical activities of light, moderate and vigorous intensity. It consists of eight questions, which assess the number of sessions and total weekly time (hours and/or minutes) spent for each activity type. The amount of time (in minutes per day) that study participants were engaged in PA of different intensity was calculated and used for data analysis.

3.5.2.4 Anthropometric measurements

Anthropometric measurements were conducted at baseline and follow up of each intervention period. Body weight was measured using a digital scale (WM203, Willawong QLD, Australia) to the closest 0.1 kg and with study participants in light clothing and barefoot, whereas standing height was measured using a wall-mounted stadiometer (SE206,

Seven Hills, NSW, Australia) to the nearest 0.1cm. Waist circumference (WC) was measured directly over the skin at the umbilicus level, using a flexible steel tape calibrated in cm with mm graduations (Luftkin W606PM, Maryland, USA) to the nearest 0.1cm. Body mass index (BMI) was calculated by dividing weight (kg) to height squared $(m)^2$ (Quetelet's equation). Study participants were categorized as underweight (BMI <18.5 kg/m^2), normal weight (BMI 18.5-24.9 kg/m^2), overweight (BMI 25.0-29.9 kg/m^2) or obese (BMI ≥30 kg/m^2) based on the World Health Organization (WHO) cut-off points for BMI (WHO, 1995). Moreover, participants were classified as normal (WC <94 cm in men and <80 cm in women), high CVD risk (WC 94-102 cm in men and 80-88 cm in women) and very high CVD risk (WC >102 cm in men and 88 cm in women) based on WHO gender-specific WC cut-off points (WHO, 2008).

3.5.2.5 *Biochemical analyses*

Blood biomarkers were measured from early-morning venous blood samples collected from the participants by a trained researcher at baseline and follow-up of each intervention period. Participants attended La Trobe University following a 10-hour overnight fast. Collected venous blood was centrifuged (Hettic Rotina 420r, Massachusetts, USA) at 2350 rpm for 10 min at 4 ^{0}C and the extracted plasma and/or serum was apportioned into aliquots of 500 μL each and stored at -80 ^{0}C until analysis.

HDL- cholesterol efflux

The HDL-c efflux in serum plasma (collected in serum separating tubes) was measured using the Cholesterol Efflux Fluorometric Assay Kit (BioVision; Milpitas, CA, USA) following the manufacturer's instructions. Briefly, 5x104 J774A.1 cells were seeded into 96-well tissue culture plates and grown in supplemented phenol red-free DMEM for 24

hours. Cells were washed with serum-free, phenol red-free DMEM and labelled for 1 hour in 1:1 ratio labelling reagent to serum-free, phenol red-free DMEM. The labelling medium was removed before the cells were incubated in equilibration medium for 18-19 hours. Samples were pre-treated with serum treatment reagent to remove interferents (LDL/VLDL) prior to addition to the cells according to the protocol. 2 µL of each pre-treated human serum sample, assayed in duplicate, was added, and incubated with the cells for 4 hours at 37°C. Supernatant was transferred to black walled 96-well plates and fluorescence measured using a FLUOstar Omega spectrophotometer (BMG LabTech, Vic, Australia) with excitation and emission wavelengths 485 and 520 nm, respectively. Cells were lysed and the lysate was transferred to black-walled 96-well plates and fluorescence measured. Percentage cholesterol efflux was measured using the following equation: *% Cholesterol Efflux = (RFU of supernatant / RFU of cell lysate + RFU of supernatant) x 100. The* Intra-assay coefficient variation (CV) was 2.2%.

Total cholesterol, LDL, HDL, Triglycerides

Serum cholesterol concentrations were measured using the Alinity c Cholesterol Assay kit (Abbott GmbH & Co; Wiesbaden, Germany) according to the manufacturer's instructions. Briefly, cholesterol esters were enzymatically hydrolyzed by cholesterol esterase to cholesterol and free fatty acids (Intra-assay CV was 1.0%). The Alinity c Direct LDL Assay kit (Sekisui Diagnostics P.E.I. Inc; Charlottetown, Canada) was used for the direct, quantitative determination of LDL cholesterol in human serum (intra-assay CV was 0.8%). Serum HDL-c concentrations were measured using the Ultra HDL Assay kit (Abbott GmbH & Co; Wiesbaden, Germany). This assay is a homogeneous method in which HDL-c concentrations are measured without the need for off-line pretreatment or centrifugation steps (Intra-assay CV was 1.0%). Finally, the Alinity c Triglyceride Assay (Abbott GmbH

& Co; Wiesbaden, Germany) was used for the quantitation of TG in human serum. With this method, triglycerides are enzymatically hydrolyzed by lipase to free fatty acids and glycerol (Intra-assay CV was 1.8%).

3.6   Sample size calculation

The sample size was calculated to detect a 5% change in the primary outcome, HDL-c efflux, based on results of previous research (Hernaez et al., 2014); a sample size of 50 participants was considered adequate to provide >80% statistical power to detect significant between-group differences of 5%, a standard deviation (SD) of 11% in HDL-c efflux levels and a drop-out rate of 20%.

3.7   Statistical analyses

All statistical analyses were conducted using the SPSS statistical software for Windows (IBM, version 24.0; IBM, Armonk, NY, USA). Normality of continuous variables was assessed using the Kolmogorov-Smirnov test. Repeated-measures ANOVA (analysis of variance) was used to examine a) treatment effects (between-group differences, i.e., HPOO *vs* LPOO, at each time point of measurement), b) time effects (within-group changes in each intervention arm from baseline to follow-up), and c) Treatment * Time interaction effects (differences in the changes from baseline to follow-up between the two intervention arms). We performed both per protocol (PP) and intention–to-treat (ITT) analyses. The PP analysis included only those participants who had complete data from baseline to follow-up in the first and/or second intervention period. Multiple imputations of missing data were conducted for the ITT analysis. Because both methods of data analyses provided concordant results in terms of mean values, mean changes and statistical significance, results from the ITT analysis are presented in this article. Adjustments for gender and age were made in all statistical analyses. Continuous variables are presented either as mean ±

SD, as estimated marginal means and standard errors (SE) or as mean change and 95% Confidence Interval of change (CI). In addition, categorical variables are presented as frequency (n) and percentage (%). Statistical significance is set at P< 0.05 and all reported *P* values are two-tailed.

3.8 Results

Fifty volunteers (n=33 females and n=17 males) were enrolled in the study from July 2018 to October 2019 and were assigned into treatment arms. Four participants discontinued the intervention, due to inability to comply and three participants withdrew for personal reasons. In total, 43 participants completed the study (Figure 3.1).

3.8.1 Baseline characteristics of study participants

Baseline characteristics of the total sample (*n=50*) and by treatment arm in terms of socio-demographics, anthropometric and biochemical indices are presented in Table 3.1. The mean age of participants was 39±14 years (age range, 20 to 70 years), while the majority were females (66%) and born in Australia (70%). The mean BMI and WC of participants was 24.7±3.5 kg/m^2 and 86.9±11.2 cm, respectively. In addition, 48% of the study population was classified as overweight/obese, while 16% of study participants were considered at high CVD risk and 24% very high CVD risk according to their WC measurements. In terms of biochemical indices, mean serum HDL-c efflux concentration was 53.1±4.8 % for the total cohort. The lipid profile of the cohort was also assessed: mean circulating TG, TC, HDL and LDL cholesterol was 1.0±0.5 mmol/L, 5.0±0.5 mmol/L, 1.5±0.3 mmol/L and 3.0±0.9 mmol/L, respectively. No significant differences were observed between treatment arms in any of the aforementioned descriptive characteristics, thus indicating homogeneity at baseline.
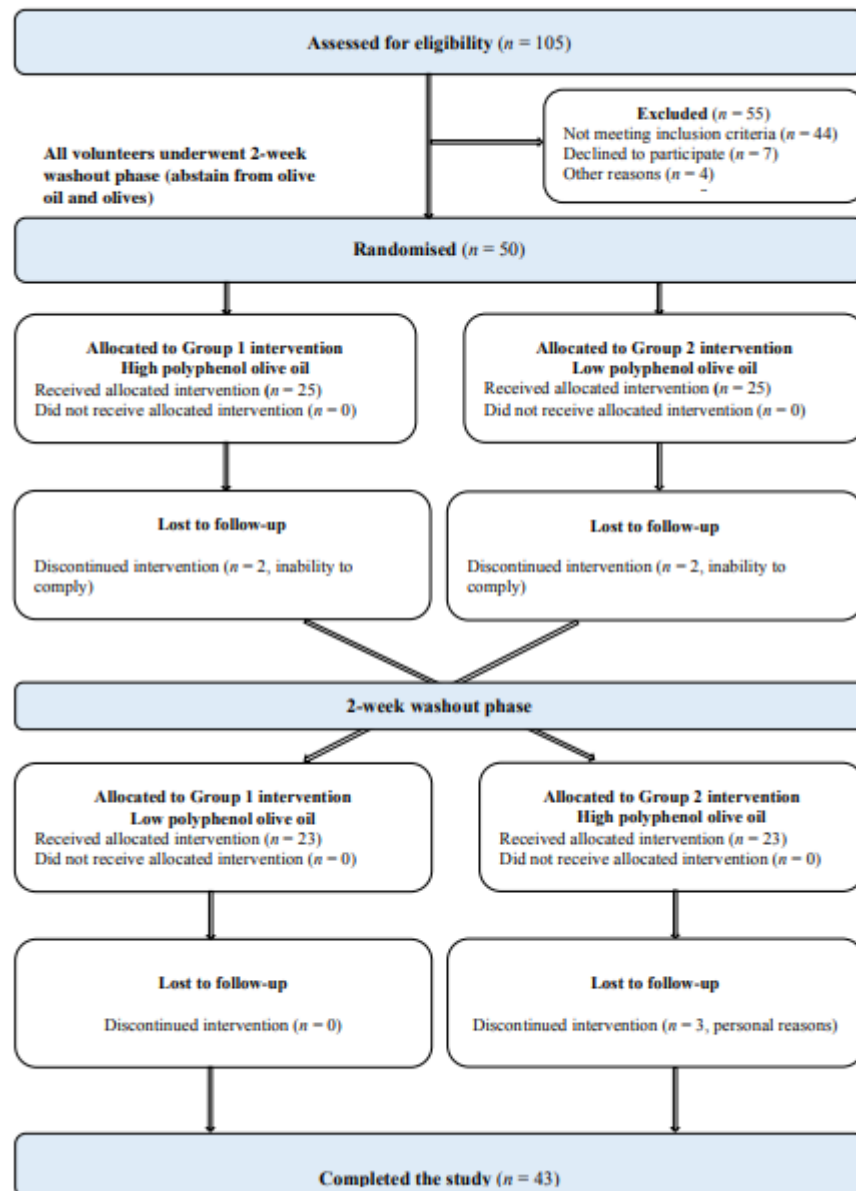
Figure 3.1 OLIVAUS study participant flow diagram

Table 3.1   Baseline descriptive characteristics of study

	Total sample (n=50)	Low Polyphenol OO (n=25)	High Polyphenol OO (n=25)	P-value*
Socio-demographics	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Age (years)	38.5 (13.9)	38.1 (14.8)	39.0 (13.2)	0.818
Education (years)	17.3 (3.5)	17.3 (4.2)	17.2 (2.8)	0.968
Gender	**(%)**	**(%)**	**(%)**	
Females	66.0	64.0	68.0	0.765
Males	34.0	36.0	32.0	
Country of Birth	**(%)**	**(%)**	**(%)**	
Australia, NZ, Pacific Islanders	70.0	68.0	72.0	0.321
Europe	10.0	4.0	16.0	
South America	8.0	12.0	4.0	
Middle East & Asia	12.0	16.0	8.0	
Anthropometrics	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Height (cm)	168.9 (9.6)	170.6 (10.4)	167.2 (8.6)	0.220
Weight (kg)	70.7 (12.8)	72.7 (13.7)	68.5 (11.8)	0.249
BMI (kg/m^2)	24.7 (3.5)	24.9 (3.7)	24.4 (3.2)	0.617
Waist circumference (cm)	86.9 (11.2)	88.2 (11.9)	85.6 (10.6)	0.434
Weight status categories[†]	**(%)**	**(%)**	**(%)**	
Underweight	2.0	0.0	4.0	0.252
Normal weight	50.0	56.0	44.0	
Overweight	44.0	36.0	52.0	
Obese	4.0	8.0	0.0	
Waist circumference categories[‡]	**(%)**	**(%)**	**(%)**	
Normal	50.0	44.0	56.0	0.632
High risk	16.0	20.0	12.0	
Very high risk	34.0	36.0	32.0	

	Total sample (n=50)	Low Polyphenol OO (n=25)	High Polyphenol OO (n=25)	P-value*
Biochemical indices	Mean (SD)	Mean (SD)	Mean (SD)	
HDL-c efflux (%)	53.1 (4.8)	52.7 (4.6)	53.3 (5.0)	0.663
TG (mmol/L)	1.0 (0.5)	1.1 (0.7)	0.9 (0.3)	0.334
TC (mmol/L)	5.0 (1.0)	4.9 (1.1)	5.2 (0.9)	0.370
HDL-c (mmol/L)	1.5 (0.3)	1.5 (0.3)	1.6 (0.3)	0.150
LDL-c (mmol/L)	3.0 (0.9)	3.0 (0.9)	3.1 (0.8)	0.582

* P-values for testing between-group differences in continuous variables were derived from the independent samples t-test.

P-values for examining associations between categorical variables were derived from the Chi-square test;

SD, standard deviation; OO, olive oil; BMI, body mass index;

HDL-c efflux, high density lipoprotein cholesterol efflux; TG, triglycerides;

TC, total cholesterol; HDL-c, high density lipoprotein cholesterol,

LDL-c, low density lipoprotein cholesterol.

[†] Weight status categories: Underweight, BMI<18.5 kg/m^2; Normal weight, $18.5 \leq$ BMI < 25 kg/m^2; Overweight, $25 \leq$ BMI < 30 kg/m^2; Obese, BMI $\geq$ 30 kg/m^2.

[‡] Waist circumference categories: Normal, WC <80 cm in women & <94 cm in men; High risk, 80-88 cm in women & 94-102 cm in men; Very high risk: WC > 88 cm in women and > 102 cm in men.

3.8.2 Effect of LPOO and HPOO on dietary intake and physical activity

Table 3.2 illustrates dietary energy, macro- and micronutrient intake changes from baseline to follow-up, as well as the relevant differences between treatment arms. The changes in dietary intake were not significantly different between the two treatment arms. A significant increase in dietary energy intake was observed within both the LPOO (by 1806.1 kJ/day, 95% CI: 1075.4 to 2536.8) and HPOO (by 1766.6 kJ/day, 95% CI: 1035.9 to 2497.3) treatment arms. Similarly, consumption of LPOO and HPOO led to a significant increase in total fat (by 49.3 g/day, 95% CI: 41.1 to 57.4 and 46.0 g/day, 95% CI: 37.8 to 54.1,

respectively), SFA (by 7.4 g/day, 95% CI: 4.0 to 10.8 and 6.5 g/day, 95% CI: 3.1 to 9.9, respectively), MUFA (by 36.8 g/day, 95% CI: 33.2 to 40.3 and by 35.1 g/day, 95% CI: 31.6 to 38.6, respectively) and PUFA intake (by 3.1 g/day, 95% CI: 1.0 to 5.1 and by 3.0 g/day, 95% CI: 1.0 to 5.1, respectively) from baseline to follow-up. Regarding PA, no within-group changes or between-group differences were observed in daily energy expenditure in leisure-time physical activity over the intervention period (data not shown).

Table 3.2   **Effect of low polyphenol OO vs. high polyphenol OO on mean changes in dietary energy, macro- and micronutrient intake.**

	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Energy intake (KJ/day)				
Low Polyphenol OO (n=50)	8712.8 (328.3)	10518.9 (344.4)	**1806.1 (1075.4 to 2536.8)**	0.940
High Polyphenol OO (n=50)	8892.6 (328.3)	10659.2 (344.4)	**1766.6 (1035.9 to 2497.3)**	
Treatment*effect p-value	0.700	0.774		
Protein intake (g/day)				
Low Polyphenol OO (n=50)	102.0 (5.5)	100.7 (5.2)	-1.3 (-14.3 to 11.8)	0.924
High Polyphenol OO (n=50)	97.4 (5.5)	97.0 (5.3)	-0.4 (-13.4 to 12.7)	
Treatment*effect p-value	0.558	0.619		
CHO intake (g/day)				
Low Polyphenol OO (n=50)	214.8 (10.1)	213.4 (11.1)	-1.5 (-23.5 to 20.6)	0.972
High Polyphenol OO (n=50)	219.9 (10.1)	217.8 (11.1)	-2.0 (-24.0 to 20.0)	
Treatment*effect p-value	0.726	0.776		
Total fat intake (g/day)				
Low Polyphenol OO (n=50)	79.9 (4.0)	129.2 (4.5)	**49.3 (41.1 to 57.4)**	0.571
High Polyphenol OO (n=50)	84.3 (4.0)	130.3 (4.5)	**46.0 (37.8 to 54.1)**	
Treatment*effect p-value	0.441	0.870		

	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
SFA intake (g/day)				
Low Polyphenol OO (n=50)	27.7 (1.5)	35.1 (1.9)	**7.4 (4.0 to 10.8)**	0.707
High Polyphenol OO (n=50)	28.8 (1.5)	35.3 (1.9)	**6.5 (3.1 to 9.9)**	
Treatment*effect p-value	0.620	0.953		
MUFA intake (g/day)				
Low Polyphenol OO (n=50)	30.6 (1.7)	67.3 (1.9)	**36.8 (33.2 to 40.3)**	0.514
High Polyphenol OO (n=50)	31.8 (1.7)	67.0 (1.9)	**35.1 (31.6 to 38.6)**	
Treatment*effect p-value	0.605	0.877		
PUFA intake (g/day)				
Low Polyphenol OO (n=50)	14.6 (1.0)	17.7 (1.0)	**3.1 (1.0 to 5.1)**	0.971
High Polyphenol OO (n=50)	15.7 (1.0)	18.7 (1.0)	**3.0 (1.0 to 5.1)**	
Treatment*effect p-value	0.483	0.469		
Fibre intake (g/day)				
Low Polyphenol OO (n=50)	29.7 (1.7)	30.7 (1.8)	0.9 (-3.1 to 4.9)	0.314
High Polyphenol OO (n=50)	29.6 (1.7)	33.5 (1.8)	3.8 (-0.2 to 7.8)	
Treatment*effect p-value	0.963	0.268		

All statistical analyses were adjusted for gender and age. Results in bold indicate statistical significance ($p < 0.05$). OO, olive oil; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SEM, standard error of the mean; CI, confidence interval

3.8.3 Effect of LPOO and HPOO on anthropometrics

The changes observed in anthropometric indices of OLIVAUS participants from baseline to follow-up after each 3-week intervention, as well as the differences between the two treatment arms are presented elsewhere (Sarapis et al., 2020). In brief, no significant between-group differences were observed in any of the examined outcomes. A small but significant increase in body weight by 0.4 kg (95% CI 0.2 to 0.7) was observed only in the LPOO treatment arm. No within-group changes were observed in any of the other outcomes (i.e., BMI and WC).

3.8.4 Effect of LPOO and HPOO on HDL-c efflux and serum lipids

The effect of the two OO interventions on HDL-c efflux is illustrated in Figure 3.2. No significant differences between treatment arms were observed regarding the changes in HDL-c efflux from baseline to follow-up. A non-significant increase in HDL-c efflux was observed within both the LPOO and HPOO groups by 0.54% (95% CI -0.29 to 1.37) and 0.10 (95% CI -0.74 to 0.94), respectively for the total sample. No between-group differences were observed in circulating TG, TC, HDL-c and LDL-c for the total sample from baseline to follow up (Table 3.3). However, compared to baseline, serum HDL-c significantly increased after LPOO and HPOO intake, by 0.13 mmol/L (95% CI 0.04 to 0.22) and 0.10 mmol/L (95% CI 0.02 to 0.19), respectively. There was a small but significant increase in LDL-c by 0.14 mmol /L (95% CI 0.001 to 0.28) following the HPOO intervention, however non-significant differences were observed between the two treatment arms. No significant within-group changes or between group differences were observed in TG and TC.
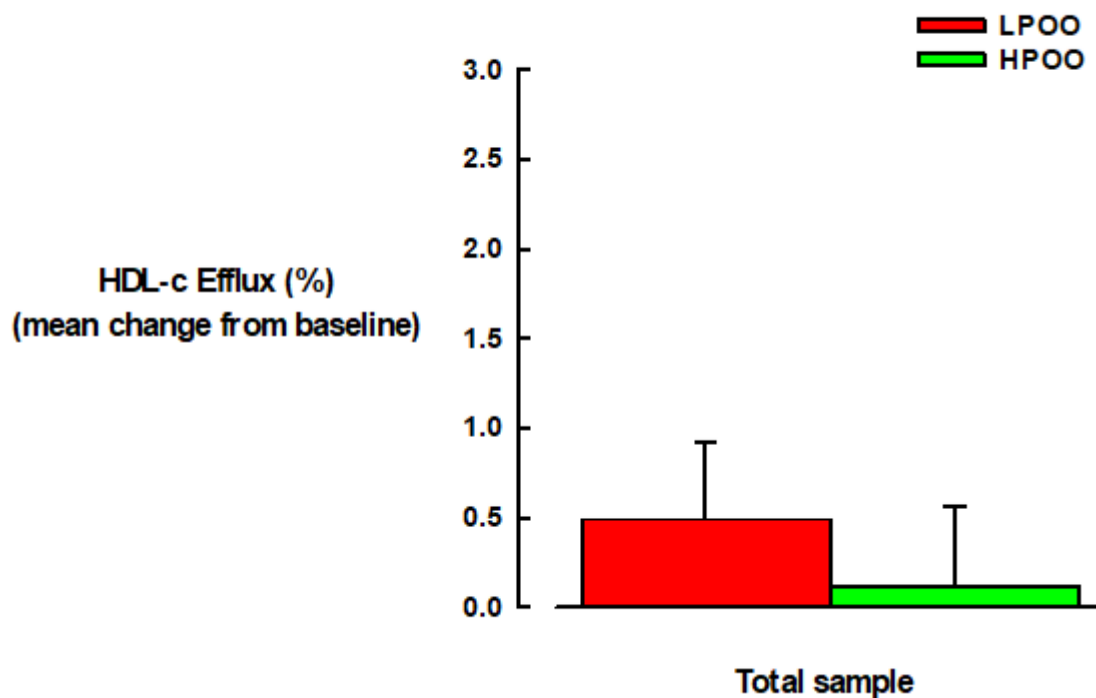
Figure 3.2   **Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil**

Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil (HPOO; 320 mg/kg polyphenols; N=43) and low polyphenol olive oil (LPOO; 86 mg/kg polyphenols; N=44) on high density lipoprotein cholesterol (HDL-c) Efflux. No within-group changes or between-group differences were observed in HDL-c efflux. Results are presented as mean changes ± standard errors from baseline to follow-up.

Table 3.3   Effect of low polyphenol OO vs. high polyphenol OO on mean changes in serum lipids

Biomarker	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Triglycerides (mmol/L)				
Low Polyphenol OO (n=50)	0.97 (0.07)	0.89 (0.09)	-0.08 (-0.21 to 0.05)	0.425
High Polyphenol OO (n=50)	0.92 (0.07)	0.91 (0.09)	-0.01 (-0.14 to 0.12)	
Treatment*effect p-value	0.613	0.860		
Total Cholesterol (mmol/L)				
Low Polyphenol OO (n=50)	5.01 (0.13)	5.08 (0.13)	0.08 (-0.11 to 0.27)	0.856
High Polyphenol OO (n=50)	5.09 (0.13)	5.14 (0.13)	0.05 (-0.14 to 0.24)	
Treatment*effect p-value	0.646	0.749		
HDL cholesterol (mmol/L)				
Low Polyphenol OO (n=50)	1.57 (0.05)	1.70 (0.05)	**0.13 (0.04 to 0.22)**	0.650
High Polyphenol OO (n=50)	1.56 (0.05)	1.66 (0.05)	**0.10 (0.02 to 0.19)**	
Treatment*effect p-value	0.927	0.627		
LDL cholesterol (mmol/L)				
Low Polyphenol OO (n=50)	3.01 (0.11)	3.04 (0.11)	0.03 (-0.11 to 0.16)	0.256
High Polyphenol OO (n=50)	3.05 (0.11)	3.19 (0.11)	**0.14 (0.001 to 0.28)**	
Treatment*effect p-value	0.795	0.330		

All statistical analyses were adjusted for gender and age. OO, olive oil; SEM, standard error of the mean; CI, confidence interval

3.8.5 Adherence to Treatment

As previously reported (Sarapis et al., 2021) adherence to the dietary intervention in the OLIVAUS trial was overall high, despite having a multi-ethnic population that was unaccustomed to high OO intake. We measured the volume of unconsumed OO post each intervention period and no significant differences were observed between the two treatment arms. Specifically, participants' adherence was found to be 92% for both the LPOO and HPOO treatment arms during the first intervention period and 92% and 90% for the LPOO and the HPOO arms, respectively, during the second intervention period (Supplementary Table S3).

3.9 Discussion

The present double blind, cross-over, randomized controlled trial investigated the effect of 3-weeks daily consumption of either raw extra virgin HPOO (320 mg/kg, phenolic content; 60 mL, daily dose) or LPOO (86 mg/kg, phenolic content; 60 mL daily dose) on HDL-c efflux and serum lipids in Australian adults with no previously diagnosed medical condition. Our results showed no differences in any of the examined biomarkers between the two treatment arms. A non-significant increase in HDL-c efflux was observed within both the LPOO and HPOO groups for the total sample, while serum HDL-c increased significantly within both treatment arms. A small but significant increase in circulating LDL-c was also observed in the HPOO arm.

The non-significant changes observed in HDL-c efflux to OO polyphenol intake in the present study (+0.54%, LPOO; +0.10%, HPOO) are not aligned with previous literature. For example, Hernaez *et al.(2014)* demonstrated that the daily consumption of 25 mL raw HPOO (366 mg/kg, phenolic content) or LPOO (2.7 mg/kg, phenolic content) for 3-weeks significantly increased HDL-c efflux by 3.05% only in the HPOO treatment arm compared

with the LPOO arm (-2.34%) in healthy men (Hernaez et al., 2014). A significant increase in the capacity of HDL to promote cholesterol efflux by 14.81% has also been reported in healthy adults, after 12 weeks of EVOO intake (793 mg/kg, phenolic content; 25 mL daily dose) in a noncontrolled single-arm study (Helal et al., 2013). Supporting these data, another study showed that the consumption of a functional virgin OO (FVOO) enriched with OO and thyme phenolic compounds (500 mg/kg, total phenolic content; 25mL daily dose) for 3-weeks, compared to a virgin olive oil (80 mg/kg, phenolic content) enhanced the ability of HDL to induce cholesterol efflux from macrophages via the increased expression of cholesterol efflux-related genes in hypercholesterolemic adults (Farràs et al., 2019).

Previous evidence suggests that OO polyphenols may improve HDL functionality via several cellular pathways. Specifically, OO polyphenols have been shown to enhance apoA-1 stability in HDL, a key protein involved in cholesterol efflux and an anti-atherogenic and antioxidant factor of HDL (Arora et al., 2016), reduce oxidative modifications of HDL and increase the size of the HDL particle (Hernaez et al., 2014). Berrougui *et al*. (Berrougui et al., 2015) recently demonstrated that increased HDL phenolic content has been associated with improvements in the physiochemical properties of HDL and fluidity of the phospholipidic monolayer, and therefore HDL functionality. Similarly, the EUROLIVE study reported that the intake of phenol-rich OOs resulted in phenolic compound binding to HDL, contributing to HDL functionality enhancement, and particularly cholesterol efflux (Hernaez et al., 2014). Interestingly, HDL monolayer fluidity has been recognized as a hallmark of HDL functionality (Pedret et al., 2018). Increased levels of free cholesterol or low levels of phospholipids on the HDL membrane may result in a less functional lipoprotein due to reduced HDL fluidity (Favari et al., 2015).

Furthermore, HDL oxidation plays an important role on HDL functionality since oxidized HDL is less fluid, and therefore less likely to perform cholesterol efflux from macrophages (Fernández-Castillejo et al., 2017). Previous studies have reported that phenolic compounds have the ability to inhibit lipoprotein oxidation through the scavenging of free radicals due to their antioxidant properties (Cicerale et al., 2012; Franco et al., 2014). Finally, Castillejo *et al*. (Fernández-Castillejo et al., 2017) recently reported that a larger HDL particle size has been found to enhance cholesterol efflux capacity via changes in the HDL monolayer fluidity.

The molecular mechanisms by which phenolic compounds promote cholesterol efflux still need to be further investigated. These mechanisms involve the passive diffusion process as well as pathways that are mediated by the transmembrane transporters ABCA1, ABCG1 and the scavenger receptor B1 (SR-B1). Specifically, ABCA1 facilitates cholesterol efflux from cells to lipid-poor apoA-1, while ABCG1 and the SR-B1 receptor are responsible for the efflux of cholesterol from macrophages to HDL (Kennedy et al., 2005; Wang et al., 2001). EVOO phenolic compounds, especially hydroxytyrosol, has been shown to stimulate ABCA1 protein expression, which is a key factor in cholesterol efflux and to increase apoA-1 -mediated cholesterol efflux (Cedó et al., 2020). Sola *et al.* (Solá et al., 2011) reported an increase in apoA-1 concentrations after virgin olive oil consumption in high-CVD risk individuals; Violante *et al.* (Violante et al., 2009) also demonstrated that consumption of EVOO for 3 months increased serum apoA-1 in hypercholesterolemic subjects. Given that our participants were healthy without any previously diagnosed medical condition could potentially explain the non-significant changes in HDL-c efflux following both LPOO and HPOO intake. In addition, the concentration of hydroxytyrosol (HT), which is the most biologically active phenolic compound found in VOO (Suárez et

al., 2011), was higher in the LPOO (5.3 mg/kg, HT) compared to HPOO (3.3 mg/kg, HT) in the current study, possibly explaining the more pronounced increase in HDL-c efflux in the LPOO treatment arm.

It is noteworthy that, 3-weeks of both extra virgin HPOO and LPOO consumption in the present study significantly increased circulating HDL-c, hence demonstrating the cardioprotective effect of OO on other CVD risk markers. In this context, two recent-meta-analyses, one conducted by our team, reported that phenolic-rich OO intake increases HDL-c levels (George et al., 2019; Tsartsou et al., 2019). Furthermore, it has been reported that the daily consumption of OOs rich in polyphenols reduces LDL-c and improves lipoprotein associated atherogenic ratios (Fernández-Castillejo et al., 2016). The PREDIMED study reported that one-year intervention with a MedDiet enriched with VOO improved various LDL atherogenic related characteristics but did not improve the plasma LDL-c concentrations in a sub-sample of adults at high CVD risk (Hernáez et al., 2017). Our results are consistent with this non beneficial effect of VOO on circulating LDL-c.

With regards to the dietary intake changes and any potential confounding effect caused by differences in dietary intake, the OLIVAUS study showed that both treatment oils equally increased the intake of energy and macronutrients (i.e., fatty acids), while no significant within group changes or between group differences were observed in the intake of foods high in polyphenols (Supplementary Table S1 and Table S2). It is noteworthy that both the LPOO and HPOO tested in the current study had the same nutritional composition in terms of fat-soluble vitamins and fatty acids, so the observed improvements in the examined biomarkers were exclusively associated with the intervention oils' phenolic content. Furthermore, the considerable amount of polyphenols (i.e., 86 mg/kg) present in the LPOO that was used in the current study could potentially explain the non-significant differences

between the two intervention arms with regards to the changes in HDL-c efflux and the examined serum lipids.

The findings of the present study should be interpreted in light of its strengths and limitations. The greatest strength of this study was its randomized, cross-over double-blind, controlled design, which confers strong inter-individual variability. This design also enabled us to isolate the effects of OO polyphenols on the examined outcomes since it allowed us to control for potential confounding effects caused by the intake of other dietary polyphenols. Another strength was that study participants remained consistent with their habitual diet, hence allowing us to directly assess the effects of OO consumption. One of the limitations of the present study was that cholesterol efflux was measured *ex vivo*. Considering that our HDL-c efflux assay involved the use of cell lines, our results might not reflect the real *in vivo* status. Another limitation was that the intervention's comparator was an OO with a moderately high polyphenol content (i.e., 86 mg/kg, phenolic content), which made it more difficult to observe between-group differences. Lastly, despite the inclusion of a washout period before the initiation of the intervention and between the intervention periods, there is no guarantee that any potential carry-over effect on the examined biomarkers was completely avoided. However, pairwise comparisons that examined potential carry-over effects were insignificant for all biomarkers.

3.10 Conclusions

The OLIVAUS study examined the effect of OO polyphenols on HDL-c efflux capacity and serum lipids in healthy Australian adults. No significant differences between the two OO treatment arms were observed in any of the examined outcomes. A non-significant increase in serum HDL-c efflux was observed within both the LPOO and extra virgin HPOO treatment arms after consumption of the two kinds of OO for 3 weeks each.

Furthermore, a significant increase in circulating HDL-c was observed within both treatment arms. Considering that HDL functionality has been found to be a better anti-atherogenic marker than circulating HDL cholesterol, CVD prevention strategies should focus on increasing not only HDL quantity but also HDL functionality. However, the non-significant findings of our study indicate that additional research is warranted to further understand the effect of OO phenolic compounds of different concentrations, on HDL mediated cholesterol efflux pathways, especially in multi-ethnic populations with different food habits, such as Australians.

3.11 Supplementary material

Data related to food groups high in phenolic content (i.e., whole grain cereals, fruits, vegetables, legumes, nuts/seeds, soy products, oils, fruit juices, alcoholic drinks and coffee) that participants consumed during the intervention, were extracted from FoodWorks nutritional analysis software. We analyzed whether there were any significant within-group changes or between-group differences in the intake of these food groups from baseline to follow-up. Phenol-explorer was used to calculate the phenolic content of the above-mentioned food groups and therefore to determine their inclusion in the statistical analyses (Supplementary Table S1). Results derived from ITT analyses demonstrated no significant between group-differences or within group changes in the intake of the abovementioned food items/beverages from baseline to follow-up. However, a significant increase in oil intake was observed within both treatment arms as expected due to the increased intake of the intervention oils (Supplementary Table S2).

Table S1: Food groups high in phenolic content (Phenol Explorer)

Food Groups	Phenolic Content (mg/100 mL)*
Grains	
Whole grains	167.24
Refined grains	49.18
Vegetables	
Red/orange vegetables	260.71
Green vegetables	129.58
Other vegetables (white, yellow, pink, black, purple)	50.19
Fruits	
Citrus/berries	16.51
Legumes	
Lentils, chickpeas, beans	114.92
Nuts	
Almonds, peanuts, walnuts, macadamia, pines, pistachio	44.83
Oils	
Cereal, vegetable, nut, seed	21.1
Beverages	
Coffee	14.85
Fruit Juice	6.98
Tea Infusions	3.14
Alcoholic drinks (wine, beer, spirits)	1.01

*Phenolic content: flavonoids, lignans, polyphenols, other polyphenols, stilbenes

Table S2: Effect of Low Polyphenol Olive Oil (OO) vs. High Polyphenol Olive Oil (OO) on dietary intake (Food groups)

Dietary intake	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Grains (Serves)				
Low Polyphenol OO (n=50)	7.2 (0.5)	7.2 (0.5)	0.05 (-0.9 to 1.0)	0.811†
High Polyphenol OO (n=50)	7.5 (0.5)	7.4 (0.5)	-0.1 (-1.1 to 0.9)	
Treatment*effect p-value	0.581	0.796		
Refined Grains (Serves)				
Low Polyphenol OO (n=50)	4.1 (0.4)	4.1 (0.4)	0.0 (-0.9 to 0.8)	0.931†
High Polyphenol OO (n=50)	4.4 (0.4)	4.3 (0.4)	-0.1 (-0.9 to 0.7)	
Treatment*effect p-value	0.600	0.679		
Whole Grains (Serves)				
Low Polyphenol OO (n=50)	3.1 (0.3)	3.2 (0.4)	0.1 (-0.4 to 0.8)	0.063‡
High Polyphenol OO (n=50)	3.1 (0.3)	3.1 (0.4)	0.0 (-0.6 to 0.6)	
Treatment*effect p-value	0.392	0.462		
Fruits (Serves)				
Low Polyphenol OO (n=50)	1.3 (1.1)	1.4 (1.1)	0.1 (-0.2 to 0.3)	0.431‡
High Polyphenol OO (n=50)	1.6 (1.1)	1.5 (1.1)	-0.1 (-0.3 to 0.2)	
Treatment*effect p-value	0.219	0.642		
Citrus/melons/berries (Serves)				
Low Polyphenol OO (n=50)	0.3 (0.1)	0.3 (0.0)	0.0 (-0.1 to 0.1)	0.855‡
High Polyphenol OO (n=50)	0.3 (0.1)	0.3 (0.0)	0.0 (-0.1 to 0.1)	

Dietary intake	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Treatment*effect p-value	0.240	0.321		
Fruit juice (Serves)				
Low Polyphenol OO (n=50)	0.1 (0.0)	0.1 (0.0)	0.0 (-0.1 to 0.1)	0.967[‡]
High Polyphenol OO (n=50)	0.1 (0.0)	0.2 (0.0)	0.1 (-0.1 to 0.2)	
Treatment*effect p-value	0.372	0.399		
Vegetables (Serves)				
Low Polyphenol OO (n=50)	4.1 (0.3)	4.2 (0.5)	0.1 (-1.0 to 1.2)	0.635[‡]
High Polyphenol OO (n=50)	4.1 (0.5)	4.8 (0.5)	0.7 (-0.4 to 1.8)	
Treatment*effect p-value	0.928	0.610		
Dark Green Vegetables (Serves)				
Low Polyphenol OO (n=50)	0.5 (0.1)	0.7 (0.2)	0.2 (-0.2 to 0.6)	0.394[‡]
High Polyphenol OO (n=50)	0.5 (0.1)	0.9 (0.2)	**0.4 (0.0 to 0.8)**	
Treatment*effect p-value	0.485	0.686		
Red/orange vegetables (Serves)				
Low Polyphenol OO (n=50)	1.3 (0.2)	1.2 (0.2)	-0.1 (-0.6 TO 0.4)	0.103[‡]
High Polyphenol OO (n=50)	1.2 (0.2)	1.6 (0.2)	0.4 (-0.1 TO 0.8)	
Treatment*effect p-value	0.697	0.078		
Legumes (Serves)				
Low Polyphenol OO (n=50)	0.5 (0.2)	0.5 (0.2)	0.0 (-0.3 to 0.4)	0.544[‡]
High Polyphenol OO (n=50)	0.5 (0.1)	0.5 (0.1)	-0.1 (-0.4 to 0.3)	

Dietary intake	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Treatment*effect p-value	0.719	0.739		
Nuts/Seeds (Serves)				
Low Polyphenol OO (n=50)	0.6 (0.1)	0.6 (0.1)	0.0 (-0.2 to 0.3)	0.333[‡]
High Polyphenol OO (n=50)	0.7 (0.1)	0.7 (0.1)	0.0 (-0.3 to 0.2)	
Treatment*effect p-value	0.321	0.992		
Soy Products (Serves)				
Low Polyphenol OO (n=50)	0.1 (0.1)	0.1 (0.0)	0.0 (-0.1 to 0.1)	0.444[‡]
High Polyphenol OO (n=50)	0.1 (0.1)	0.1 (0.0)	0.0 (-0.1 to 0.1)	
Treatment*effect p-value	0.259	0.797		
Oil (tsp)				
Low Polyphenol OO (n=50)	9.1 (0.6)	19.7 (0.7)	**10.6 (9.3 to 12.0)**	0.589[†]
High Polyphenol OO (n=50)	10.0 (0.7)	20.1 (0.7)	**10.1 (8.8 to 11.4)**	
Treatment*effect p-value	0.337	0.703		
Alcoholic drinks (stand. drinks)				
Low Polyphenol OO (n=50)	1.1 (0.4)	0.5 (0.2)	-0.5 (-1.4 to 0.4)	0.774[‡]
High Polyphenol OO (n=50)	0.6 (0.4)	0.8 (0.2)	0.2 (-0.7 to 1.0)	
Treatment*effect p-value	0.928	0.840		
Caffeine intake (mg/day)				
Low Polyphenol OO (n=50)	199.4 (54.1)	182.0 (35.6)	-17.4 (-122.3 to 87.6)	0.612[†]
High Polyphenol OO (n=50)	242.8 (56.0)	186.7 (36.8)	-56.1 (-164.6 to 52.5)	

Dietary intake	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Treatment*effect p-value	0.578	0.926		

All statistical analyses were adjusted for gender and age. Results in bold indicate P <0.05, therefore statistically significant. The normality of the distribution of each food intake variable was examined with the Kolmogorov-Smirnov test.

[†]P-values for testing between-group differences in normally distributed data were derived from repeated measures ANOVA.

[‡] P-values for testing between-group differences in non-normally distributed data were derived from the non-parametric Friedman test for repeated measures.

3.12   References

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Chapter 4: Antioxidant and anti-inflammatory effect of high polyphenol extra virgin olive oil

This chapter reports the findings in relation to two of the secondary outcomes of the OLIVAUS study; namely oxidative status and inflammation.

After a brief introduction, the chapter content is an exact copy of the manuscript entitled: *"Extra Virgin Olive Oil high in polyphenols improves antioxidant status in adults. A double-blind, randomized, controlled, cross-over study (OLIVAUS)"*, which was published in 2021 in European Journal of Nutrition (Impact Factor, 5.614). The article has been reproduced with permission from Springer Nature and reformatted for thesis presentation. A full copy of the publication can be found in the Appendix K.

Contributions:

Conceptualization/study design, C.I., E.S.G., C.J.T., and W.M.; investigation, K.S.; data curation, K.S. and G.M.; statistical analysis, K.S., L.P and G.M.; writing—original draft preparation, K.S.; review and editing, all authors; supervision, G.M., C.J.T. and C.I.; project administration, K.S., E.S.G., J.C.W. and W.M.; funding acquisition, C.I., E.S.G., W.M., H.L.M., and C.J.T. All authors have read and agreed to the published version of the manuscript.

4.1 Chapter overview

Chronic vascular inflammation is now well accepted as a key contributor to the pathophysiology of cardiovascular disease (CVD). Moreover, oxidative stress and inflammation are closely related to endothelial dysfunction, which is a determinant in the development of atherosclerosis and an early predictor for cardiovascular events.

There is strong evidence supporting the relationship between diet and cardiovascular health. Specifically, numerous Mediterranean diet (MedDiet) intervention studies have linked the consumption of phenolic compounds found in several foods (i.e., olive oil) with improvements in cardiovascular risk factors including oxidative stress and inflammation. For instance, results from a large dietary intervention trial, the PREDIMED study, support that optimal polyphenol intake or specific MedDiet food sources of polyphenols may reduce the risk of all-cause mortality. However, the majority of these studies have been conducted with Mediterranean populations for whom MedDiet is the prevalent dietary pattern. Furthermore, there is currently conjecture whether many of the olive oil-in specific-attributed benefits may in fact be mediated by other constituents of this diet with antioxidant and anti-inflammatory properties. These issues highlight the need for further research to understand the contribution of olive oil (OO) polyphenols, alone, to improved cardiovascular outcomes in multiethnic populations with different habitual dietary patterns to Mediterranean populations.

In view of the potential antioxidant and anti-inflammatory benefits of OO polyphenols, the following paper provides results from the effect of two kinds of OO differing in their phenolic content, on markers of oxidative status (oxidised low-density lipoprotein and total antioxidant capacity) and inflammation (high sensitivity C-reactive protein) in healthy Australian adults.

4.2 Publication details

Extra Virgin Olive Oil high in polyphenols improves antioxidant status in adults. A double-blind, randomized, controlled, cross-over study (OLIVAUS)

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4.3 Abstract

Purpose: Olive oil polyphenols have been associated with cardiovascular health benefits. This study examined the antioxidant and anti-inflammatory effect of extra-virgin high polyphenol olive oil (HPOO) vs. low polyphenol olive oil (LPOO) in healthy Australian adults.

Methods: In a double-blind cross-over trial, 50 participants (aged 38.5±13.9 years, 66% females) were randomized to consume 60 mL/day of HPOO (320 mg/kg polyphenols) or LPOO (86 mg/kg polyphenols) for three weeks. Following a 2-week wash-out period, participants crossed-over to the alternate treatment. Plasma oxidized low-density lipoprotein (ox-LDL), total antioxidant capacity (TAC), high-sensitivity C-reactive protein (hs-CRP) and anthropometrics were measured at baseline and follow-up.

Results: Fourty-three participants completed the study. Although there were no significant differences between treatments in the total sample, plasma ox-LDL decreased by 6.5 mU/mL (95% CI -12.4 to -0.5) and TAC increased by 0.03 mM (95% CI 0.006 to 0.05) only in the HPOO arm. Stratified analyses were also performed by cardiovascular disease risk status defined by abdominal obesity (WC > 94cm in males, > 80cm in females) or inflammation (hs-CRP > 1 mg/L). In the subgroup with abdominal obesity, ox-LDL decreased by 13.5 mU/mL (95% CI -23.5 to -3.6) and TAC increased by 0.04 mM (95% CI 0.006 to 0.07) only after HPOO consumption. In the subgroup with inflammation, hs-CRP decreased by 1.9 mg/L (95% CI -3.7 to -0.1) only in the HPOO arm.

Conclusions: Although there were no significant differences between treatments, the changes observed after HPOO consumption demonstrate the antioxidant and anti-

inflammatory effect of this oil, which is more pronounced in adults with high cardiometabolic risk. (Clinical Trial Registration: ACTRN12618000706279).

4.4 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, accounting for 21% of deaths in 2017 ("Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017," 2018). According to the latest Australian data, a similarly high proportion of mortality (26% of all deaths) was attributed to CVD, in 2017-2018 (ABS, 2018) . Factors such as oxidative stress and chronic vascular inflammation are closely related to endothelial dysfunction, which is a determinant in the development of atherosclerosis and an early predictor for cardiovascular events (Steven et al., 2019). Both oxidative stress and inflammation may cause injury to endothelial cells, promoting a pro-inflammatory response, as evidenced by the increased expression of endothelial dysfunction markers such as adhesion molecules and cytokines (e.g., Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)), with the latter being responsible for the secretion of C-reactive protein (CRP) (Siti et al., 2015). A large body of evidence has established that oxidized low density lipoprotein (ox-LDL) is a highly immunogenic particle which plays a major role in the initiation and progression of atheromatic plaque formation within the arterial wall, and is therefore considered as a hallmark in the pathogenesis of atherosclerosis (Fitó et al., 2005; Gao et al., 2017; Ishigaki et al., 2009). However, the oxidative damage process can be interrupted by the presence of various antioxidants (endogenous/exogenous and antioxidant enzymes) (Sies, 2007). In this regard, plasma total antioxidant capacity

(TAC), which reflects the overall antioxidant status *in vivo*, has been shown to be inversely associated with risks of chronic diseases including CVD (Wang et al., 2013).

Extensive evidence indicates that certain dietary patterns are cardioprotective (Collins C, 2017). One of the most globally researched and evidence-based dietary approaches for the prevention and management of chronic diseases is the traditional Mediterranean diet (MedDiet), which has been shown to improve cardiovascular risk factors, including markers of oxidative stress and inflammation (Estruch et al., 2006; Farràs et al., 2020; Sofi et al., 2010). The Mediterranean dietary pattern is a plant-based diet, rich in unsaturated fatty acids and antioxidants present in a variety of staple foods, such as fruits, vegetables, nuts, legumes, wholegrain cereals and extra virgin olive oil (EVOO) (Donini et al., 2015; Farràs et al., 2020). Olive oil (OO) is the principal source of dietary fat and a unique culinary component of the MedDiet. OO contains variable concentrations of polyphenols (also referred to as biophenols), that can be influenced by factors such as olive cultivar, region and soil, ripeness of the fruit, as well as the extraction procedure (Ghanbari et al., 2012). Notably, virgin OOs (VOOs), obtained by mechanical extraction methods (i.e. direct-press or centrifugation), preserve high phenolic content, whereas refined OOs (ROOs), subjected to both physical and/ or chemical processing, have a lower phenolic content (Sarapis et al., 2020)

Olive oil polyphenols have been associated with several cardiovascular health benefits, mainly due to their antioxidant and anti-inflammatory properties (George et al., 2019; Sarapis et al., 2020; Souza et al., 2017). In this context, the EUROLIVE Study, a European multicenter study, demonstrated that daily consumption of polyphenol-rich OO (366 mg/kg, phenolic content; 25 mL, daily dose) significantly reduced blood levels of ox-LDL (Castaner et al., 2011). Two recent meta-analyses, one conducted by our own group,

synthesized the available evidence from intervention studies examining high phenolic OO (HPOO) versus low phenolic OO (LPOO) consumption on various cardiovascular risk markers, and reported a beneficial effect of HPOO on reducing markers related to oxidative stress and inflammation (i.e., ox-LDL and CRP, respectively) compared to the LPOO treatment arm (George et al., 2019; Hohmann et al., 2015). In 2019, a network meta-analysis reported a dose–response relationship between higher intakes of OO phenolic compounds and lower ox-LDL values (Schwingshackl et al., 2019). However, most of the intervention studies included in the abovementioned meta-analyses have been conducted in Mediterranean populations that are accustomed to high OO intake, an observation that highlights the need for additional research in multiethnic populations with different habitual food consumption. Moreover, despite the evidence on the unique cardioprotective properties of polyphenols in EVOO, these are not currently recognized by CVD guidelines, thus underlining the need for additional evidence of higher methodological quality. Hence, the present study aimed to investigate the effect of daily consumption of (60 mL) raw extra virgin HPOO, compared to LPOO, for 3 weeks, on secondary outcome measures such as oxidative status and inflammation biomarkers in Australian adults with no previously diagnosed medical conditions.

4.5 Materials and Methods

4.5.1 Study population

The "OLIVAUS study" (Marx et al., 2019) was conducted according to the Guidelines for Good Clinical Practice (GCP), the guidelines laid down in the Declaration of Helsinki and the CONSORT reporting guidelines. All procedures involving human subjects were approved by the Human Research Ethics Committee of La Trobe University (HEC17-067) and written informed consent was obtained from all volunteers. The trial protocol has been

registered with the Australia New Zealand Clinical Trials Registry ACTRN12618000706279.

Participants were recruited in Melbourne, Australia via social media, La Trobe University staff email database advertising, word of mouth and study posters on display at the campus. A standardized screening procedure was followed to identify eligible participants, who were required to be within the age range of 18-75 years and a body mass index (BMI) 18.5-40 kg/m^2. Exclusion criteria included non-English speaking individuals, pregnant or lactating women, smokers, individuals on a special type of diet for medical reasons (e.g., gluten free for coeliac disease) and/or with a high habitual OO intake (>1 tablespoon/day). Exclusion also applied if individuals were taking vitamins or antioxidant supplements as part of a regular regime and were unable to discontinue their use for the duration of the trial (except for iron, calcium and Vitamin D). Finally, study subjects taking prescribed medication (e.g., antihypertensive agents, lipid-lowering drugs, nonsteroidal anti-inflammatory drugs) and those with diagnosed chronic diseases (diabetes, hyperlipidaemia, hypertension, inflammatory conditions), gut-related diseases or any other condition that could impair adherence, were also excluded. Additional details on the procedures followed to identify eligible study participants is provided elsewhere (Marx et al., 2019).

4.5.2 Study design and procedure

The OLIVAUS study was a double-blind, cross-over, randomised controlled trial (RCT) aiming to evaluate the effect of extra virgin HPOO compared to a commercially available LPOO on both novel i.e., high density lipoprotein cholesterol (HDL-c) efflux (primary outcome) and routinely measured CVD risk markers, including oxidative status and inflammation. The primary outcome (HDL-c efflux) will be reported elsewhere as this is not within the scope of this paper. Prior to the main study, a pilot study was conducted in

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order to test the feasibility of the study protocol and the data collection tools (Sarapis et al., 2019). Enrolled participants were randomly assigned in a 1:1 ratio, to one of the two treatment arms, i.e., extra virgin HPOO or LPOO. The randomization into one of the two treatment arms was performed in blocks of six using a computerized random number generator in excel software. An independent senior researcher not otherwise involved in the study generated the sequence.

Study participants were requested to consume a daily dose of 60 mL of either type of raw OO over two intervention periods of 3 weeks each, in conjunction with their habitual diet. The two types of OO varied only in their phenolic content, (320 mg/kg in HPOO vs. 86 mg/kg in LPOO) but did not differ with respect to their other nutrient composition, including fatty acid profile. Two washout periods, of 2 weeks each, during which study participants were instructed to avoid olives and OO consumption, preceded the first and the second intervention periods of OO administration. We chose a 2-week washout period as this was sufficient to eliminate the carry-over effect of OO polyphenols between interventions, considering the short half-life of OO's phenolic compounds (Miró-Casas et al., 2001). In addition, the intervention in the present study was designed with a daily dose of 60 mL OO, which reflects the habitual intake in Mediterranean populations where the cardioprotective benefits of virgin OO have previously been reported (George et al., 2019; Hohmann et al., 2015; Schwingshackl et al., 2019).

Participants were provided with OO at the beginning of each intervention period. The OO was supplied in dark coloured glass containers to minimise phenolic content loss due to sunlight. To ensure blinding of the researchers to the OO type, each bottle was assigned a different code number that was concealed from study participants and research team members. The code was disclosed only after the completion of the statistical analyses. To

assess the level of adherence to the intervention, participants were instructed to return the containers at the end of each intervention period so that the daily amount of unconsumed OO could be measured and recorded. Study participants were also instructed to keep a written record of daily OO consumed during each intervention period using a provided log sheet. This information was collected by research team members after the end of each intervention period. Full details of the study protocol, including a comparison of the concentrations of total polyphenols and polyphenol subclasses in each of the two types of OOs, are provided elsewhere (Marx et al., 2019).

4.5.3   Measurements

4.5.3.1   *Socio-demographics, use of medication and dietary supplements*

Socio-demographic data were collected from eligible participants during a scheduled interview at our trial clinic room located at La Trobe University. Trained researchers conducted all interviews using a standardized questionnaire. Specifically, the socio-demographic data collected during this interview included age, gender, language(s) spoken at home, level of education, ethnicity and parental country of birth. Any medications and dietary supplements taken by the study participants were also recorded.

4.5.3.2   *Dietary intake*

A 3-day food diary was used to collect information on the dietary intake of study participants during two weekdays and one weekend day (preferably non-consecutive) at baseline and follow-up of each 3-week intervention period. Specifically, study participants were instructed to record details on their intake of food and beverages, including information on the quantity, type/brand and cooking methods of the consumed items. The level of detail required to be recorded in the diary, as well as additional strategies on how

to incorporate raw, uncooked OO in their habitual diet was provided to study participants at a pre-baseline meeting by a trained nutritionist. The completed food diaries were returned and checked by the research team members for potential wrong or missing entries during the scheduled interviews with the study participants. FoodWorks[®]9 software (Xyris Software Pty Ltd, Queensland, Australia) was used for assessing dietary intake and extracting data on energy, micro- and macro-nutrients as well as the consumption of food groups and individual food items.

4.5.3.3 *Anthropometric measurements*

Anthropometric measurements were conducted four times during the study, i.e. at baseline and follow up of each intervention period. Body weight and standing height were measured with study participants in light clothing and barefoot, using a digital scale (WM203, Willawong QLD, Australia) to the closest 0.1 kg and a wall-mounted stadiometer (SE206, Seven Hills, NSW, Australia) to the nearest 0.1cm, respectively. Waist circumference (WC) was measured to the nearest 0.1cm, using a flexible steel tape calibrated in cm with mm graduations (Luftkin W606PM, Maryland, USA) directly over the skin at the umbilicus level. Body mass index (BMI) was calculated using Quetelet's equation (weight (kg) / height $(m)^2$). Using World Health Organization (WHO) cut-off points for BMI, study participants were classified as underweight (BMI <18.5 kg/m^2), normal weight (BMI 18.5-24.9 kg/m^2), overweight (BMI 25.0-29.9 kg/m^2) or obese (BMI $\geq$30 kg/m^2) (WHO, 1995). Furthermore, gender-specific WC cut-off points proposed by WHO were also used to categorise study participants for CVD risk: normal (WC <94 cm in men and <80 cm in women), High CVD risk (WC 94-102 cm in men and 80-88 cm in women) and very high CVD risk (WC >102 cm in men and 88 cm in women) (WHO, 2008).

4.5.3.4 *Biochemical analyses*

At baseline and follow up of each intervention period (in the morning following a 10-hour overnight fast), venous blood was collected from participants by a trained researcher at La Trobe University using standard venipuncture. Using the Centers for Disease Control/American Heart Association (CDC/AHA) cut-off points for CRP, study participants were classified as low cardiometabolic risk (CRP <1 mg/L), intermediate (CRP, 1-3 mg/L) and high cardiometabolic risk (CRP > 3 mg/L) (Pearson et al., 2003).

Collected venous blood was centrifuged (Hettic Rotina 420r, Massachusetts, USA) at 2350 rpm for 10min at 4^0C and the extracted plasma and/or serum was apportioned into aliquots of 500 μL each and stored at -80^0C until analysis. The TAC in serum plasma (collected in lithium heparin tubes) was measured using the Cell Biolabs, Inc. OxiSelect Total Antioxidant Capacity (TAC) Assay Kit (San Diego, CA, USA). This colorimetric assay, performed in 96 well microtiter plates, is based on the reduction of copper (II) to copper (I) by antioxidants in the plasma, such as uric acid. Upon reduction, the copper (I) ion reacts with a coupling chromogenic reagent. Absorbance was measured at 490nm using the Omega Fluostar Plate Reader. Absorbance values are proportional to the sample's total reductive capacity. Antioxidant capacity was determined by comparison to uric acid standards. All samples and standards were measured in duplicate. Results were expressed as mM Uric Acid Equivalents (UAE). The detection limit was 0.0039mM. Intra-assay coefficient of variation (CV%) was 2.3% (range 0.1-7.7%). Laboratory analysis for TAC assay was conducted by AL, KP and OF. Plasma ox-LDL concentrations (collected in K3 EDTA tubes) were measured using the Mercodia oxidized-LDL ELISA kit (Mercodia AB, Uppsala, Sweden). This ELISA assay is a solid phase two-site enzyme immunoassay in which 2 monoclonal antibodies are directed against separate antigenic determinants on the

oxidized apoB molecule. The detection limit was 0.6 mU/L. Intra-assay CV was 6.3% (range 5.5-7.3%). Finally, the Alinity c CRP Vario assay (SENTINEL CH, Milano, Italy) was used for the quantitative immunoturbidimetric determination of hs-CRP in human serum. The detection limit was 0.4 mg/L (intra-assay CV was 0.8%). Laboratory analysis for ox-LDL assay was performed by MG.

4.6 Sample size calculation

Power calculations indicated that a sample size of 40 was adequate to provide sufficient statistical power to detect a statistically significant between-group difference of 5% and a standard deviation (SD) of 11% in HDL-c efflux levels, which was the primary outcome of the OLIVAUS study, with power 80% and 5% level of significance (Hernaez et al., 2014). The total sample size was set at 50 study participants, in order to account for an attrition rate of 20%. A post hoc power calculation that was based on the available 50 participants, showed that this sample size provided more than 90% statistical power to detect significant between-group differences both in TAC and ox-LDL. However, the sample size of 50 participants did not provide enough statistical power in the case of hs-CRP, as the calculated statistical power was 31%.

4.7 Statistical analyses

All statistical analyses were conducted using the SPSS statistical software for Windows (IBM, version 24.0; IBM, Armonk, NY, USA). For all continuous variables, the Kolmogorov-Smirnov test was performed to examine the normality of their distribution. A general linear model, i.e. repeated-measures ANOVA (analysis of variance) was used to examine the between-group differences (treatment effect; i.e., extra virgin HPOO *vs* LPOO) of mean values at each time point of measurement, the within-group changes (time effect) from baseline to follow-up in each intervention arm, and the differences in the

changes from baseline to follow-up between the two intervention arms (Treatment x Time interaction effect). Both per protocol (PP) and intention–to-treat (ITT) analyses were performed. The PP analyses were conducted in study participants who had full data from baseline to follow-up in the first or the second intervention period. For the ITT analyses, multiple imputations were conducted in order to compensate for all missing values. Considering that the PP and the ITT analyses provided similar results, (i.e. mean values, mean changes and statistical significance), the results arising from the latter are presented in this article. In all statistical analyses, adjustments were made for gender and age. Statistical analyses were conducted on the total sample, but stratified analyses were also performed in order to examine the effect of the implemented treatments in participants with high CM risk, defined based on the WC and hs-CRP levels. Data is presented either as mean ± SD, as estimated marginal means and standard errors (SE) or as mean change and 95% Confidence Interval of change (CI) for continuous variables and as frequency (n) and percentage (%) for categorical ones. All reported P values are two-tailed, and the level of statistical significance is set at P< 0.05.

4.8   Results

Fifty volunteers (n=33 females, and n=17 males) were eligible and enrolled in the study from July 2018 through to October 2019. Seven participants discontinued the intervention, due to inability to comply (n=4) and for personal reasons (n=3) and therefore, there was complete data for 43 participants. Fig. 1 provides the study participant flow diagram.

4.8.1   Baseline characteristics of study participants

Table 4.1 presents the descriptive characteristics of study participants in terms of their socio-demographics, anthropometric and biochemical indices in the total sample (*n=50*) and by treatment arm. Study participants had a mean age of 38.5±13.9 years (total range 20

to 70 years) and mean education of 17.3±3.5 years. In addition, most study participants were females (66%) and born in Australia (70%). The mean BMI was 24.7±3.5 kg/m^2 and mean WC was 86.9±11.2 cm. In addition, 44% of study participants were overweight and 4% were obese. Based on their WC measurements, 16% of study participants had a high cardiometabolic risk and 24% had very high cardiometabolic risk. Regarding the oxidative status profile of study participants, their mean TAC and mean ox-LDL was 0.5±0.1 mM and 75.4±21.2 mU/mL, respectively. Furthermore, mean circulating hs-CRP for the cohort was 1.7±2.8 mg/L, while 70% of study participants were categorised as low risk (hs-CRP < 1 mg/L), 12% intermediate risk (hs-CRP, 1-3 mg/L) and 18% high risk (hs-CRP > 3 mg/L) based on their inflammation status. Finally, there were no significant differences between treatment arms in any of the abovementioned baseline characteristics, thus indicating homogeneity at baseline.

Figure 4.1   OLIVAUS study participant flow diagram

Table 4.1   Baseline descriptive characteristics of study participants

	Total sample (n=50)	Low Polyphenol OO (n=25)	High Polyphenol OO (n=25)	P-value*
Socio-demographics	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Age (years)	38.5 (13.9)	38.1 (14.8)	39.0 (13.2)	0.818
Education (years)	17.3 (3.5)	17.3 (4.2)	17.2 (2.8)	0.968
Gender	**(%)**	**(%)**	**(%)**	
Females	66.0	64	68	0.765
Males	34.0	36	32	
Country of Birth	**(%)**	**(%)**	**(%)**	
Australia, NZ, Pacific Islanders	70.0	68.0	72.0	0.321
Europe	10.0	4.0	16.0	
South America	8.0	12.0	4.0	
Middle East & Asia	12.0	16.0	8.0	
Anthropometrics	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Height (cm)	168.9 (9.6)	170.6 (10.4)	167.2 (8.6)	0.220
Weight (kg)	70.7 (12.8)	72.7 (13.7)	68.5 (11.8)	0.249

	Total sample (n=50)	Low Polyphenol OO (n=25)	High Polyphenol OO (n=25)	P-value*
BMI (kg/m^2)	24.7 (3.5)	24.9 (3.7)	24.4 (3.2)	0.617
Waist circumference (cm)	86.9 (11.2)	88.2 (11.9)	85.6 (10.6)	0.434
Weight status categories[†]	**(%)**	**(%)**	**(%)**	
Underweight	2.0	0.0	4.0	0.252
Normal weight	50.0	56.0	44.0	
Overweight	44.0	36.0	52.0	
Obese	4.0	8.0	0.0	
Waist circumference categories[‡]	**(%)**	**(%)**	**(%)**	
Normal	50.0	44.0	56.0	0.632
High risk	16.0	20.0	12.0	
Very high risk	34.0	36.0	32.0	
Biochemical indices	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
TAC (mM)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	0.826
ox-LDL (mU/mL)	75.4 (21.2)	70.5 (16.4)	80.4 (24.4)	0.099
hs-CRP (mg/L)	1.7 (2.8)	1.9 (2.6)	1.6 (3.1)	0.663
hs-CRP categories[§]	**(%)**	**(%)**	**(%)**	
Low risk	70.0	64.0	76.0	0.596

	Total sample (n=50)	Low Polyphenol OO (n=25)	High Polyphenol OO (n=25)	P-value*
Intermediate risk	12.0	16.0	8.0	
High risk	18.0	20.0	16.0	

* p-values for testing between-group differences in continuous variables were derived from the independent samples t-test. p-values for examining associations between categorical variables were derived from the Chi-square test; SD, standard deviation; OO, olive oil; NZ, New Zealand; BMI, body mass index; TAC, total antioxidant capacity; ox-LDL, oxidized low density lipoprotein; hs-CRP, high sensitivity C-reactive protein.

[†] Weight status categories: Underweight, BMI<18.5 kg/m^2; Normal weight, $18.5 \le$ BMI < 25 kg/m^2; Overweight, $25 \le$ BMI < 30 kg/m^2; Obese, BMI $\ge$ 30 kg/m^2.

[‡] Waist circumference categories: Normal, WC <80 cm in women & <94 cm in men; High risk, 80-88 cm in women and 94-102 cm in men; Very high risk: WC> 88 cm in women and > 102 cm in men.

[§] hs-CRP categories: Low risk, hs-CRP <1mg/L; Intermediate risk, 1-3 mg/L; High risk, hs-CRP > 3   mg/L

4.8.2 Effect of LPOO and HPOO on dietary intake

The changes observed in dietary energy and macronutrient intake from baseline to follow-up after each 3-week intervention, as well as the differences between treatment arms are presented elsewhere (Sarapis et al., 2020). In brief, the changes from baseline to follow-up were not significantly different between the two treatment arms. However, dietary energy intake increased significantly in participants following LPOO (by 1806.1 kJ/day, 95% CI: 1075.4 to 2536.8) and HPOO (by 1766.6 kJ/day, 95% CI: 1035.9 to 2497.3). Consumption of LPOO and HPOO also significantly increased intake of total fat (by 49.3 g/day, 95% CI: 41.1 to 57.4 and 46.0 g/day, 95% CI: 37.8 to 54.1, respectively), SFA (by 7.4 g/day, 95% CI: 4.0 to 10.8 and 6.5 g/day, 95% CI: 3.1 to 9.9, respectively), MUFA (by 36.8 g/day, 95% CI: 33.2 to 40.3 and by 35.1 g/day, 95% CI: 31.6 to 38.6, respectively) and PUFA (by 3.1 g/day, 95% CI: 1.0 to 5.1 and by 3.0 g/day, 95% CI: 1.0 to 5.1, respectively).

Table 4.2 demonstrates the changes observed in micronutrient intake from baseline to follow-up and the relevant differences between the two intervention arms. The changes were not significant between-groups, but there was a significant increase within groups in a-tocopherol intake (by 7.5 mg/day, 95% CI: 5.7 to 9.3 and by 7.0 mg/day, 95% CI: 5.2 to 8.8) and vitamin E intake (by 7.9 mg/day, 95% CI: 2.1 to 13.8 and by 12.4 mg/day, 95% CI: 6.6 to 18.2) after LPOO and HPOO consumption, respectively. No significant within-group changes or between-group differences were observed in the other examined macronutrients (protein, carbohydrates and dietary fibre) or micronutrients (β-carotene, vitamin C, selenium and zinc). The changes in intake of other food groups that are high in phenolic content (i.e., whole grain cereals, fruits, vegetables, legumes, nuts/seeds, soy products, oils, fruit juices, alcoholic drinks, tea and coffee) as determined by phenol

explorer (Neveu et al., 2010) were also examined. No significant within-group changes or between-group differences were observed in the daily intake of the abovementioned food and beverage items over the intervention period (data not shown).

4.8.3 Effect of LPOO and HPOO on anthropometrics

The changes observed in anthropometric indices from baseline to follow-up after each 3-week intervention, as well as the differences between the two treatment arms are presented elsewhere (Sarapis et al., 2020). In brief, no significant between-group differences were observed in any of the examined outcomes. A small but significant increase in body weight by 0.4 kg (95% CI: 0.2 to 0.7) was observed only in the LPOO treatment arm. No within-group changes were observed in any of the other outcomes (i.e., BMI and WC).

Table 4.2   Effect of low polyphenol OO vs. high polyphenol OO on mean changes in micronutrient intake.

	Baseline Mean (SEM)	Follow-up Mean (SEM)	Mean change (95% CI) (Time effect)	P-value (Treatment*time effect)
β-carotene intake (ug/day)				
Low Polyphenol OO (n=50)	4379.1 (552.0)	4733.9 (686.8)	354.8 (-828.1 to 1537.8)	0.368
High Polyphenol OO (n=50)	3869.4 (552.0)	4986.6 (686.8)	1117.2 (-65.8 to 2300.1)	
P-value (Treatment effect)	0.515	0.795		
Vitamin C intake (mg/day)				
Low Polyphenol OO (n=50)	122.3 (9.4)	114.6 (22.6)	-7.7 (-53.7 to 38.3)	0.196
High Polyphenol OO (n=50)	112.9 (9.4)	147.8 (22.6)	34.9 (-11.1 to 80.9)	
P-value (Treatment effect)	0.479	0.301		
α-tocopherol intake (mg/day)				
Low Polyphenol OO (n=50)	12.5 (0.9)	20.0 (0.8)	**7.5 (5.7 to 9.3)**	0.693
High Polyphenol OO (n=50)	13.2 (0.9)	20.2(0.8)	**7.0 (5.2 to 8.8)**	
P-value (Treatment effect)	0.580	0.864		
Vitamin E (mg/day)				
Low Polyphenol OO (n=50)	17.2 (2.0)	25.1 (2.9)	**7.9 (2.1 to 13.8)**	0.282
High Polyphenol OO (n=50)	16.0 (2.0)	28.4 (2.9)	**12.4 (6.6 to 18.2)**	
P-value (Treatment effect)	0.677	0.416		
Selenium intake (ug/day)				

	Baseline Mean (SEM)	Follow-up Mean (SEM)	Mean change (95% CI) (Time effect)	P-value (Treatment*time effect)
Low Polyphenol OO (n=50)	87.9 (5.1)	90.2 (6.5)	2.2 (-9.0 to 13.4)	0.409
High Polyphenol OO (n=50)	88.2 (5.1)	97.1 (6.5)	8.8 (-2.4 to 20.1)	
P-value (Treatment effect)	0.966	0.453		
Zinc intake (mg/day)				
Low Polyphenol OO (n=50)	10.7 (0.5)	11.5 (0.6)	0.821 (-0.3 to 2.0)	0.327
High Polyphenol OO (n=50)	11.5 (0.5)	11.6 (0.6)	0.018 (-1.1 to 1.2)	
P-value (Treatment effect)	0.243	0.981		

All statistical analyses were adjusted for gender and age. Results in bold indicate statistical significance (p <0.05). OO, olive oil; SEM, standard error of the mean; CI, confidence interval.

4.8.4 Effect of LPOO and HPOO on markers of oxidative status and inflammation

The effect of the two OO interventions on oxidative status markers is illustrated in Figure 4.2 and 4.3. The changes in ox-LDL and TAC from baseline to follow up were not significantly different between the two treatment arms. However, for the total sample following HPOO consumption, compared to baseline, plasma ox-LDL decreased significantly by 6.5 mU/mL (95% CI: -12.4 to -0.5) (Fig. 2), and TAC increased by 0.03 mM (95% CI: 0.006 to 0.05) (Fig. 3). In addition, stratified analyses of study participants with higher WC measures (>94 cm in males, >80 cm in females), thus indicating cardiometabolic risk, exhibited a total decrease in ox-LDL by 13.5 mU/mL (95% CI: -23.5 to -3.6) (Fig. 2) and an increase in TAC by 0.04 mM (95% CI: 0.006 to 0.07) (Fig. 3) after HPOO intake.
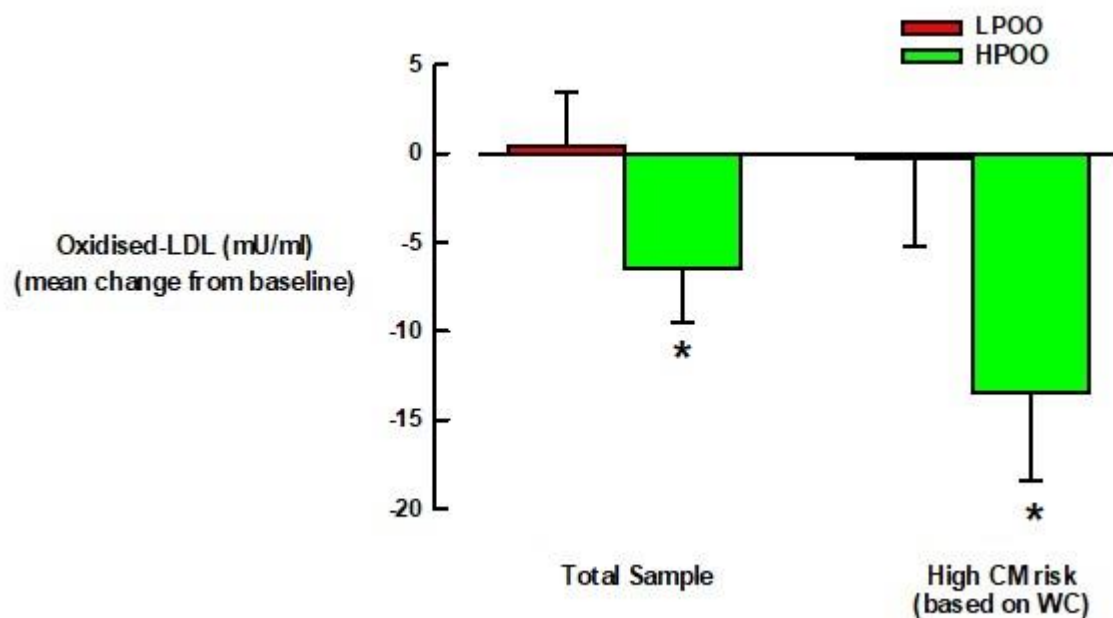
Figure 4.2   Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil

Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil (HPOO) and low polyphenol olive oil (LPOO) on oxidized-LDL (ox-LDL). Results are presented as mean changes ± standard errors from baseline to follow-up for the total sample (n=50) and for participants with high cardiometabolic risk (CM) (n=25, LPOO group; n=25, HPOO group) based on waist circumference (WC) measures (i.e., > 94 cm in males, > 80 cm in females). HPOO, 320mg/kg polyphenols; LPOO, 86 mg/kg polyphenols. *p< 0.05, significant within-group change from baseline to follow-up.
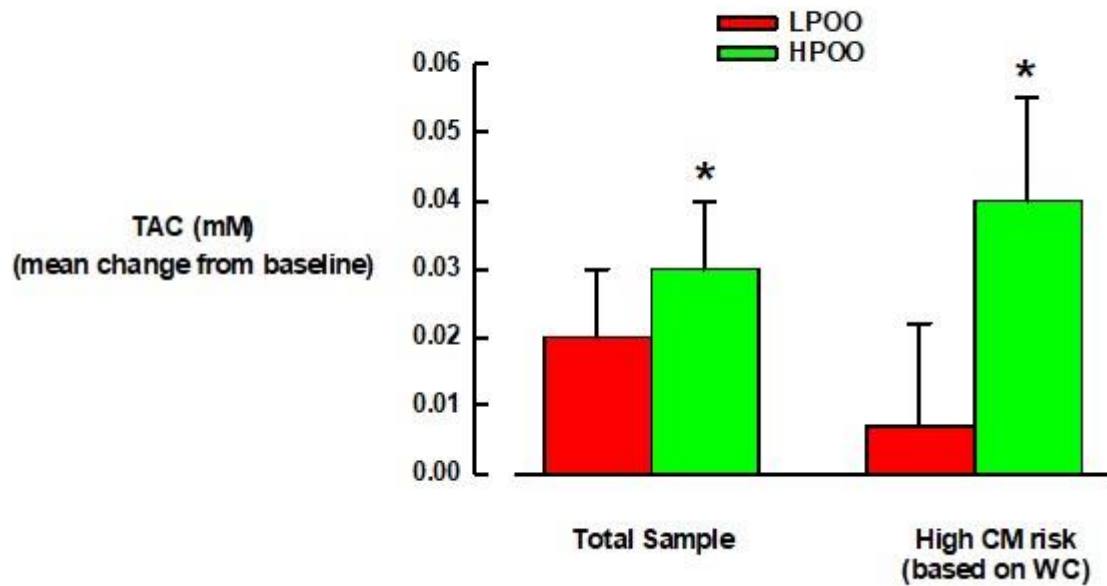
Figure 4.3 **Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil**

Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil (HPOO) and low polyphenol olive oil (LPOO) on total antioxidant capacity (TAC). Results are presented as mean change ± standard errors from baseline to follow-up for the total sample (n=48, LPOO group; n=46, HPOO group) and for participants with high cardiometabolic risk (CM) (n=23, LPOO group; n=22, HPOO group) based on waist circumference (WC) measures (i.e., > 94 cm in males, > 80 cm in females). HPOO, 320mg/kg polyphenols; LPOO; 86 mg/kg polyphenols. *p< 0.05, significant within-group change from baseline to follow-up.

No within-group changes or between-group differences were observed in circulating hs-CRP of the total sample from baseline to follow up (Fig. 4). However, stratified analyses of participants with intermediate-high cardiometabolic risk based on their inflammation status (hs-CRP >1 mg/L), demonstrated a significant decrease in hs-CRP by 1.9 mg/L (95% CI: -3.7 to -0.1) after HPOO consumption (Figure 4.4), but this change was not found to differ compared to the non-significant change observed in the LPOO treatment arm. No within-group changes or between-group differences were observed in hs-CRP levels after stratified analyses of study participants with higher WC measures (Figure 4.4).
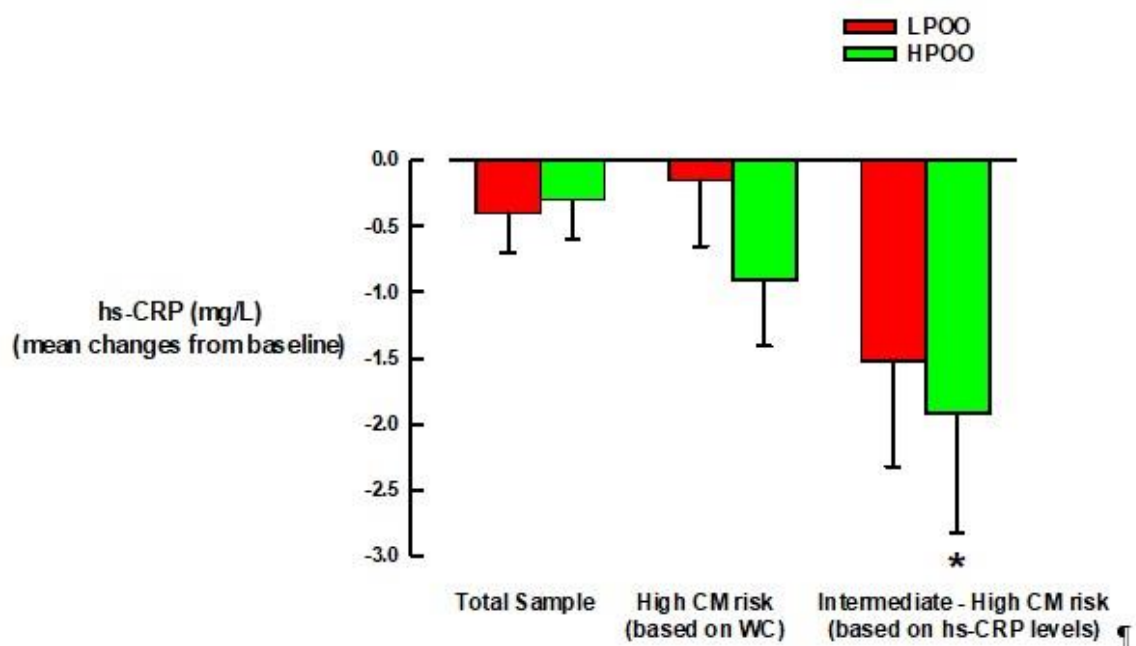
Figure 4.4   Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil

Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil (HPOO) and low polyphenol olive oil (LPOO) on high-sensitivity C-reactive protein (hs-CRP). Results are presented as mean change ± standard errors from baseline to follow-up for the total sample (n=50), participants with high cardiometabolic risk (CM) (n=25, LPOO

n=25, HPOO group) based on waist circumference (WC) measures (i.e., > 94 cm in males, > 80 cm in females;) and for participants with intermediate-high CM risk (n=16, LPOO group; n=14, HPOO group) based on hs-CRP levels (i.e. > 1 mg/L). HPOO, 320mg/kg polyphenols; LPOO; 86 mg/kg polyphenols. *p< 0.05 significant within-group change from baseline to follow-up.

4.8.5 Adherence to Treatment

Adherence to treatment was overall high in the study cohort and did not differ significantly between the two treatment arms as shown in Table S1. Based on the OO volume returned by participants after each intervention period, adherence was found to be 92% for both the LPOO and HPOO treatment arm after the first intervention period and 92% for the LPOO and 90% for the HPOO treatment arm after the second intervention period.

4.9 Discussion

The present double blind, cross-over, randomized controlled trial investigated the effect of daily consumption of 60 mL raw extra virgin HPOO (320 mg/kg, phenolic content) in comparison with LPOO (86 mg/kg, phenolic content), each for 3 weeks, on circulating biomarkers of oxidative status and inflammation in Australian adults with no previously diagnosed medical condition. No between-group differences were observed in any of the examined biomarkers between the two treatment arms. However, HPOO consumption induced a significant increase in TAC and a reduction in plasma ox-LDL in the total cohort. Furthermore, when the statistical analyses were stratified to include only participants with a high cardiometabolic risk based on their WC measures, HPOO consumption induced an even more pronounced increase in TAC and a greater reduction in plasma ox-LDL. A significant decrease was also observed in circulating hs-CRP for those participants with intermediate-high cardiometabolic risk based on their inflammation status. No significant within-group changes were observed after LPOO consumption.

The changes observed in ox-LDL (by 6.5 mU/mL and 13.5 mU/mL in the total sample and high cardiometabolic risk subsample based on WC measures, respectively) in the present study are comparable to the changes reported in previous clinical trials which have examined the effect of VOO intake on oxidative status markers. In this context, Marrugat et al, (Marrugat et al., 2004) demonstrated that a 3-week intervention of three OOs (daily dose of 25 mL) that differed only in their phenolic content (ROO, 0 mg/kg phenolic content; common OO, 68 mg/kg phenolic content; VOO, 150 mg/kg phenolic content) demonstrated a significant decrease in ox-LDL by 14.5 mU/mL after VOO consumption in healthy adults. In another RCT, Hernaez et al, (Hernáez et al., 2015) reported that the daily consumption of 25 mL raw HPOO (366 mg/kg, phenolic content) for 3-weeks led to a significant decrease in ox-LDL by 3.2 mU/mL compared to LPOO (2.7 mg/kg, phenolic content) in healthy men. Furthermore, Moreno-Luna et al, (Moreno-Luna et al., 2012) found that daily consumption of 60 mL HPOO (564 mg/kg, phenolic content) for 8 weeks induced a significant reduction in ox-LDL compared to ROO (polyphenol free), in young women with mild hypertension.

The differences in the daily doses of the treatment OOs, their phenolic content and the duration of the intervention between the aforementioned clinical trials and our study provides an explanation of the different reported effects (Schwingshackl et al., 2019; Tsartsou et al., 2019). However, in most of these previous clinical trials, OO was consumed as part of the MedDiet, further suggesting that many of the OO attributed benefits might be also related to other foods in this diet that are considered good sources of nutrients with antioxidant properties. In this context, a recent network meta-analysis reported that consumption of OO with a moderate polyphenol concentration (~60 mg/kg) as part of the MedDiet is sufficient to induce antioxidant effects (Tsartsou et al., 2019). In the present

study, however, this was not the case, since OO was not consumed as part of the Mediterranean dietary pattern. This in conjunction with the cross-over study design enabled us to isolate the effects of a single ingredient in the MedDiet, i.e., EVOO.

The mechanisms by which OO polyphenols can protect LDL from oxidation may be reflected in their ability to counteract both metal- and radical-dependent oxidation and to act as chain-breaking antioxidants for peroxidation (Fitó et al., 2000). In addition to the potential direct antioxidant properties of OO polyphenols, these have been found to preserve the activity of other antioxidants present in OO (i.e. tocopherols), thus increasing the resistance of LDL against oxidation (Covas et al., 2000; Visioli et al., 1995).Considering that antioxidants exert their effect against oxidation synergistically, no single antioxidant could represent overall antioxidant status *in vivo*. For this reason, several biomarkers have been used to reflect the human body's antioxidant status, including plasma TAC (Wang et al., 2013). Previous studies have found low plasma levels of TAC in individuals with coronary heart disease (Nojiri et al., 2001), thus providing some initial indications on the link between overall antioxidant status and CVD risk (Wang et al., 2012). In the present study, plasma TAC increased significantly after HPOO consumption by 0.03 mM in the total sample and by 0.04 mM in participants with high cardiometabolic risk based on their WC measures. This finding provides further evidence of the ability of OO polyphenols to enhance endogenous antioxidant status. Similar to our results, a single-arm clinical trial with healthy adults found that daily consumption of 50 mL EVOO rich in phenolic compounds (510 mg/kg, phenolic content) for 4 weeks increased plasma TAC levels by 0.3 mM (Oliveras-López et al., 2014). Furthermore, the VOLOS study showed that 7 weeks of daily consumption of 40 mL EVOO containing 166 mg/kg polyphenols, increased TAC significantly by 0.08 mM compared to LPOO (2 mg/kg, phenolic content,)

in adults with mild dyslipidemia (Visioli et al., 2005).

In addition to oxidation, increased blood levels of inflammatory molecules, such as CRP and various inflammatory cytokines, have been closely linked to endothelial dysfunction and serve as additional risk predictors for CVD (Wongwarawipat et al., 2018). It is recognized that foods rich in phenolic compounds, including EVOO, have cardioprotective effects due to their anti-inflammatory properties (George et al., 2019; Schwingshackl et al., 2019; Tsartsou et al., 2019). The mechanisms by which polyphenols can exert their anti-inflammatory effect seems to be mediated via their regulatory role in the production and secretion of a wide variety of pro-inflammatory molecules (Rosillo et al., 2016). In the present study, HPOO consumption was found to significantly reduce hs-CRP levels by 1.9 mg/L in adults classified as intermediate-high cardiometabolic risk based on their hs-CRP levels (i.e., >1 mg/L). In another study, Moreno-Luna et al, (Moreno-Luna et al., 2012) demonstrated that women with mild hypertension had reduced CRP concentrations (also by 1.9 mg/L) after daily intake of 60 mL HPOO (564 mg/kg, phenolic content) for 8 weeks compared to ROO (polyphenol free). Furthermore, Fito et al, (Fitó et al., 2008) reported reduced CRP levels by 1.1 mg/L in stable coronary disease patients after 3-weeks of 50 mL VOO (161 mg/kg, phenolic content) consumption in comparison with LPOO (14.67 mg/kg, phenolic content). On the other hand, a 4-week intervention that compared the daily intake of 25 mL EVOO (577 mg/kg, phenolic content) with ROO (polyphenols not detectable) did not produce any changes in CRP, IL-6 or TNF-a in individuals with type 2 diabetes and overweight (Santangelo et al., 2016).

In terms of dietary intake changes, in the current study, both LPOO and HPOO treatment equally increased intake of specific micro-nutrients (i.e., vitamin E, a-tocopherol, selenium, zinc) that have established antioxidant and/or anti-inflammatory properties (Wang et al.,

2013). This finding was expected due to the similar nutrient composition of the two treatment OOs, with the only exception being their phenolic content. The implication of these dietary intake changes is that the only dietary factor that can explain the significant and favorable within-group changes observed after extra virgin HPOO consumption was polyphenols, since their intake was 4-folds higher in the HPOO compared to the LPOO treatment arm (320 mg/kg *vs*. 86 mg/kg). Furthermore, the non-significant between-group differences with regards to the changes in ox-LDL, TAC and CRP can be explained by the phenolic content of the LPOO examined in the present study. In this regard, although the concentration of polyphenols in the LPOO was lower compared to extra virgin HPOO, it was still high enough to produce some clinically significant health benefits, also in synergy to the intake of the other important bioactive nutrients that are present in the intervention OOs. Moreover, the increased energy intake and body weight after the addition of 60 mL of the intervention oils in participants' daily diet, would be expected to lead to a non-beneficial effect of the test oils on the examined outcomes. However, this was not the case in our study, indicating a potential counterbalancing effect of OO polyphenols on weight gain, whilst confirming their antioxidant and anti-inflammatory activity.

The findings of the present study should be interpreted taking into consideration its strengths and limitations. The main strength is the study's randomized, double-blind cross-over design, which allows each participant to act as their own control by consuming both intervention OOs, thus reducing inter-individual variability. This design also enabled us to isolate the effects of OO polyphenols on the examined outcomes, since it allowed us to control for potential confounding effect caused by differences in the intake of other nutrients with antioxidant and/or anti-inflammatory properties. Another strength is that study participants remained in line with their habitual diet, hence allowing to directly assess

the benefits of OO consumption. One of the limitations of the current study is that although participant adherence to the intervention was overall high, measurements of compliance relied on self-reporting and were therefore subjective. Another limitation is that the present study was not adequately powered to detect significant changes in hs-CRP, considering that the study sample size was originally calculated on the basis of the expected differences only in the primary outcome (i.e., HDL-efflux). Lastly, despite the inclusion of a washout period before the initiation of the intervention and between the intervention periods, there is no guarantee that any potential carry-over effect on the examined biomarkers was completely avoided. However, pairwise comparisons that examined potential carry-over effects were insignificant for all biomarkers.

4.10 Conclusions

The OLIVAUS study examined the effect of OO polyphenols on oxidative status and inflammation biomarkers in Australian adults. There were no significant differences between the two OO treatment arms in any of the examined outcomes. There was however a significant within-group reduction in plasma ox-LDL and hs-CRP and an increase in plasma TAC after the daily consumption of 60 mL of extra virgin HPOO for 3-weeks, which was more pronounced in individuals with increased WC measures and hs-CRP levels, thus indicating elevated metabolic risk, at pre-intervention. Considering the strong link of oxidative stress and inflammation with CVD risk, these findings highlight the preventive potential of extra virgin HPOO in healthy individuals and especially in those with a higher risk for CVD and therefore most in need for primary prevention initiatives. Finally, considering that all previous intervention studies were conducted in Mediterranean populations, the results of our study provide new evidence for a potentially widely accessible dietary intervention that can be also incorporated in the multicultural Australian

context as a means for the primary prevention of CVD.

4.11 Supplementary Material

A summary of the OO volume returned by study participants following LPOO and HPOO consumption is presented in Supplementary Table S3. Overall, the actual remaining volume of OO was not significantly different between the two treatment arms. After the first intervention period, the remaining mean volume of LPOO and HPOO was 106.4±152.2 mL and 100.4± 123.6 mL, respectively. The remaining mean volume of LPOO was 105.9±133.2 mL compared to 123.1±164.7 mL of HPOO after the second intervention period.

Based on the measured actual remaining OO, compliance was found to be 92% for both the LPOO and HPOO group after the first intervention period, while 92% for the LPOO group and 90% for the HPOO group after the second intervention period. Nevertheless, compliance was not found to differ significantly between the two groups.

Table S3. Summary of olive oil (OO) volume returned by study participants following the two diet interventions.

	Low polyphenol OO	High Polyphenol OO	
	Mean (SD)	Mean (SD)	P-value*
1st intervention			
Actual remaining OO (mL)	106.4 (152.2)	100.4 (123.6)	0.888
Compliance based on actual remaining (%)	92	92	
2nd Intervention			
Actual remaining OO (mL)	105.9 (133.2)	123.1 (164.7)	0.708
Compliance based on actual remaining (%)	92	90	

*P-values derived from the non-parametric Mann-Whitney test. SD, standard deviation; OO, olive oil. Low or high polyphenol OO (1260 ml, total volume) was supplied at the commencement of each 3-week intervention period.

4.12   References

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Chapter 5: The Effect of High Polyphenol Extra Virgin Olive Oil on Blood Pressure and Arterial Stiffness

This chapter reports the findings in relation to two of the secondary outcomes of the OLIVAUS study; namely blood pressure and arterial stiffness.

After a brief introduction, the chapter content is an exact copy of the manuscript entitled: *"The Effect of High Polyphenol Extra Virgin Olive Oil on Blood Pressure and Arterial Stiffness in Healthy Australian Adults: A Randomised Controlled, Cross-Over Study",* which was published in 2020 in *Nutrients* (*Impact Factor* 4.777). The published article has been reformatted for thesis presentation. A full copy of the publication can be found in the Appendix L.

This paper was recognized by the editors of *Nutrients* as the most notable article in the category – Nutritional Epidemiology - published between July to November 2020.

Contributions:

Conceptualization/study design, C.I., E.S.G., C.J.T., G.K., A.P. and W.M.; investigation, K.S. and J.H.; data curation, K.S. and G.M.; statistical analysis, K.S., G.M. and L.A.P.; supervision, G.M., C.J.T. and C.I.; project administration, K.S., E.S.G., J.C.W. and W.M.; funding acquisition, C.I., E.S.G.,W.M., H.L.M., C.J.T. and L.A.P. KS drafted the original manuscript. All authors provided intellectual input and critically reviewed the manuscript.

5.1 Chapter overview

Hypertension and arterial stiffness are considered major risk factors in the pathogenesis of cardiovascular disease (CVD). Numerous clinical and observational studies have shown that haemodynamic variables such as peripheral (brachial) and central (aortic) systolic and diastolic blood pressure (BP) are significant predictors of cardiovascular events (i.e., coronary artery disease, stroke, heart failure and atrial fibrillation). Furthermore, impaired viscoelasticity in the larger central arterial system significantly contributes to CVD and is positively associated with hypertension.

Undoubtedly, dietary choices affect health and wellbeing. Previous studies have reported that EVOO, the principal source of dietary fat in the Mediterranean diet (MedDiet) provides a cardioprotective effect through mediating improvements in cardiovascular risk factors, including BP; however, few studies have investigated the contribution of the polyphenol component of EVOO, in particular, to these improvements, thus indicating the need for further research. The present study is one of the first human trials to examine the effect of olive oil (OO)-derived polyphenols on central (aortic) BP and arterial stiffness by using non-invasive applanation tonometry.

The following paper provides results following a dietary intervention with two kinds of OO differing in their phenolic content on peripheral and central systolic and diastolic BP. The effect of the OO polyphenols on measures of arterial stiffness was also investigated and relevant results are presented in this paper.

5.2 Publication details

The Effect of High Polyphenol Extra Virgin Olive Oil on Blood Pressure and Arterial Stiffness in Healthy Australian Adults (OLIVAUS): a randomized, controlled, cross-over study

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5.3 Abstract

Extra virgin olive oil (EVOO) is suggested to be cardioprotective, partly due to its high phenolic content. We investigated the effect of extra-virgin high polyphenol olive oil (HPOO) versus low polyphenol olive oil (LPOO) on blood pressure (BP) and arterial stiffness in healthy Australian adults. In a double-blind, randomized, controlled cross-over trial, 50 participants (age 38.5±13.9 years, 66% female) were randomised to consume 60 mL/day of either HPOO (360 mg/kg polyphenols) or LPOO (86 mg/kg polyphenols) for three weeks. Following a two-week wash-out period, participants crossed-over to consume the alternate oil. Anthropometric data, peripheral BP, central BP and arterial stiffness was measured at baseline and follow-up. No significant differences were observed between treatments. However, a significant decrease in peripheral and central systolic BP (SBP) by 2.5 mmHg (95% C.I: -4.7 to -0.3) and 2.7 mmHg (95% C.I: -4.7 to -0.6) respectively, was observed after HPOO consumption. Neither olive oil changed diastolic BP (DBP) or measures of arterial stiffness. The reductions in SBP after HPOO consumption provides evidence for a potentially widely accessible dietary intervention to prevent cardiovascular disease in the Australian sociocultural context. Longer intervention studies and/or higher doses of EVOO polyphenols are warranted to elucidate the potential effect on DBP and arterial stiffness.

Keywords: olive oil; extra virgin olive oil; polyphenols; cardiovascular disease; blood pressure; hypertension; arterial stiffness.

5.4 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity worldwide. Established risk factors such as hypertension, dyslipidaemia, diabetes and obesity contribute to 9.7 million annual deaths related to CVD globally (Tzoulaki et al., 2016). The most recent Australian data, collected between 2017 and 2018, indicates 27% of all deaths (43,477 deaths) were attributed to CVD (ABS, 2018). A similarly high proportion of Australian adults (~34%) have diagnosed hypertension (AIHW, 2003). Previous studies have indicated that changes in peripheral and central hemodynamics such as in peripheral (brachial) and central (aortic) BP and pressure wave reflections contribute to the development of adverse cardiovascular events (Li et al., 2019). Moreover, stiffening of the central elastic arteries, such as the aorta and the pulmonary arteries, is an accepted independent predictor of CVD risk and is positively associated with systolic hypertension (Shirwany & Zou, 2010; Zieman et al., 2005). Several surrogate markers reflecting vascular health are used in clinical practice. Pulse wave velocity (PWV), estimated by non-invasive applanation tonometry and pulse wave analysis, is considered the gold-standard marker of arterial stiffness (Van Bortel et al., 2012). Furthermore, systemic arterial wave reflections, as measured by augmentation index (AIx), provide additional clinical information on CVD, while assessment of central BP and pulse pressure (PP) provides further predictive value beyond the corresponding brachial BP ("Correction to: 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines," 2018; Kennedy et al., 2018; Shirwany & Zou, 2010).

Extensive evidence indicates that certain dietary patterns are cardioprotective (Collins C, 2017). The traditional Mediterranean diet (MedDiet), has been shown to improve CVD risk factors including lipidemic and glycaemic profile, markers of inflammation and oxidative stress (Estruch et al., 2006; Sofi et al., 2010). The MedDiet is plant-rich, with staple foods consisting of wholegrain cereals, vegetables, fresh fruit, seafood, legumes, nuts and red wine (Davis et al., 2017; Estruch et al., 2006). Previous studies have demonstrated that MedDiet food components improve vascular health. Moderate consumption of red wine has been found to reduce BP and improve arterial stiffness in healthy individuals (Mahmud & Feely, 2002) and patients with coronary artery disease (Karatzi et al., 2005; Papamichael et al., 2008). Furthermore, regular consumption of olive oil (OO), which is the principal source of dietary fat in the MedDiet, has demonstrated BP-lowering effects (Fito et al., 2005; Psaltopoulou et al., 2004).

The reported cardio-protective benefits of OO have been mostly attributed to the presence of variable concentrations of bioactive compounds, including polyphenols (also referred to as biophenols), mainly known for their anti-inflammatory and anti-oxidant properties (Estruch et al., 2006; George et al., 2019; Marx et al., 2019). One of the determinants of final OO polyphenol concentration is the oil extraction procedure (Ghanbari et al., 2012; Marx et al., 2019; Souza et al., 2017). In particular, extra virgin olive oil (EVOO) is obtained by mechanical extraction techniques under conditions that preserve high polyphenol concentrations, whereas refined OO is subject to both physical and/or chemical processing, which significantly lowers the phenolic content (Souza et al., 2017). Although there is some evidence linking dietary polyphenol intake, including those in virgin OOs, with decreased CVD risk (Godos et al., 2017; Moreno-Luna et al., 2012), this favourable effect of polyphenols is not currently taken into consideration by dietary guidelines, thus

indicating a need for further relevant evidence.

Our research team recently conducted a meta-analysis of the published literature to determine the effects of HPOO consumption compared with LPOO, on cardiovascular markers (George et al., 2019). This meta-analysis indicated that HPOO can improve outcomes related to cholesterol (total and high-density lipoprotein (HDL)) and oxidative stress (oxidized low-density lipoprotein (LDL) and malondialdehyde) compared to the LPOO treatment arm. However, no significant changes were observed with respect to SBP and DBP after either OO consumption (daily dose ranged between 25 and 75 mL), while none of the included studies reported measures of arterial stiffness. In 2019, a network meta-analysis reported that EVOO may reduce oxidized LDL (ox-LDL) and LDL cholesterol compared to ROO and LPOO, respectively, while a dose-response relationship was observed between higher intakes of OO phenolic compounds and lower SBP and ox-LDL values. As also stated in another recent review, most intervention studies investigating the effect of HPOO on CVD risk markers have been conducted in Mediterranean populations that have high habitual OO intake (Hohmann et al., 2015), thus highlighting the need for additional research on multiethnic populations with different habitual food cultures. Hence, the aim of the current study was to examine the effect of daily consumption of (60 mL) raw extra virgin HPOO compared to LPOO, for 3 weeks, on peripheral and central BP and arterial stiffness in Australian adults with no previously diagnosed medical condition.

5.5 Materials and Methods

5.5.1 Study population

The "OLIVAUS study" (Marx et al., 2019) was conducted according to the Guidelines for

Good Clinical Practice (GCP), the guidelines laid down in the Declaration of Helsinki and the CONSORT reporting guidelines. All procedures involving human subjects were approved by the Human Research Ethics Committee of La Trobe University (HEC17-067) and written informed consent was obtained from all volunteers. The trial protocol has been registered with the Australia New Zealand Clinical Trials Registry ACTRN12618000706279.

All participants were recruited in Melbourne, Australia via social media and La Trobe University email database advertising, word of mouth and posters on campus. A standardized screening procedure was followed in order to identify eligible participants, who were required to be within the age range of 18-75 years and a body mass index (BMI) 18.5-40 kg/m^2. Exclusion criteria included non-English speaking individuals, pregnant or lactating women, smokers, individuals on a special type of diet for medical reasons (e.g., gluten free for coeliac disease) and/or with a high habitual OO intake (>1 tablespoon/day). Exclusion also applied if individuals were taking vitamins or antioxidant supplements as part of a regular regime and were unable to discontinue their use for the duration of the trial (with the exception of iron, calcium and Vitamin D). Finally, study subjects taking prescribed medication (e.g., antihypertensive agents, lipid-lowering drugs, nonsteroidal ant-inflammatory drugs) and those with diagnosed chronic diseases (diabetes, hyperlipidaemia, hypertension, inflammatory conditions), gut-related diseases or any other condition that could impair adherence, were also excluded.

5.5.2 Study design and procedure

The OLIVAUS study was a double-blind, cross-over, randomised controlled trial (RCT) aiming to evaluate the effect of extra virgin HPOO consumption on CVD risk markers in

comparison with a commercially available OO which was low in polyphenols (LPOO). Prior to the main study, a pilot study was conducted with five study participants in order to test the feasibility of the study protocol and the data collection tools (Sarapis et al., 2019a, 2019b). Enrolled participants were randomly assigned in a 1:1 ratio, to one of two treatment arms, i.e. Group (1) extra virgin HPOO / LPOO or Group (2) LPOO / extra virgin HPOO, using the block-randomization method of a software program for sequence. Blocks of 6 participants were generated by a senior researcher, who was not directly involved in the participant recruitment or data collection phase. Allocation of each participant was emailed to the research team at the commencement of the study, by a researcher who was not involved in any participant contact.

Study participants were requested to consume a daily dose of 60 mL of either type of raw OO over 2 intervention periods of 3 weeks each, in conjunction with their habitual diet. The two types of OO varied only in their phenolic content, (i.e. 360 mg/kg in HPOO vs. 86 mg/kg in LPOO) but did not differ with respect to the rest of their nutrient composition, including their fatty acid profile. Two washout periods, of 2 weeks each, during which study participants were instructed to avoid olives and OO consumption, preceded the first and the second intervention periods of OO administration. The intervention in the present study was designed with a daily dose of 60 mL OO, which reflects the habitual intake in populations where the cardioprotective benefits of virgin OO have been previously reported (George et al., 2019; Hohmann et al., 2015; Schwingshackl et al., 2019).

Participants were provided with OO bottles at the beginning of each intervention period. The OOs were supplied in dark coloured glass containers to minimise phenolic content loss due to sunlight. To ensure blinding of the researchers to the OO type, each bottle was

assigned a different code number that was concealed from study participants and research team members. This was disclosed only after the completion of the statistical analyses. To assess the level of adherence to the intervention, participants were instructed to return the containers at the end of each intervention period so that the daily amount of unconsumed OO could be measured and recorded. Study participants were also instructed to keep a written record of daily OO consumed during each intervention period using a checklist provided to them. This information was recorded by research team members after the end of each intervention period. Full details of the study protocol, including a comparison of the concentrations of total polyphenols and polyphenol subclasses in each of the two types of OOs, are provided elsewhere (Marx et al., 2019).

5.5.3 Measurements

5.5.3.1 Socio-demographics, use of medication and dietary supplements

Socio-demographic data were collected from eligible participants during a scheduled interview at our trial clinic room located at La Trobe University. Trained researchers conducted all interviews using a standardized questionnaire. Specifically, the socio-demographic data collected during this interview included age, gender, language(s) spoken at home, level of education, ethnicity and parental country of birth. Any medications and dietary supplements taken by the study participants were also recorded.

5.5.3.2 Dietary intake

A 3-day food diary was used to collect information on the dietary intake of study participants during two weekdays and one weekend day (preferably non-consecutive) at baseline and the follow up of each intervention period. Specifically, study participants were instructed to record details on their intake of food and beverages, including information on

the quantity, type/brand and cooking methods of the consumed items. The level of detail required to be recorded in the diary, as well as additional strategies on how to incorporate raw, uncooked OO in their habitual diet was provided to study participants at a pre-baseline meeting by a trained nutritionist. The completed food diaries were returned and checked by the research team members for potential wrong or missing entries during the scheduled interviews with the study participants. All dietary intake data was analyzed for energy, macro- and micronutrients' content using FoodWorks®9 software (Xyris Software Pty Ltd, Queensland, Australia).

5.5.3.3 *Physical Activity*

Physical activity (PA) was assessed using the Active Australia Survey (AAS) questionnaire (Australian Institute of Health and Welfare, 2003), a tool that has been validated in the Australian population. This questionnaire is designed to assess participation in a range of leisure-time physical activities of light, moderate and vigorous intensity. It consists of eight questions, which assess the number of sessions and total weekly time (hours and/or minutes) spent for each activity type. Study participants were required to complete and submit the AAS questionnaire during the week preceding the interviews at the first baseline and at the last follow-up meeting. The amount of time (in minutes per day) that study participants were engaged in physical activity of different intensity was calculated and used for data analysis.

5.5.3.4 *Anthropometric measurements*

Anthropometric measurements were conducted four times during the study, i.e. at baseline and follow up of each intervention period. Body weight and standing height were measured with study participants in light clothing and barefoot, using a digital scale (WM203,

Willawong QLD, Australia) to the closest 0.1 kg and a wall-mounted stadiometer (SE206, Seven Hills, NSW, Australia) to the nearest 0.1cm, respectively. Waist circumference (WC) was measured to the nearest 0.1cm, using a flexible steel tape calibrated in cm with mm graduations (Luftkin W606PM, Maryland, USA) directly over the skin at the umbilicus level. Body mass index (BMI) was calculated using Quetelet's equation (weight (kg) / height $(m)^2$). Using World Health Organization (WHO) cut-off points for BMI, study participants were classified as underweight (BMI <18.5 kg/m^2), normal weight (BMI 18.5-24.9 kg/m^2), overweight (BMI 25.0-29.9 kg/m^2) or obese (BMI ≥ 30 kg/m^2) (WHO, 1995). Furthermore, gender-specific WC cut-off points proposed by WHO were also used to categorise study participants for CVD risk: normal (WC <94 cm in men and <80 cm in women), High CVD risk (WC 94-102 cm in men and 80-88 cm in women) and very high CVD risk (WC >102 cm in men and 88 cm in women) (WHO, 2008).

5.5.4 Haemodynamic indices

5.5.4.1 Blood pressure

Peripheral (brachial) and central (aortic) blood pressure (BP) were measured using applanation tonometry with a SphygmoCor XCEL device (Model XCEL, AtCor Medical, Sydney, Australia), at baseline and follow up examinations of each intervention period. Following a minimum of 5 min rest in the supine position, peripheral brachial systolic BP (SBP) and diastolic BP (DBP) was measured using a blood pressure cuff affixed to the upper left arm. Three consecutive BP recordings were made and the average of the last two recordings was used for data analysis. In addition, central SBP and DBP, as well as PP measures were automatically derived via the brachial BP cuff. The BP categories recommended by the American College of Cardiology (ACC) /American Heart Association (AHA) were used to classify study participants into those with Normal BP (SBP/DBP

<120/80 mmHg), Elevated BP (SBP 120-129 mmHg and DBP <80 mmHg), Hypertension Stage I (SBP 130-139 mmHg or DBP 80-89 mmHg) and Hypertension Stage II (SBP ≥140 mmHg or DBP ≥90 mmHg) ("Correction to: 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines," 2018).

5.5.4.2 *Arterial stiffness*

Measures of peripheral and central arterial stiffness, using pulse wave analysis (PWA) and pulse wave velocity (PWV), were obtained non-invasively with the SphygmoCor XCEL device (Model XCEL, AtCor Medical, Sydney, Australia). This was carried out using the standard procedure as outlined in our previous paper (Kennedy et al., 2018). PWA is a noninvasive, valid and reliable technique to investigate mechanical properties of the arterial tree, using central blood pressures and analysis of systemic arterial wave reflection. Peripheral arterial stiffness indices of augmentation pressure (AP) and augmentation index (AIx) were derived automatically by the device as part of the standard BP measurement procedure. The AP was calculated as the difference between the first and second systolic peak, while the AIx was calculated as the percentage contribution that the AP makes to the overall PP *(AIx=AP/PP x 100)*. PWV was measured using a tonometer to capture the carotid waveform, while a femoral cuff was placed high on the left thigh in order to capture the femoral waveform. The PWV was then calculated by dividing the distance between the carotid and femoral measurement sites by the transit time. This method is considered the gold standard technique for assessing central arterial stiffness.

5.6 Sample size calculation

Power calculations showed that a sample size of 40 was adequate to provide sufficient statistical power to detect a statistically significant between-group difference of 5% and a standard deviation (SD) of 11 in HDL-C efflux levels (i.e. the primary outcome of the OLIVAUS study), with power 80% and 5% level of significance (Hernaez et al., 2014). The total sample size was set at 50 study participants, in order to also account for an attrition rate of 20%. Although, the selected sample was adequate for the examination of HDL-C efflux, this might not be the case for the secondary outcomes of the OLIVAUS study, including BP and measures of arterial stiffness.

5.7 Statistical analyses

All statistical analyses were conducted using the SPSS statistical software for Windows (IBM, version 24.0; IBM, Armonk, NY, USA). For all continuous variables, the Kolmogorov-Smirnov test was performed to examine the normality of their distribution. A general linear model, i.e. repeated-measures ANOVA (analysis of variance) was used to examine the between-group differences (treatment effect; i.e., extra virgin high *vs* low polyphenol OO) of mean values at each time point of measurement, the within-group changes (time effect) from baseline to follow-up in each intervention arm, and the differences in the changes from baseline to follow-up between the two intervention arms (treatment x time interaction effect). Both per protocol (PP) and intention–to-treat (ITT) analyses were performed. The PP analyses were conducted in study participants who had full data from baseline to follow-up in the first or the second intervention period. For the ITT analyses, multiple imputations were conducted in order to compensate for all missing values. Five imputed models derived from this process. Considering that the PP and the ITT analyses provided similar results, (i.e. mean values, mean changes and statistical

significance), the results coming from the latter are presented in this article. In all statistical analyses adjustments were made for gender and age. Data is presented either as mean ± SD, as estimated marginal means and standard errors (SE) or as mean change and 95% Confidence Interval of change (CI) for continuous variables and as frequency (n) and percentage (%) for categorical ones. All reported *P* values are two-tailed, and the level of statistical significance is set at P< 0.05.

5.8 Results

Fifty volunteers (n=33 females, and n=17 males) from 105 interested individuals who agreed to be screened, were eligible and enrolled in the study from July 2018 through to October 2019. Seven participants discontinued the intervention, due to inability to comply (n=4) and for personal reasons (n=3) and therefore, 43 participants completed the study. Figure 5.1 provides the study participant flow diagram.

5.8.1 Baseline characteristics of study participants

Table 5.1 presents the descriptive characteristics of study participant socio-demographics, anthropometrics and hemodynamic indices in the total sample (n=50) and by gender. Study participants had a mean age of 38.5±13.9 years (the age range was between 20 and 70 years) and their mean years of education was 17.3±3.5. In addition, the majority of study participants were females (66%), had a tertiary education (86%) and were born in Australia (70%). No significant gender differences were observed in any of these socio-demographic characteristics. The mean BMI and WC was 24.7±3.5 kg/m^2 and 86.9 ± 11.2 cm respectively, with no significant differences between genders. In addition, 44% of study participants were overweight and 4% were obese. Based on their WC measurements, 16% had a high cardiometabolic risk and 24% had very high risk. Although there were no

significant differences between genders observed in BMI and WC, compared to females, male study participants were taller (179.3±6.8 cm vs. 163.6±5.8 cm, P<0.001) and had a higher body weight (79.6±9.6 kg vs. 66.1±11.9 kg, P<0.001). At baseline, the mean peripheral SBP and DBP for the cohort was 120.0±13.4 mmHg and 69.9±8.4 mmHg respectively, while 18% of study participants were categorised as having elevated BP, 20% had Stage 1 Hypertension and 8% had Stage 2 Hypertension. Mean central SBP and DBP was 106.8±13.3 mmHg and 70.6±8.7 mmHg respectively, mean heart rate was 61.5±10.2 bpm and PWV was 9.5±1.4 m/s. There were no significant differences between genders in any of these hemodynamic indices.
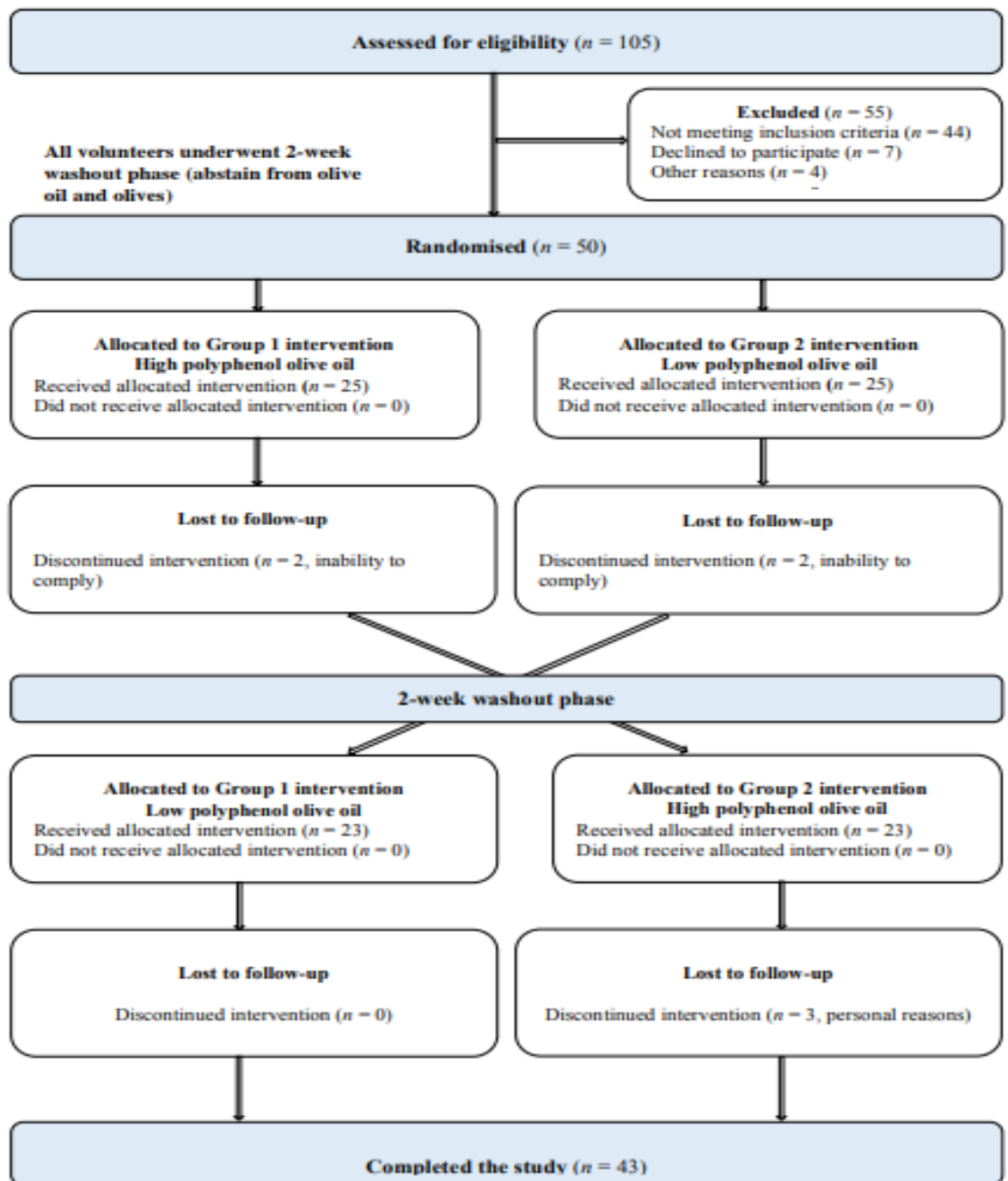
Figure 5.1   OLIVAUS study participant flow diagram

Table 5.1   Descriptive characteristics of study participants

Variable	Total sample (n=50)	Males (n=17)	Females (n=33)	P-value*
Age (years)	38.5 (13.9)	33.4 (11.6)	41.2 (14.4)	0.058
Education (years)	17.3 (3.5)	17.4 (3.7)	17.2 (3.5)	0.895
Highest Level of Education	**n (%)**	**n (%)**	**n (%)**	0.241
Secondary School	2 (4.0)	0 (0.0)	2 (6.1)	
Tertiary	43 (86.0)	17 (100.0)	26 (78.8)	
Trade	2 (4.0)	0 (0.0)	2 (6.1)	
Other	3 (6.0)	0 (0.0)	3 (9.1)	
Country of Birth				0.798
Australia, NZ, Pacific Islanders	35 (70.0)	11 (64.7)	24 (72.7)	
Europe	5 (10.0)	2 (11.8)	3 (9.1)	
South America	4 (8.0)	1 (5.9)	3 (9.1)	
Middle East & Asia	6 (12.0)	3 (17.6)	3 (9.1)	
Anthropometrics	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Height (cm)	168.9 (9.6)	179.3 (6.8)	163.6 (5.8)	<0.001
Weight (Kg)	70.7 (12.8)	79.6 (9.6)	66.1 (11.9)	<0.001
BMI (kg/m^2)	24.7 (3.5)	24.7 (2.4)	24.6 (3.9)	0.915
Waist circumference (cm)	86.9 (11.2)	88.9 (8.7)	85.9 (12.3)	0.364

Variable	Total sample (n=50)	Males (n=17)	Females (n=33)	P-value*
Hemodynamic Indices				
Peripheral BP	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Systolic Pressure (mmHg)	120.0 (13.4)	121.7 (9.1)	119.1 (15.2)	0.454
Diastolic Pressure (mmHg)	69.9 (8.4)	69.7 (8.9)	70.0 (8.3)	0.904
Central aortic BP				
Systolic Pressure (mmHg)	106.8 (13.3)	106.9 (8.5)	106.8 (15.3)	0.971
Diastolic Pressure (mmHg)	70.6 (8.7)	70.3 (9.1)	70.8 (9.7)	0.843
Pulse Pressure (mmHg)	36.0 (8.9)	36.2 (7.9)	35.9 (9.5)	0.930
Heart rate (bpm)	61.5 (10.2)	58.1 (8.9)	63.2 (10.4)	0.092
Systemic Arterial Stiffness				
Augmented pressure (mmHg)	6.8 (6.8)	4.8 (4.2)	7.8 (7.6)	0.077
Augmented index (%)	16.6 (14.9)	12.2 (9.4)	18.9 (16.8)	0.077
Pulse wave velocity (m/s)	9.5 (1.4)	9.5 (1.3)	9.5 (1.5)	0.933

* p-values were derived from the Student's t-test for continuous variables and from the chi-square test for categorical variables. Results in bold indicate $p < 0.05$, and are therefore statistically significant. BMI, body mass index; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; y: Weight status categories: Underweight, BMI < 18.5 kg/m^2; Normal weight, $18.5 \leq$ BMI < 25 kg/m^2; Overweight, $25 \leq$ BMI < 30 kg/m^2; Obese, BMI $\geq$30 kg/m^2. z: Waist circumference categories: Normal, WC < 80 cm in women and <94 cm in men; High risk, 80–88 cm in women and 94–102 cm in men; Very high risk: WC > 88 cm in women and >102 cm in men. §: Peripheral BP categories: Normal BP, SBP < 120 and DBP < 80 mmHg; Elevated BP, SBP > 120–129.9 and DBP < 80 mmHg; Hypertension Stage 1, SBP 130–139.9 or DBP 80–89.9 mmHg; Hypertension Stage 2, SBP > = 140 or DBP > = 90.

5.8.2 Effect of LPOO and HPOO on dietary intake and physical activity

The changes observed in dietary energy, macro- and micronutrient intake from baseline to follow-up, as well as the differences between treatment arms are summarized in Table 5.2. The changes from baseline to follow-up were not significantly different between the two treatment arms. However, dietary energy intake increased significantly in participants following LPOO (by 1806.1 kJ/day, 95% CI: 1075.4 to 2536.8) and HPOO (by 1766.6 kJ/day, 95% CI: 1035.9 to 2497.3). Consumption of LPOO and HPOO also significantly increased intake of total fat (by 49.3 g/day, 95% CI: 41.1 to 57.4 and 46.0 g/day, 95% CI: 37.8 to 54.1, respectively), SFA (by 7.4 g/day, 95% CI: 4.0 to 10.8 and 6.5 g/day, 95% CI: 3.1 to 9.9, respectively), MUFA (by 36.8 g/day, 95% CI: 33.2 to 40.3 and by 35.1 g/day, 95% CI: 31.6 to 38.6, respectively) and PUFA (by 3.1 g/day, 95% CI: 1.0 to 5.1 and by 3.0 g/day, 95% CI: 1.0 to 5.1, respectively). In addition, no significant within-group changes or between-group differences were observed in the other examined macronutrients (protein, carbohydrates and dietary fibre), nor in micronutrients such as sodium, potassium, magnesium and calcium, including caffeine. Regarding physical activity, no within-group changes or between-group differences were observed in the time study participants were engaged in physical activities of moderate-to-vigorous intensity over the intervention period (data not shown).

Table 5.2   **Effect of low polyphenol OO vs. high polyphenol OO on mean changes in dietary energy, macro- and micronutrient intake.**

	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Energy intake (KJ/day)				
Low Polyphenol OO (n=50)	8712.8 (328.3)	10518.9 (344.4)	**1806.1 (1075.4 to 2536.8)**	0.940
High Polyphenol OO (n=50)	8892.6 (328.3)	10659.2 (344.4)	**1766.6 (1035.9 to 2497.3)**	
Treatment*effect p-value	0.700	0.774		
Protein intake (g/day)				
Low Polyphenol OO (n=50)	102.0 (5.5)	100.7 (5.2)	-1.3 (-14.3 to 11.8)	0.924
High Polyphenol OO (n=50)	97.4 (5.5)	97.0 (5.3)	-0.4 (-13.4 to 12.7)	
Treatment*effect p-value	0.558	0.619		
CHO (g/day)				
Low Polyphenol OO (n=50)	214.8 (10.1)	213.4 (11.1)	-1.5 (-23.5 to 20.6)	0.972
High Polyphenol OO (n=50)	219.9 (10.1)	217.8 (11.1)	-2.0 (-24.0 to 20.0)	
Treatment*effect p-value	0.726	0.776		
Total fat intake (g/day)				
Low Polyphenol OO (n=50)	79.9 (4.0)	129.2 (4.5)	**49.3 (41.1 to 57.4)**	0.571
High Polyphenol OO (n=50)	84.3 (4.0)	130.3 (4.5)	**46.0 (37.8 to 54.1)**	
Treatment*effect p-value	0.441	0.870		

	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
SFA intake (g/day)				
Low Polyphenol OO (n=50)	27.7 (1.5)	35.1 (1.9)	**7.4 (4.0 to 10.8)**	0.707
High Polyphenol OO (n=50)	28.8 (1.5)	35.3 (1.9)	**6.5 (3.1 to 9.9)**	
Treatment*effect p-value	0.620	0.953		
MUFA intake (g/day)				
Low Polyphenol OO (n=50)	30.6 (1.7)	67.3 (1.9)	**36.8 (33.2 to 40.3)**	0.514
High Polyphenol OO (n=50)	31.8 (1.7)	67.0 (1.9)	**35.1 (31.6 to 38.6)**	
Treatment*effect p-value	0.605	0.877		
PUFA intake (g/day)				
Low Polyphenol OO (n=50)	14.6 (1.0)	17.7 (1.0)	**3.1 (1.0 to 5.1)**	0.971
High Polyphenol OO (n=50)	15.7 (1.0)	18.7 (1.0)	**3.0 (1.0 to 5.1)**	
Treatment*effect p-value	0.483	0.469		
Fibre intake (g/day)				
Low Polyphenol OO (n=50)	29.7 (1.7)	30.7 (1.8)	0.9 (-3.1 to 4.9)	0.314
High Polyphenol OO (n=50)	29.6 (1.7)	33.5 (1.8)	3.8 (-0.2 to 7.8)	
Treatment*effect p-value	0.963	0.268		
Sodium intake (mg/day)				
Low Polyphenol OO (n=50)	2611.1 (269.5)	2287.1 (168.9)	-324.0 (-878.2 to 230.3)	0.994
High Polyphenol OO (n=50)	3096.7 (269.5)	2775.5 (168.9)	-321.2 (-875.5 to 233.1)	

	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Treatment*effect p-value	0.206	**0.044**		
Potassium intake (mg/day)				
Low Polyphenol OO (n=50)	3486,7 (227.0)	3389.3 (170.5)	-97.3 (-631.1 to 436.4)	0.488
High Polyphenol OO (n=50)	3334.4 (227.0)	3501.9 (170.5)	167.5 (-366.2 to 701.3)	
Treatment*effect p-value	0.636	0.642		
Magnesium intake (mg/day)				
Low Polyphenol OO (n=50)	574.2 (92.9)	446.0 (18.9)	-128.2 (-308.4 to 52.0)	0.271
High Polyphenol OO (n=50)	433.6 (92.9)	447.6 (18.9)	14.0 (-166.2 to 194.2)	
Treatment*effect p-value	0.287	0.953		
Calcium intake (mg/day)				
Low Polyphenol OO (n=50)	1005.0 (92.3)	1056.2 (95.6)	51.1 (-205.1 to 307.3)	0.916
High Polyphenol OO (n=50)	977.3 (92.3)	1009.2 (95.6)	31.9 (-224.3 to 288.1)	
Treatment*effect p-value	0.832	0.729		

All statistical analyses were adjusted for gender and age. Results in bold indicate statistical significance ($p < 0.05$). OO, olive oil; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SEM, standard error of the mean; CI, confidence intervals.

5.8.3 Effect of LPOO and HPOO on anthropometrics

Table 5.3 summarizes the changes observed in anthropometric indices from baseline to follow-up and the relevant differences between the two intervention arms. There was a small but significant increase in body weight by 0.4 kg (95% CI: 0.2 to 0.7) following the LPOO intervention, but this change was not found to differ compared to the non-significant change observed in HPOO group. No within-group changes or between-group differences were observed in BMI and WC after the daily consumption of the two intervention oils.

Table 5.3   Effect of low polyphenol OO vs. high polyphenol OO on mean changes in anthropometric indices.

	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Weight (kg)				
Low Polyphenol OO (n=50)	70.8 (1.5)	71.2 (1.5)	**0.4 (0.2 to 0.7)**	0.163
High Polyphenol OO (n=50)	70.7 (1.5)	70.9 (1.5)	0.2 (-0.1 to 0.4)	
Treatment*effect p-value	0.993	0.902		
Height (cm)				
Low Polyphenol OO (n=50)	168.9 (0.9)	169.0 (0.9)	0.1 (-0.2 to 0.4)	0.890
High Polyphenol OO (n=50)	168.9 (0.9)	169.0 (0.9)	0.1 (-0.1 to 0.4)	
Treatment*effect p-value	0.974	0.992		
BMI (kg/m^2)				
Low Polyphenol OO (n=50)	24.7 (0.4)	24.8 (0.4)	0.1 (-0.01 to 0.2)	0.305
High Polyphenol OO (n=50)	24.7 (0.4)	24.7 (0.4)	0.02 (-0.1 to 0.1)	
Treatment*effect p-value	0.993	0.897		
Waist circumference (cm)				
Low Polyphenol OO (n=50)	87.1 (1.3)	87.4 (1.2)	0.3 (-0.1 to 0.7)	0.501
High Polyphenol OO (n=50)	87.1 (1.3)	87.3 (1.2)	0.1 (-0.2 to 0.5)	
Treatment*effect p-value	1.000	0.919		

All statistical analyses were adjusted for gender and age. Results in bold indicate statistical significance ($p < 0.05$). OO, olive oil; SEM, standard error of the mean; CI, confidence interval.

5.8.4 Effect of LPOO and HPOO on peripheral BP, central BP and arterial stiffness

The effect of the two intervention OOs on peripheral and central BP are illustrated in Figure 5.2. The changes from baseline to follow-up were not significantly different between the two treatment arms. However, compared to baseline, peripheral (brachial) and central (aortic) SBP was significantly reduced after HPOO by 2.5 mmHg (95% CI: -4.7 to -0.3) and by 2.7 mmHg (95% CI: -4.7 to -0.6), respectively. No other significant within-group changes or between group differences were observed in peripheral and central DBP, as well as in the rest of the examined hemodynamic (i.e. PP and HR) and arterial stiffness indices (i.e. AP, AIx and PWV) (Table 5.4).

Table 5.4   **Effect of low polyphenol OO vs. high polyphenol OO on mean changes in hemodynamic and arterial stiffness indices.**

Blood Pressure (BP)	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Pulse Pressure (mmHg)				
Low Polyphenol OO (n=50)	35.7 (1.0)	36.4 (1.0)	0.7 (-0.8 to 2.1)	
High Polyphenol OO (n=50)	36.3 (1.0)	35.9 (1.0)	-0.4 (-1.9 to 1.1)	0.296
Treatment*effect p-value	0.653	0.723		
Pulse rate (bpm)				
Low Polyphenol OO (n=50)	61.1 (1.3)	59.4 (1.4)	-1.7 (-3.9 to 0.4)	
High Polyphenol OO (n=50)	61.0 (1.3)	60.6 (1.4)	-0.4 (-2.6 to 1.7)	0.403
Treatment*effect p-value	0.954	0.553		
Augmented Pressure (mmHg)				
Low Polyphenol OO (n=50)	6.5 (0.7)	6.0 (0.7)	-0.5 (-1.6 to 0.6)	0.987
High Polyphenol OO (n=50)	6.9 (0.7)	6.3 (0.7)	-0.5 (-1.6 to 0.5)	
Treatment*effect p-value	0.692	0.714		
Augmented Index (%)				
Low Polyphenol OO (n=50)	16.2 (1.7)	14.6 (1.8)	-1.7 (-4.2 to 0.8)	0.807
High Polyphenol OO (n=50)	16.6 (1.7)	15.4 (1.8)	-1.2 (-3.7 to 1.3)	
Treatment*effect p-value				

Blood Pressure (BP)	Baseline Mean (SEM)	Follow-up Mean (SEM)	Change (Time-effect) (95% CI)	Treatment*time interaction (p-value)
Pulse Rate (bpm)				
Low Polyphenol OO (n=50)	9.6 (0.1)	9.5 (0.1)	-0.03 (-0.3 to 0.2)	0.926
High Polyphenol OO (n=50)	9.5 (0.1)	9.4 (0.1)	-0.05 (-0.3 to 0.2)	
Treatment*effect p-value	0.679	0.608		

All statistical analyses were adjusted for gender and age. OO, olive oil; SEM, standard error of the mean; CI, confidence interval.
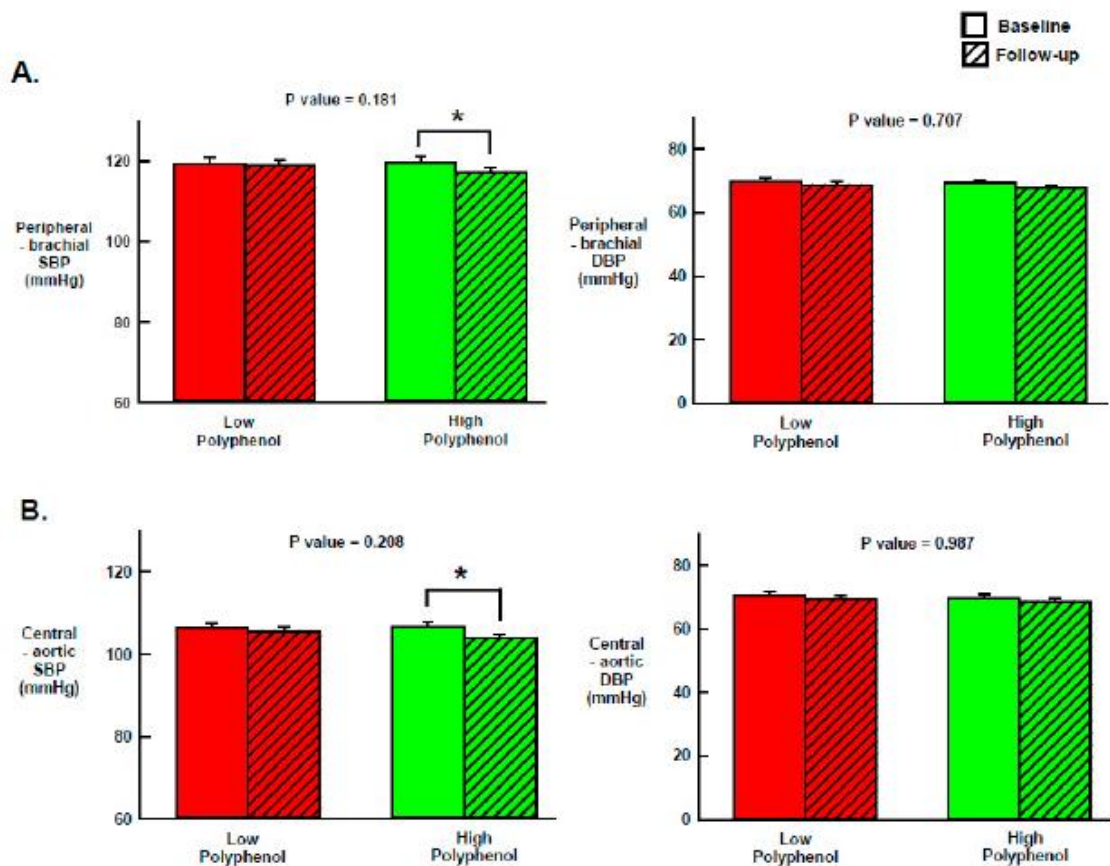
Figure 5.2   Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil

Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil (HPOO; 360 mg/kg polyphenols) and low polyphenol OO (LPOO; 86 mg/kg polyphenols) on mean peripheral (**A**) and central (**B**) blood pressure. N = 50 participants. SBP, systolic blood pressure; DBP, diastolic blood pressure. Data are mean standard deviation. The P values in the Figure indicate the between-group differences in the changes from baseline to follow-up (treatment*time effect) for each blood pressure measure. The asterisk (*) indicates significance ($p < 0.05$) of within-group changes from baseline to follow-up.

5.9   Compliance to treatment

Compliance to treatment was high, as reflected in the OO volume returned by participants after each intervention period. Based on the measured actual remaining OO, compliance was found to be 92% for both the LPOO and HPOO group after the first intervention period,

while 92% for the LPOO group and 90% for the HPOO group after the second intervention period. Nevertheless, compliance was not found to differ significantly between the two groups (Supplementary Table S3).

5.10 Discussion

The present double-blind, cross-over, randomised controlled trial investigated the effect of daily consumption of 60 mL raw extra virgin HPOO in comparison with LPOO, each for 3-weeks, on BP and arterial stiffness in Australian adults. The key finding was that peripheral and central SBP decreased significantly by 2.5 mmHg and 2.7 mmHg, respectively after extra virgin HPOO (phenolic content 360 mg/kg) consumption. However, no significant differences were observed between the two interventions with regards to the changes in peripheral and central SBP. No significant within-group changes or between-group differences were either observed on diastolic BP, and measures of arterial stiffness.

The significant decrease reported from our study in peripheral SBP is consistent with the limited number of RCTs that have examined the effect of HPOO consumption on peripheral BP, but after providing different doses of OO, different phenolic content of the administered oil, and varying intervention duration. In this regard, Moreno-Luna et al. (Moreno-Luna et al., 2012) described that daily consumption of 60 mL HPOO with the highest phenolic content reported in published literature (i.e. 564 mg/kg) for 8 weeks, significantly reduced peripheral SBP and DBP (by 7.9 mmHg and 6.6 mmHg, respectively) compared to refined OO with no polyphenols, in young women with mild hypertension. Using an OO with high polyphenol concentration comparable to our study, Bondia-Pons et al. (Bondia-Pons et al., 2007) reported that 9-weeks daily consumption of 25 mL of OO (366 mg/kg of polyphenols), significantly decreased peripheral SBP (~2.4 to 4.4 mmHg),

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in healthy non-Mediterranean men living in Europe. Other authors have described that the daily consumption of 25 mL of HPOO (366 mg/kg of polyphenols) for 3 weeks reduced significantly peripheral SBP (by 4.2 mmHg), in healthy adults (Martin-Pelaez et al., 2017). In agreement with our results, the two aforementioned studies did not show any significant changes in peripheral DBP. These findings are supported by a recent meta-analysis, reporting that consumption of OOs with at least 150 mg/kg polyphenols significantly reduces peripheral SBP but not peripheral DBP (Hohmann et al., 2015), although there is evidence coming from one clinical trial indicating that OO with less phenolic content might also exert SBP-lowering effects. In this context, the NUTRAOLEUM study showed that daily consumption of 30 mL of virgin OO (phenolic content, 124 mg/kg) for 3 weeks significantly reduced peripheral SBP by 2.0 mmHg but not peripheral DBP, in healthy adults (Sanchez-Rodriguez et al., 2018).

It is noteworthy, that other clinical trials also examining the effect of HPOO on peripheral SBP and DBP reported either significant results only for peripheral DBP or no significant findings on BP. In this regard, the EUROLIVE study demonstrated that 3-weeks of daily consumption of 25 mL EVOO, containing 366 mg/kg of polyphenols, significantly reduced peripheral DBP, but had no effect on SBP in healthy men (Castaner et al., 2011), while another recent meta-analysis showed no significant pooled effect of the consumption of HPOO (the phenolic content of the OOs examined in these studies ranged from 150 to 800 mg/kg) on peripheral SBP and DBP (George et al., 2019).

To the best of our knowledge, this is the first study reporting a significant reduction in central SBP after consumption of HPOO. This is of importance, considering that raised central BP has been positively associated with cardiovascular risk and mortality

(Papamichael et al., 2008; Protogerou et al., 2007). The effect of different bioactive nutrients (e.g. omega 3 fish oils, Vitamin C, Vitamin E) on central hemodynamic markers (i.e. central SBP and DBP), either in the acute postprandial state or after long-term use, has been previously reported (Tanaka & Safar, 2005). However, there is currently no evidence stemming from long-term RCTs regarding the effects of OO polyphenols alone, on these markers. Considering the scarcity of evidence and although not directly comparable with our study, authors reported significant postprandial reductions in both central SBP and DBP, ranging from 3 to 5 mmHg, after the consumption of meals combining OO and red wine by healthy study participants (Papamichael et al., 2008). However, the combined meal design of this previous study makes it difficult to give attribution to the OO and/or wine for the favourable effects observed on central BP. The mechanisms by which virgin OO minor compounds might exert their beneficial effects on central hemodynamic markers remains unclear. Therefore, further studies are warranted to reach final conclusions about the effect of OO polyphenols on central SBP and/or DBP.

Dietary intake and body weight changes observed in our study deserve comment in the context of the favourable effect of HPOO on peripheral and central SBP. In this regard, the addition of 60 mL of OO in participants' habitual diet resulted in significant increases of caloric intake leading to weight gain in both intervention groups. Previous cross-sectional and prospective studies have reported that body weight gain is directly associated with increases in arterial BP in normotensive subjects (McCarron & Reusser, 1996), with a 1 kg increase in body weight predicting a 0.63 mmHg and 0.42 mmHg increase in SBP and DBP respectively (Xie et al., 2016). Based on the above, the increases in body weight of 0.2 kg and 0.4kg observed in the HPOO and LPOO group respectively, would be expected to lead to corresponding increases in BP. However, that was not the case in our study, indicating a

potential counterbalancing effect of OO polyphenols on weight gain. In this regard, the phenolic content of 360 mg/kg in HPOO, which was much higher compared with the effective threshold of 150 mg/kg reported by a recent meta-analysis (Hohmann et al., 2015), could provide a basis for interpreting the significant reduction in peripheral and central SBP observed in this group, despite the non-significant weight gain of 0.2 kg.

Further to dietary energy intake, our study also recorded the intake of macro- (i.e. protein and dietary fibre) and micronutrients (i.e. sodium, potassium, magnesium and calcium) that have established effects on BP levels (D'Elia et al., 2020; Li et al., 2020; Pamuk et al., 2020; Schutten et al., 2018). In this regard, there were no differences between the two intervention arms, indicating that the only dietary factor which could account for the observed favourable effect to reduce SBP, was the higher phenolic content in HPOO compared to LPOO. However, it is not clear why the higher phenolic content in HPOO has a significant lowering effect only on SBP but not on DBP. It could be speculated that both quantity (i.e. dose) and quality (i.e. chemical structure) of polyphenols in EVOO may exert differential effects on the vascular system (Silva et al., 2015; Tripoli et al., 2005). Nevertheless, further clinical trials are required to examine the effect of OOs with different phenolic profile on BP, arterial stiffness and other cardiometabolic risk markers.

To the best of our knowledge, the OLIVAUS study is the first human clinical trial to investigate the effect of OO polyphenols on measures of arterial stiffness through applanation tonometry. Stiffening of the arterial wall in the larger central arterial system represents an important CVD risk marker (Shirwany & Zou, 2010) and the early detection of such abnormalities can inform relevant preventive or treatment initiatives. However, the present study did not detect within- group changes or between-group differences in any

measures of arterial stiffness after either OO intervention. The absence of significant findings may be partly attributed to our study being adequately powered for its primary outcome, while this might not be the case for the secondary outcomes, including measures of arterial stiffness. Despite the scarcity of evidence in this field, there is a large body of published literature documenting the effect of polyphenols on biochemical markers of endothelial function, which also represent other surrogate measures of arterial stiffness. In this context, Sanchez-Rodriquez et al. (Sanchez-Rodriguez et al., 2018) investigated the effect of three virgin OOs enriched with polyphenols (124 mg/kg, 490 mg/kg and 487 mg/kg) and triterpenes (86 ppm, 86 ppm and 389 ppm, respectively) on endothelial function biomarkers in healthy adults. These investigators reported significant reductions in plasma levels of the vasoconstrictor hormone endothelin-1 at the end of the three interventions and regardless of triterpene content. In another clinical trial in women with mild hypertension, daily consumption of extra virgin HPOO (564 mg/kg polyphenols) for 8 weeks significantly decreased plasma levels of asymmetrical dimethylarginine (ADMA), which is a surrogate marker of poor endothelial function (Sonmez et al., 2010). The participants also had significantly increased concentrations of vasodilating nitric oxide (NO) molecule after the intervention, supporting a beneficial effect of high polyphenol OO on endothelial function (Moreno-Luna et al., 2012; Sonmez et al., 2010)

The findings reported in our study should be interpreted in light of its strengths and limitations. The main strength of the present study is its randomised, double blind, cross-over design that reduces interindividual variability and increases the external validity of the study findings. The use of applanation tonometry represents another strength since it is state-of-the-art, non-invasive method to measure BP and arterial stiffness. In addition, in a multiethnic population that is not accustomed to a high consumption of OO, our

participants' compliance was overall high throughout both intervention periods. On the other hand, one of the limitations of the present study is that the sample size was calculated on the basis of the expected differences only in its primary outcome (i.e. HDL-efflux). Another limitation could be the potential effect of seasonality on the examined outcomes, due to the fact that the participants were enrolled in the study gradually (i.e., from July 2018 through to October 2019). Lastly, despite the inclusion of a washout period before the initiation of the intervention and between the intervention periods, there is no guarantee that any potential carry-over effect on the examined hemodynamic markers was completely avoided. However, pairwise comparisons that examined potential carry-over effects were insignificant for all hemodynamic markers.

5.11 Conclusions

To our knowledge, the OLIVAUS study is the first to examine the effect of OO polyphenols on peripheral and central SBP and DBP as well as on measures of arterial stiffness in Australian adults. Although there were no significant differences between OO treatments in any of the examined outcomes, there was a significant reduction in peripheral and central SBP after daily consumption of extra virgin HPOO for 3 weeks. This provides evidence for a potentially widely accessible dietary intervention that can reduce CVD risk in a multicultural context, such as in Australia. However, additional clinical trials of longer duration and use of EVOO with different phenolic content and profile are required to shed more light on the potential effect of OO polyphenols on other CVD risk markers, including DBP and arterial stiffness.

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Chapter 6:   Conclusions and Future Recommendations

6.1   Summary of main findings

Cardiovascular disease (CVD) is of growing concern worldwide including Australia (AIHW, 2021). This doctorate aimed to assess the efficacy of two kinds of OO with different phenolic content (LPOO; 86mg/kg, phenolic content vs. HPOO; 320mg/kg, phenolic content) on CVD risk markers in healthy Australian adults.

One hundred and five participants were screened for eligibility for the OLIVAUS study. Data from 50 eligible participants (mean age, 38.5 ± 13.9 years; 66% females and 44% males) were collected in the nutrition clinical rooms at La Trobe University in Melbourne Australia over a 10-week period. Baseline characteristics of study participants indicated non-significant differences between the two treatment arms in all of the measured descriptive characteristics (i.e., socio-demographic, anthropometric, biochemical and hemodynamic parameters[13]), thus indicating homogeneity at baseline.

This research analyzed data that supports the examination of the effects of LPOO and HPOO on dietary intake changes. Regarding energy and nutrients intake, results indicated that the observed changes were not significantly different between the two treatment arms. Both intervention groups demonstrated a significant increase in dietary energy and total fat

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[13] Hemodynamic parameters include peripheral (brachial) and central (aortic) systolic and diastolic blood pressure, pulse pressure, augmentation pressure, augmentation index, pulse wave velocity and measures of arterial stiffness.

intake. These results are not unexpected considering that the addition of 60 ml of OO in the participant's daily diet, led to the intake of an extra 1800 kJ/day. Considering that study participants in both intervention arms were advised to avoid replacing their habitually consumed raw fats with the intervention OOs supplied, the caloric and fat surplus coming from the supplied OO consumption provides an explanation of the relevant dietary intake increases. A significant increase in SFA, MUFA, PUFA and specific micronutrient (i.e., a-tocopherol and vitamin E) intake was also observed within both intervention arms-an observation that could be explained by the high content of the intervention OOs in the abovementioned macro and micronutrients. However, considering that the supplied OOs had the same nutrient composition (i.e., fat-soluble vitamins and fatty acids) and that both intervention arms consumed the same amount (i.e., 60 mL) of OO, it is not surprising that the relevant increases were equal in both arms, thus also explaining the non-significant between-group changes. Regarding intake of foods with high phenolic content (i.e., whole grain cereals, fruits, vegetables, legumes, nuts/seeds, soy products, oils, fruit juices, alcoholic drinks and coffee) as part of the study participants' habitual diet, the OLIVAUS study also showed no significant within-group changes or between-group differences. This indicates that OO was the only dietary source that affected polyphenol's dietary intake in the two treatment arms, which also highlights that any effect on the examined outcomes mainly stems from the phenolic content of the consumed OOs.

Changes in PA levels were also examined in the OLIVAUS study. It is widely recognized that PA is inversely associated with cardiometabolic risk parameters (i.e., BP, serum lipids), in a dose-response manner (Carroll & Dudfield, 2004). No within-group changes or between-group differences were observed in daily energy expenditure in leisure-time PA over the intervention period (data not presented in this doctoral thesis). These findings

demonstrate that the only factor which could account for any observed favourable effects on the examined metabolic outcomes, was the intervention OOs.

In terms of the effect of OO polyphenols on anthropometric indices (i.e., body weight, BMI and WC), no significant between-group differences were observed in any of the examined outcomes. A small increase in body weight was observed within both treatment arms (as expected due to the increased energy intake), which was significant only in the LPOO arm. The increase in study participants' body weight, could possibly have a mediating effect on the examined cardiometabolic risk outcomes. However, this was not the case in our study, indicating a potential counterbalancing effect of OO polyphenols on the potential unfavorable effect of weight gain on cardiometabolic risk markers. No significant within-group changes were observed in any of the other anthropometric indices (i.e., in BMI and WC).

The effect of the two intervention oils on cholesterol transport and concentration biomarkers, i.e., HDL-c efflux (primary outcome) and serum lipoproteins was also examined. Results demonstrated non-significant differences between treatment arms in the changes observed in HDL-c efflux from baseline to follow-up. However, an increase in HDL-c efflux albeit non-significant was observed within both the LPOO and HPOO groups, which was marginally greater in the LPOO treatment arm. Previous evidence indicates that phenol-rich OO may improve significantly HDL-c efflux in both healthy individuals (Helal et al., 2013) and those at high cardiometabolic risk (Farràs et al., 2019). Furthermore, OO phenolic compounds, especially hydroxytyrosol (HT) and its derivatives,

have been shown to stimulate key factors involved in cholesterol efflux, such as ABCA1[14] protein expression and in vitro apoA-1 (Cedó et al., 2020). The concentration of HT, which is the most biologically active phenolic compound found in OO (Suárez et al., 2011), was higher in the LPOO (5.3 mg/kg, HT) compared to HPOO (3.3 mg/kg, HT) in the current study, possibly explaining the more pronounced increase in HDL-c efflux in the LPOO treatment arm.

In terms of circulating lipids and lipoproteins, no between-group differences were observed in TG, TC, HDL-c and LDL-c post intervention for the total sample. In agreement with previous literature (George et al., 2019; Tsartsou et al., 2019), the OLIVAUS intervention resulted to a significant increase in serum HDL-c within both treatment arms over the 10-week intervention period, thus supporting the cardioprotective effects of OO intake. This finding is also confirmed by a recent systematic review, which reported an increase in HDL-c levels after OO consumption and a statement indicating that OO is superior compared to other plant oils in improving HDL-c levels (Ghobadi et al., 2019). Regarding LDL-c, although no significant differences were observed between the two treatment arms, the OLIVAUS study reported a small but significant increase in LDL serum levels, but only within the HPOO treatment arm. This finding is in contradiction to other similar trials that have reported either no changes or a reduction in the concentrations of circulating LDL-c. In this context, the PREDIMED study reported that one-year intervention with VOO that was consumed by study participants as part of a Mediterranean dietary pattern, did not

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[14] One of the two major pathways involved in reverse cholesterol transport is the ATP-binding cassette receptor ABCA1. The latter facilitates the efflux of phospholipids and free unesterified cholesterol from cells to lipid-poor apolipoprotein A-1 (apoA-1) through a process that involves the binding of apoA-1 to the ABCA1 transporter.

result to a reduction of plasma LDL-c concentrations in adults at high risk for CVD (Hernáez et al., 2017). On the contrary, a recent network meta-analysis that investigated metabolic changes in circulating lipid biomarkers in relation to the phenolic content of the OO, demonstrated reduction in LDL-c concentrations and lipoprotein ratios, following the daily consumption of OOs rich in polyphenols (Tsartsou et al., 2019) .

In view of the antioxidant and anti-inflammatory properties of OO polyphenols, the OLIVAUS study also examined the effect of the two intervention OOs on markers of oxidative status (i.e., ox-LDL and TAC) and inflammation (i.e., hs-CRP). Results indicated a significant reduction in ox-LDL in the total sample following HPOO intake. In addition, stratified analyses of study participants at high cardiometabolic risk (i.e., those with WC measures >94 cm in males and >80 cm in females) demonstrated a further reduction in ox-LDL only within the HPOO group. Both treatment arms illustrated an increase in TAC, which was significant only after HPOO consumption, and even more pronounced in high cardiometabolic risk participants. These results are comparable to the findings reported in previous RCTs that investigated the effect of OO with different phenolic content on oxidative status markers, hence confirming the antioxidant potential of OO polyphenols (George et al., 2019; Hernáez et al., 2015; Marrugat et al., 2004; Moreno-Luna et al., 2012; Schwingshackl et al., 2019).

In terms of the effect of HPOO and LPOO on the examined inflammatory biomarkers, a markedly significant decrease in hs-CRP was observed in a subgroup of participants with intermediate or high cardiometabolic risk (i.e., in those with hs-CRP > 1 mg/L), only within the HPOO arm. It is widely recognized that foods rich in phenolic compounds, including EVOO, have cardioprotective effects due to their anti-inflammatory properties (George et al., 2019; Schwingshackl et al., 2019; Tsartsou et al., 2019). The mechanisms by which

polyphenols can exert their anti-inflammatory effect appears to be mediated via their regulatory role in the production and secretion of a wide variety of pro-inflammatory molecules (Rosillo et al., 2016).

Finally, the effect of the dietary intervention on hemodynamic indices, i.e., peripheral (brachial) and central (aortic) systolic and diastolic BP and measures of arterial stiffness was also investigated. Based on the results, no significant between-group differences in any of the examined outcomes were observed from baseline to follow-up. However, a significant decrease in peripheral SBP was observed within the HPOO treatment arm in the total study sample. This finding is consistent with the limited number of RCTs that have examined the effect of HPOO consumption on peripheral BP. A significant reduction in central SBP after consumption of HPOO was also reported in our study. This is highly important, considering that increased central BP has been positively correlated with cardiovascular risk and mortality (Papamichael et al., 2008; Protogerou et al., 2007). Furthermore, a significant reduction in central diastolic BP was also observed within both the LPOO and HPOO treatment arms in a subgroup of pre- and hypertensive individuals, which was more pronounced following HPOO consumption. Despite these findings, the mechanisms by which OO minor compounds might exert their beneficial effects on central BP still remain unclear. Therefore, further studies are warranted to reach final conclusions about the effect of OO polyphenols on central SBP and/or DBP.

Finally, the OLIVAUS study is the first human clinical trial (to the best of our knowledge) to investigate the effect of OO-derived polyphenols on measures of arterial stiffness through applanation tonometry. Stiffening of the arterial wall in the larger central arterial system represents an important CVD risk marker (Shirwany & Zou, 2010) and the early detection of such abnormalities can inform relevant preventive or treatment initiatives.

However, the present study did not detect within- group changes or between-group differences in any measures of arterial stiffness after either OO intervention.

It should be noted that there is a great heterogeneity among RCTs (including the OLIVAUS study) documented in the literature, in terms of the different doses and/or phenolic content of the administered OOs, while there is also a wide variability in the duration of the implemented interventions (Bondia-Pons et al., 2007; Martin-Pelaez et al., 2017; Moreno-Luna et al., 2012; Sanchez-Rodriguez et al., 2018). In this regard, the optimal dose and phenolic content of OO, as well as the ideal duration of an intervention required to demonstrate the potential benefit of OO phenolic compounds on certain cardiometabolic risk markers remains unclear warranting further research.

6.2   Strengths and limitations

The OLIVAUS study examined the effects of two dietary interventions (HPOO vs. LPOO) on CVD risk markers in free living Australian healthy participants. The main strength of this research is its randomized, double-blind crossover design, which allows each participant to act as their own control by consuming both intervention OOs, thus reducing inter-individual variability (Sibbald & Roberts, 1998). This design also enabled us to isolate the effects of OO polyphenols on the examined outcomes, since it allowed us to control for potential confounding effect caused by differences in the intake of other food sources of nutrients with antioxidant and/or anti-inflammatory properties.

Both the LPOO and HPOO tested in the current study had the same nutritional composition in terms of fat-soluble vitamins and fatty acids with the exemption of their phenolic content (86mg/kg vs. 320 mg/kg, respectively), thus confirming that intervention effects on the examined outcomes were primarily associated with the intake of OO polyphenols. Another

strength of this study is that participants did not change their habitual diet, therefore allowing the direct assessment of the benefits of OO consumption, while the high study completion rate (86%) indicates that the dietary intervention was also feasible in a multicultural population, such as the Australian, that is not accustomed to high OO consumption.

Moreover, the inclusion of hemodynamic specific outcomes which have been reported to play a significant role in the development of CVD, such as measures of arterial stiffness and central aortic hemodynamic parameters (Doupis et al., 2016; Pannier et al., 2002), is another strength of the OLIVAUS study, since these outcomes are not commonly assessed by using non-invasive methods, such as applanation tonometry [15]. A number of anthropometric indices and biomarkers were also collected, providing a more holistic and comprehensive assessment of the effect of the dietary intervention on other cardiovascular related risk factors.

One of the limitations is that the present study was underpowered to detect significant changes in some secondary outcomes (i.e., arterial stiffness), since the sample size calculations were based on the primary outcome (i.e., HDL-c efflux). Furthermore, despite the inclusion of a washout period before the initiation of the intervention and between the intervention periods, there is no guarantee that any potential carry-over effect on the examined markers was completely avoided in all study outcomes. However, pairwise

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[15] Applanation tonometry is a state-of-the-art, non-invasive method used to measure BP and arterial stiffness

comparisons did not show any significant carry-over effect on any of the examined outcomes.

Another limitation is the fact that the study's primary outcome (i.e., HDL-c efflux) was measured *ex vivo*. Considering that our HDL-c efflux assay involved the use of cell lines (mouse macrophage cell line (J774A.1), our results might not reflect the real *in vivo* status. Cell assays are always limited because they cannot adequately reflect the complexity and dynamic nature of the human *in vivo* environment and, in this case, HDL efflux processes (e.g. cAMP is used in the assay to induce the ATP-binding cassette transport, which is just one of the main mechanisms involved) (Cuchel et al., 2017).

Regarding dietary intake, data was recorded through 3 day food diaries. Although food diaries are considered one of the most robust methods of collecting dietary intake data, reliance on self-reported methods represents another limitation, since it produces reporting bias. This bias includes both over and underreporting which is well documented as a limitation in the literature (Poslusna et al., 2009). To overcome this issue biomarkers of dietary adherence, such as excreted metabolites of polyphenol intake (e.g. urine hydroxytyrosol as a marker of adherence to the implemented intervention) (Karković Marković et al., 2019) should be employed to confirm intake and findings. However, this was beyond the scope of this thesis.

One more limitation was that the intervention's comparator was a commercially available OO with a moderately high phenolic content (i.e., 86 mg/kg, phenolic content), thus providing an explanation of the non-significant between-group differences observed in the present study. In this regard, although the concentration of polyphenols in the LPOO was lower compared to extra virgin HPOO, it was still high enough to produce some clinically

significant health benefits, also in synergy to the intake of the other important bioactive nutrients that are present in the intervention OOs.

Furthermore, it is possible that due to the nature of the intervention (i.e., distinct taste and color difference between high and LPOOs) blinding may not have been completely effective despite the fact that several measures were undertaken to ensure blinding of the researchers and participants to the OO type[16]. Although this is an inherent problem in many dietary intervention trials (Mirmiran et al., 2021), the OLIVAUS study relied on measures that allowed the assessment of the adequacy of blinding (i.e., through participant interviews[17]) at the conclusion of the study. Intervention oils' organoleptic characteristics (i.e., color, taste and viscosity) and cultural differences in OO consumption habits emerged as being a critical determinant for some participants in identifying the two kinds of OO[18].

Lastly, the duration of the intervention should also be taken into consideration when interpreting the findings reported by the OLIVAUS study. As CVD is a condition that develops over many years, dietary interventions that show improvements in cardio metabolic risk outcomes need to be sustainable in order for them to be beneficial in the

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[16] To ensure blinding of researchers and participants to the OO type, the intervention oils were supplied in dark coloured glass containers, while each bottle was assigned a different code number that was concealed from study participants and research team members. The code was disclosed only after the completion of the statistical analyses.

[17] Qualitative data was collected through interviews, where participants were asked to discuss their perception on group allocation, by providing blinding comments related to the intervention oils' organoleptic characteristics

[18] The darker color and the more intense flavor of the HPOO compared to the LPOO had possibly resulted to the unblinding of treatment to some study participants, especially those with a Mediterranean background, who were already familiar with OO.

long-term. The results presented in this doctorate are only reflective of changes after a 10-week intervention period. Longer term benefits, at 6, 12 months and beyond, need to be assessed to determine if participants can maintain dietary changes and health outcomes.

6.3   Implications and future recommendations

Dietary interventions are likely to reduce the risk of developing CVD and other comorbidities given the underlying pathophysiological mechanism driving the disease (Chiavaroli et al., 2018; Keys et al., 1986; Lankinen et al., 2019; Saneei et al., 2013). As such, improving diet is likely to prevent costly treatment of clinical conditions related to CVD.

Findings of the OLIVAUS study indicate that OO derived polyphenols may provide cardioprotective benefits that are independent of the high MUFA content of the oil. These health benefits seem to mainly stem from the antioxidant and anti-inflammatory properties of polyphenols. Specifically, the results of this research suggest that HPOO can improve biomarkers related to oxidative stress (i.e., ox-LDL), inflammation (i.e., hs-CRP) and hemodynamics (i.e., peripheral and central SBP) in healthy and high CVD risk individuals. The small effect sizes and non-significant between group differences observed in specific CVD risk indices (i.e., measures of arterial stiffness, lipid profile markers (e.g. TG, TC)) may be partly explained by there being less likelihood of statistically significant reductions in specific clinical outcomes for healthy participants with baseline values within the reference ranges (Moreno-Luna et al., 2012; Sanchez-Rodriguez et al., 2018). Further research, in individuals at high cardiometabolic risk, with chronic diseases and/or impaired endothelial function that are either not managed by pharmacotherapy or where the study interventions are for longer durations may report larger effect sizes and appear sufficient to draw definitive conclusions.

Moreover, it has been reported that differences in the class (i.e., chemical structure) and/or concentrations of OO phenolic compounds may exert differential effects on the vascular system (Silva et al., 2015; Tripoli et al., 2005). Indeed, the concentration of HT was determined by chemical analyses in the current study to be higher in the LPOO compared to HPOO, possibly explaining the more pronounced increase in HDL-c efflux in the LPOO treatment arm as previously mentioned. This observation highlights the need for further clinical trials investigating the effect of OOs with different phenolic profile on specific cardiometabolic risk markers.

There are also some considerations that need to be acknowledged regarding polyphenol concentration. In this context, the phenolic content of OO differs due to several factors, including olive fruit variety, environmental factors (i.e., soil, climate), fruit maturation and processing (Tripoli et al., 2005). Globally, regulatory frameworks and policy on food labelling specifically related to the concentration of polyphenols in foods are lacking. With additional evidence to support the proposed benefits of EVOO-derived polyphenols, it will become highly important in terms of consumers' food literary to add the exact phenolic content on food labeling.

Moreover, there is substantial literature to support that the ways in which EVOO is consumed and/or cooked may influence total polyphenol bioavailability and absorption. For example, adding EVOO to the diet has synergistic effects while exposure to prolonged heat (e.g. as part of cooking) may reduce the oil's total polyphenol content (Brenes et al., 2002). This was not the case in the OLIVAUS study, since participants were instructed to add the intervention OOs in their daily meals in a raw uncooked form. Nevertheless, further data regarding the consumption of OO might be worthwhile investigating, to ascertain the

potential effect of cooking methods on the phenolic content and bioactivity of polyphenols in OO.

Qualitative data from this research indicated that participants perceived both dietary interventions (i.e., HPOO and LPOO) beneficial and enjoyable (responses collated but not reported in this work). Both dietary interventions were well received by participants as demonstrated by high rates of compliance (92% consumption of oils provided). Flavor also emerged as being a critical aspect, with participants of Mediterranean origin more prominently emphasizing the desirable flavor of extra virgin HPOO compared to LPOO. Participants also reported that they planned to maintain the changes introduced in their diet due to the implemented intervention in the long-term. These observations provide a structure for optimizing the development of dietary interventions and strategies that are more likely to achieve optimal outcomes in terms of dietary adherence and subsequently sustainable health outcomes.

Finally, while most of the studies have been conducted in Mediterranean populations that are accustomed to a high consumption of OO (George et al., 2019; Hohmann et al., 2015; Tsartsou et al., 2019), it is also important to understand whether there are genetic differences that may predispose individuals to the health benefits associated with polyphenol ingestion. Australia is a culturally diverse nation, with 49% of the population been either born overseas or with at least one parent born overseas (ABS, 2017). Given the high prevalence of CVD in Australia (AIHW, 2021) there is an urgent need for practical and cost effective interventions of wider reach, that will promote OO consumption and assess its efficacy on the prevention and management of CVD. The current study provides promising evidence for the cardioprotective effect of OO polyphenols in a multiethnic population, that is not accustomed to high OO intake. Nevertheless, additional research of

longer duration is warranted to demonstrate the sustainability of habitual EVOO consumption and related health benefits, especially in non-Mediterranean populations.

6.4   Conclusion

The work within this doctorate demonstrated that a dietary intervention that provides 60 ml of OO can be a feasible and effective approach in reducing the levels of certain CVD risk markers in a multi-ethnic population such as the Australian. This protective effect was evident after consumption of both extra virgin HPOO and LPOO, with HPOO showing a greater effect on CVD risk parameters, i.e., oxidative stress, inflammation and peripheral/central BP in people at higher CVD risk and therefore most in need for primary prevention initiatives. Furthermore, this research highlights that a key single component of the MedDiet (i.e., EVOO) can improve cardiovascular health risk in a multi-ethnic population with different habitual food cultures.

Taking into account the findings reported in this doctorate thesis, public health initiatives should a) promote consumers' awareness on the health benefits of EVOO consumption, and b) recognize the cardioprotective properties of EVOO-derived polyphenols by CVD dietary guidelines. This will assist in providing future recommendations on the concentration and volume of OO consumption that is required to achieve the observed clinical benefits.

The results of the OLIVAUS study contribute to the accumulating evidence which demonstrates the promising effect of OO polyphenol intake on cardiovascular related outcomes in healthy adults. Considering that most of the previous intervention studies were conducted in Mediterranean populations, findings of the OLIVAUS study provide evidence for a widely accessible, low-cost dietary intervention that can reduce CVD risk. This is of

particular importance in multicultural populations that are not accustomed to the use of EVOO as the primary source of fat in their daily diet.

More research assessing the impacts of this dietary intervention in larger cohorts over longer periods of time and/or higher concentrations of OO -derived polyphenols are required to a) confirm these findings and/or b) further understand the effect of OO phenolic compounds on additional CVD related risk markers and the involved mechanistic pathways in multi-cultural populations. These conclusions, if verified in further clinical trials, may be of value in reinforcing even more the important role of OO in human nutrition.

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Appendices

List of Appendices

Appendix A

Effect of high polyphenol extra virgin olive oil on markers of cardiovascular disease risk in healthy Australian adults (OLIVAUS): A protocol for a double blind randomized, controlled, cross-over study"

ORIGINAL RESEARCH

Effect of high polyphenol extra virgin olive oil on markers of cardiovascular disease risk in healthy Australian adults (OLIVAUS): A protocol for a double-blind randomised, controlled, cross-over study

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Abstract

Background: Previous clinical studies have suggested that high polyphenol extra virgin olive oil (EVOO) provides a superior cardioprotective effect compared to low polyphenol olive oil. However, further studies are required to replicate these results in non-Mediterranean populations.

Aim: To investigate the effect of high polyphenol EVOO versus low polyphenol olive oil with known polyphenol composition on markers of cardiovascular disease risk in a healthy non-Mediterranean cohort.

Methods: In a double-blind randomised cross-over trial, the present study will examine the effect of high polyphenol EVOO versus low polyphenol olive oil in 50 healthy participants. Each intervention phase will be 3 weeks long with a 2-week washout period between each phase. Outcomes to be assessed include HDL cholesterol efflux, oxidised LDL, blood lipids, C-reactive protein, arterial stiffness, blood pressure and cognitive function. Dietary intake, physical activity levels and anthropometry will also be collected.

Discussion: Because of the rigorous trial design, novel and clinically relevant outcomes, the use of a well-characterised EVOO, and, in contrast to the current literature, the non-Mediterranean study population, the present study will provide a significant contribution to the understanding of the clinical importance of polyphenol intake in the Australian sociocultural context.

Key words: biophenol, cognition, Mediterranean diet, olive oil, oxidative stress, polyphenol.

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Introduction

The traditional Mediterranean diet, known for its cardioprotective effect, has been shown to improve cardiovascular disease (CVD) risk factors including specific measures of blood lipids (HDL cholesterol (HDL-C), triglycerides), markers of inflammation, blood pressure, fasting blood glucose and risk of diabetes.[1,2] The traditional Mediterranean diet is characterised by an abundance of plant foods (e.g. leafy greens, tomatoes, onions, herbs, wholegrain cereals, legumes and nuts), moderate amounts of fermented dairy foods, seafood, red wine and small quantities of red meat and homemade sweets.[3–5] Of particular relevance to the proposed study, is the large servings of extra virgin olive oil (EVOO; 60–80 mL daily) as the primary source of culinary fat and a unique culinary component of the

Mediterranean dietary pattern. Olive oil contains highly variable concentrations of polyphenols which can be affected by season, olive variety, region and soil, ripeness of the fruit and processing.[6] EVOO is characterised by a low-temperature, mechanical processing technique which preserves the higher polyphenol content in comparison to the refining methods such as deodorisation and chemical processing techniques used to produce refined olive oils, which subsequently have significantly lower polyphenol content.[7,8]

In healthy adults, EVOO has been shown to improve CVD risk factors including blood pressure, low grade inflammation and lipid profile.[9] The cardioprotective properties of EVOO have been primarily attributed to the high monounsaturated fat content; however, EVOO contains an array of unique polyphenols, also referred to as 'biophenols'.[10] These polyphenols have shown improvements in measures of glucose metabolism, lipid peroxidation and cholesterol markers in clinical trials.[11–14] Despite this evidence, the unique, cardioprotective polyphenols in EVOO are not currently recognised by CVD guidelines, possibly because of the need for additional high-level evidence.

To further understand the mechanisms involved in the cardioprotective effect of EVOO-derived polyphenols, further clinical research is needed to: (i) replicate previously reported improvements in routinely measured cardiovascular markers (e.g. HDL/LDL cholesterol, blood pressure) in the Australian population; (ii) determine the feasibility of a provision of 60 mL of EVOO per day in a non-Mediterranean population and (iii) investigate the effect of high polyphenol EVOO on novel CVD risk markers. Increased CVD risk has, in part, been attributed to low plasma levels of (HDL-C).[15] However, emerging evidence suggests that impaired HDL function, rather than low HDL-C, may explain HDL-associated CVD risk.[16] HDL-C efflux, as measure of HDL function, has been identified as a marker that may independently predict risk of CVD.[17]

To improve the existing evidence base in this area, the proposed trial aims to investigate the effect of a high polyphenol EVOO compared to a low polyphenol olive oil on both routinely measured (e.g. blood pressure and cholesterol) and novel markers (e.g. HDL-C efflux) on CVD risk in a healthy Australian cohort.

Furthermore, recently published clinical and animal studies have provided preliminary evidence to suggest that EVOO, as well as other polyphenol-rich interventions, may improve cognitive performance and prevent age- or experimentally induced cognitive impairment.[18,19] Hence, as a secondary outcome, the present study will also investigate the effect of high polyphenol EVOO and low polyphenol olive oil on measures of cognitive performance in this healthy cohort.

Methods

The OLIVAUS study is a double-blind, randomised, controlled cross-over trial that aims to investigate the effect of a 3-week intervention of high polyphenol EVOO compared to a retail-purchased low polyphenol olive oil on CVD risk factors in 50 healthy participants (Figure 1). Compared with a low polyphenol olive oil, we hypothesise that a high polyphenol EVOO intervention will result in improved measures of HDL-C efflux, oxidised LDL and low-grade inflammation in a healthy adult population. The trial protocol (registered 30/04/2018, updated 13/02/2019) has been prospectively registered with the Australia New Zealand Clinical Trials Registry ACTRN12618000706279 and was created in accordance with the SPIRIT statement.[20]

This trial will be conducted in accordance with the Guidelines for Good Clinical Practice and the Declaration of Helsinki and CONSORT reporting guidelines. The trial team has obtained written approval for the protocol and Patient Information and Consent Form from the La Trobe University Human Research Ethics Committee (HEC17-067).

Participants will be recruited in Melbourne, Australia using social media advertisements, and through La Trobe University using email advertisements, mailing lists, word of mouth, and posters on campus and at local medical clinics. Table S1 (Supporting Information) provides the inclusion and exclusion criteria for the present study.

Figure 1 provides a visual representation of the study flow. The participant schedule throughout the trial is shown in Table S2, including data collection time-points. Once enrolled, participants will be asked to undergo an initial washout period where they will be instructed to abstain from consuming all olive oil, olive products, and antioxidant supplements for 2 weeks prior to the scheduled baseline meeting (T1). Participants will be requested to complete a 3-day diet diary including 2 week days and 1 weekend day where they are asked to include details on the foods and beverages consumed including type, brand,
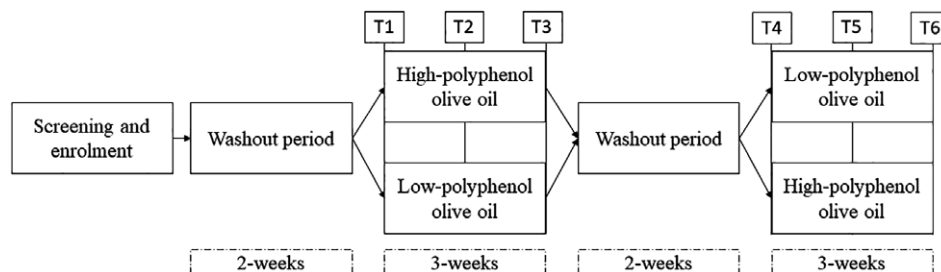
Figure 1 Study flow.

quantity in household measures and cooking methods. Participants will be asked to complete this diet diary in the days preceding the initial appointment and at the conclusion of the intervention phases. Participants will be asked to come to the baseline meeting in a fasted state. At the end of each intervention phase, participants will receive a $25 AUD gift voucher ($50 AUD in total).

The research staff will screen against the eligibility criteria during a face-to-face meeting. Following informed consent, participant numbers will be assigned sequentially and will be block randomised to receive either high polyphenol EVOO or low polyphenol olive oil. The block randomisation sequence will be developed using blocks of 6, by a senior researcher (GM), who will not have any direct involvement in the participant recruitment or data collection phase. After baseline measures are taken, a researcher who is not involved in any participant contact (JCW) will email the allocation for each participant to the team. De-identified bottles of high and low polyphenol olive oil will be randomised and coded prior to the recruitment phase and all staff will be blinded to this randomisation.

Participants will receive a 3-week supply of either the low polyphenol olive oil or high polyphenol EVOO (1.26 L) at the commencement of the first intervention (T1) and the commencement of the second intervention (T4). Participants will be required to consume 60 mL per day for each of the 3-week intervention phases. Measuring cups will be provided for participant use, where appropriate, to demonstrate the required volume. Emphasis on strategies that incorporate olive oil into their habitual diet in a raw, uncooked form will be provided by researchers. This will include dressing salads or vegetables, drizzling the oil on prepared meals such as soups or casseroles, and ensuring leftover amounts are also consumed. Participants will be supplied with the full amount of EVOO and olive oil required per 3-week intervention period.

Total polyphenol and polyphenol subclasses for each olive oil intervention were analysed by Modern Olives Laboratory Services (Lara, Australia), a Commonwealth Government accredited testing agency, using high-performance liquid chromatography. Samples were prepared and blinded for the researcher. Table S3 provides a comparison of the total polyphenol and polyphenol subclasses of each olive oil intervention. All high polyphenol EVOO was sourced from Cobram Estate Pty. Ltd. from the same harvest and lot and stored under the same conditions. An EVOO with a confirmed polyphenol count of approximately 320 ppm will be provided to participants as the high polyphenol EVOO intervention. A low polyphenol olive oil was sourced from a local supermarket where a bulk purchase of the same brand from the same lot number was made. This oil was confirmed to have a polyphenol count of approximately 86 ppm.

At the commencement of the first and second intervention phase meeting (T1 and T4), participants will attend a 1-hour appointment in the morning with research staff at the nutrition clinical rooms, Bundoora campus, La Trobe University. Data collection including 3-day diet diaries, medical history and lifestyle (e.g. physical activity) questionnaires, anthropometry, fasting blood collection, blood pressure, arterial stiffness measures and cognitive performance will take place at each face to face appointment. Basic demographic data will also be collected at baseline including age, gender and ethnicity. These are described in detail below.

The research staff will contact participants by phone or email approximately 1.5 weeks into each intervention phase to discuss progress, adherence to the intervention and to ask participants if they have experienced any adverse events during the study period.

At the end of each intervention phase (T3 and T6) participants will attend a face to face appointment where they will complete all the data collection indicated at the T1 and T3 appointment. In addition, participants will be required to return their olive oil bottles so that research staff can record the weight of any remaining oil as an additional marker of adherence.

For T3 only: Research staff will instruct the participants to undergo a 2-week washout period whereby they cease consumption of all olive oil and olive products during this period, until their next meeting (T4, start of second olive oil phase).

For T6 only: Research staff will assess blinding by asking the participant about the order they think they received the two olive oil interventions and whether there were any differences in taste.

All outcomes described below will be measured pre and post the olive oil intervention phases (T1, T3, T4, T6) as per Table S2. Blood collection will also take place at each pre- and post-time-point. Research staff will confirm that participants have fasted for 8–12 hours. If so, fasting venous blood samples will be obtained, by a researcher trained in venepuncture, from the antecubital vein using standard venous puncture techniques. If blood collection is unsuccessful research staff will arrange for blood collection at a local pathology centre within 48 hours of the scheduled appointment.

HDL-C efflux, the primary outcome, will be analysed using a Cholesterol Efflux Fluorometric Assay Kit (Biovision, Milpitas, California). Participants will be invited to participate in an optional cognitive performance assessment. If they have consented to this aspect of the trial, the participant will conduct the full cognitive assessment at each face to face appointment. The Swinburne University Computerised Cognitive Assessment Battery (SUCCAB) is a validated, computer-based cognitive battery, administered using a 5-button control box.[21] Eight tests of cognitive function will be assessed by both accuracy and response time. These tests include Simple and Choice Reaction Times, Immediate and Delayed Recognition, Congruent and Incongruent Stroop colour-words, Spatial Working Memory and Contextual Memory. This battery has been used in numerous studies to assess the cognitive effects of dietary supplementation and other interventions.[22–24]

Total, HDL and LDL cholesterol, high sensitivity C-reactive protein and triglyceride levels will be measured using standard enzyme assays. Oxidised LDL will also be analysed using a solid phase two-site enzyme immunoassay (ELISA; Mercodia, Uppsala, Sweden).

Cardiovascular function will be assessed using the non-invasive SphygomoCor XCEL system (AtCor Medical, Australia) once the participant has rested for 5 minutes in the supine position. Assessments will include standard brachial blood pressures, aortic (central) blood pressures, pulse wave analysis of peripheral arterial stiffness, and carotid-femoral pulse wave velocity analysis of central arterial stiffness.

Three-day diet diaries will be collected at each face to face appointment. Research staff will conduct a baseline interview with all participants and will confirm that they underwent the required 2-week washout period. The research staff will also review the 3-day dietary intake data to ensure sufficient detail has been recorded for nutrient analysis and to clarify any missing data on responses that look inaccurate. Participants will self-report details regarding their intake of food and liquids over a 3-day period including the quantity (via household measures), type and timing of items consumed. Furthermore, a specific section to capture timing and amount of olive oil will be incorporated. Participant weight, height and waist circumference will be measured using standard techniques, in duplicate by the research staff. If there is >10% variation between the two measures, a third measure will be obtained. The mean of the closest two measures will be used. Self-reported physical activity will be completed prior to the commencement of the trial (T1) and at the end of the trial (T6) via the Active Australia Survey,[25] a validated tool within the Australian population and consists of eight questions to assess the previous 7 days. The questionnaire captures a range of activity types including walking, work in the yard, vigorous physical activity and moderate physical activity. Adverse events will be monitored at all time-points. If a participant experiences significant adverse events, they will be withdrawn from the study. All adverse events will be reported to the trial steering committee, comprised of the Principal Investigator and trial staff. The Human Research Ethics Committee will also be notified, as appropriate. Emergency unblinding will occur for serious adverse events deemed related to the study product. All participant data will be securely stored either in onsite locked cabinets or password protected documents on secured university servers with restricted access to the study team only.

All outcomes will be analysed by using linear mixed-effects (LMEs) models with random intercepts and slopes to account for within-participant correlation over time and varying treatment effect among participants. The effect of intervention order, because of potential carry-over effect, on all outcomes will be tested and adjusted for in the LME model if necessary by including and inter-action term between the treatment and period effects. A senior statistician (LAP) will oversee the fitting of the LME models and be responsible for assessing model validity.

Participant 3-day dietary records will be analysed and dietary changes will be used as a covariate. Adjusted results will be calculated using a multiple linear regression model including the stratification factors (e.g. gender, physical activity levels). A sensitivity analysis comparing the LME analyses and pooled estimates from the multiple imputation procedures will be conducted to prevent against bias. All reported *P*-values will be 2-tailed. The levels of statistical significance will be set at $P < 0.05$ and estimates will be accompanied with 95% confidence intervals. All statistical analyses will be conducted using the SPSS statistical software for Windows (version 25); IBM, Armonk, New York. Based on the results of previous research, a sample size of 40 was considered adequate to provide sufficient statistical power to detect a statistically significant 5% difference in HDL-C efflux between the two intervention phases with 80% power and 5% level of significance.[26] To account for a 20% level of potential attrition, this sample size was expanded to 50 participants.

Results

Recruitment commenced in July 2018 and is expected to be completed by late-2019. Currently, a total of n = 21 participants have been enrolled in this trial, leading to an average recruitment rate of 7 per month. Sixty-five per cent of participants are female with a mean age of 37 years. Five of the currently recruited cohorts have completed the intervention with 100% of outcome data collected. Incomplete data have been collected on one participant due to withdrawal from the study because of inability to consume the required amount of olive oil. Ten per cent of participants that have completed the intervention consumed at least 80% of the provided oils. There have been no reported serious adverse events related to the study intervention. Reported adverse events include diarrhoea, bloating, reflux and heartburn.

Discussion

Previous clinical studies have reported that EVOO provides a cardioprotective effect through mediating improvements in cardiovascular risk factors;[1,9] however, few studies have investigated the contribution of the polyphenol component of olive oil to these improvements. The present study will compare the effect of high polyphenol EVOO to low polyphenol olive oil on markers of CVD risk that are related to cholesterol transport and metabolism, LDL oxidation, blood pressure (peripheral and central), arterial stiffness, and inflammation, as well as measures of cognitive function. By implementing a study design that will be able to differentiate between the effect of polyphenols from the other components of olive oil (e.g. monounsaturated fat will remain consistent between study arms), this trial will provide important information regarding the effect of EVOO polyphenols on a range of cardiovascular risk factors and cognition. In contrast to the current literature which has predominantly been conducted within Mediterranean

populations, this will assess the use of high polyphenol EVOO in the Australian western sociocultural context. In addition, previous research has primarily assessed the effect of a Mediterranean diet and EVOO in populations with existing comorbidities such as coronary heart disease, type 2 diabetes, cancer and cognitive decline while the present study aims to recruit healthy participants.[2,27] The present study is one of the first trials to comprehensively assess the polyphenol composition within each of the oils provided to participants. Other studies, even those which compare oils with varying polyphenol content, do not report the composition of the polyphenols contained within.[9] Finally, this study will report HDL efflux, oxidised LDL and other biomarkers of CVD that have not been extensively studied in previous dietary intervention studies. If shown to be beneficial, the present study will provide evidence for a widely accessible, low cost dietary intervention to reduce CVD risk and will significantly contribute to the existing literature on the clinical importance of polyphenol intake.

Funding source

Conflict of interest

ESG, CI, CJT and HLM have received food donations for previous trials (Cobram Estate Pty Ltd., Jalna Dairy Foods Pty Ltd., Almond Board of Australia, Simplot Australia Pty Ltd., Birds Eye, HJ Heinz Company Australia, Carmen's Kitchen).

Authorship

All authors contributed to the development of the protocol. WM and ESG lead the development of the manuscript. KS provided data on current trial results. LAP provided statistical support. GK and AP provided the information regarding the cognitive function component. CJT, HLM, CI and JCW all contributed to the manuscript. We declare that the content of this manuscript has not been published elsewhere. We would like to acknowledge the work of Mr Siddharth Shivantha (Honours student) in the early data collection phase of this trial.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Study eligibility criteria.
Table S2 Study procedure and time points in the OLIVAUS trial.
Table S3 Polyphenol composition of the HPOO and LPOO provided in the OLIVAUS trial.

Appendix B

Ethics Approval

** This is an automatically generated email, please do not reply. Contact details are listed below.**

Dear Researcher,

Please find attached information regarding your application for ethical review of research involving human participants at La Trobe University.

Application ID: HEC17-067
Application Status/Committee: Finalised - Approved

Project Title: The effect of high-polyphenol olive oil on markers of cardiovascular disease risk

Chief Investigator: Wolfgang Marx
Other Investigators: Luke Prendergast, Catherine Itsiopoulos, Colleen Thomas, Elena Papamiltiadous, Hannah Mayr, Audrey Tierney

To access the application online, log in to ResearchMaster: http://rmenet.latrobe.edu.au

NOTE: This email is not a confirmation of submission. Please check the 'Status' of the application in ResearchMaster.

If you have any further questions, please contact the:
UHEC at humanethics@latrobe.edu.au
SHE College Human Ethics Sub-Committee at chesc.she@latrobe.edu.au
ASSC College Human Ethics Sub-Committee at chesc.assc@latrobe.edu.au

Appendix C

Participants screening questionnaire

OliveOil Trial: Participant Screening Form	Version Version 4.0, 2018_20.08.2018
Participants HAVE TO be the following to be included	
◯	Aged 18-75
◯	English-speaking persons
◯	BMI: 18.5-40 kg/m2
Participants CANT be the following to be included	
◯	Pregnant or lactating women
◯	History of adverse reactions to olive oil or olives
◯	Currently prescribed warfarin, anti-coagulant therapy, statin medications, all oral hypoglycaemic agents, insulin, cyclosporine, tacrolimus, immunosuppressant agents, antihypertensive agents, and nonsteroidal anti-inflammatory drugs, hormone replacement therapy
◯	A habitual diet with ≥1 tablespoons of olive oil per day
◯	Current smoker
◯	Use of antioxidant supplements
◯	Diagnosed with any of the following conditions: hyperlipidaemia; diabetes mellitus; hypertension; inflammatory conditions (e.g. rheumatoid arthritis), intestinal disease (e.g. inflammatory bowel disease); irritable bowel syndrome, food intolerances, blood coagulation disorders, any cognitive or mood disorder, any other physiological condition or disease that could impair adherence, anti depressant medication
◯	Dieting (Special diets i.e gluten free, weight loss, etc.)

Appendix D

Patient Information and Consent Form
Patient Withdrawal Form

COLLEGE OF SCIENCE, HEALTH AND ENGINEERING

School of Allied Health
La Trobe University
Victoria 3086 Australia

MELBOURNE CAMPUSES
Bundoora
Collins Street CBD
Franklin Street CBD

REGIONAL CAMPUSES
Bendigo
Albury-Wodonga
Mildura
Shepparton

Consent Form

Declaration by Participant

- I have read the Participant Information Sheet or someone has read it to me in a language that I understand
- I understand the purposes, procedures and risks of the research described in the project.
- I give permission for my doctors, other health professionals, hospitals or laboratories outside this hospital to release information to *the researchers* concerning my disease and treatment for the purposes of this project. I understand that such information will remain confidential.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I freely agree to participate in this research project as described and understand that I am free to withdraw at any time during the study without affecting my future health care.
- I understand that I will be given a signed copy of this document to keep.
- I agree that research data provided by me or with my permission during the project may be included in a thesis, presented at conferences and published in journals on the condition that neither my name nor any other identifying information is used.

Name of participant (please print)			
Signature		Date	

Declaration by Researcher

I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Name of researcher (please print)			
Signature		Date	

Note: All parties signing the consent section must date their own signature.

School of Allied Health
La Trobe University
Victoria 3086 Australia

MELBOURNE CAMPUSES
Bundoora
Collins Street CBD
Franklin Street CBD

REGIONAL CAMPUSES
Bendigo
Albury-Wodonga
Mildura
Shepparton

Withdrawal of Participation Form

Declaration by Participant

I wish to withdraw from participation in the above research project and understand that such withdrawal will not affect my relationship with *La Trobe University.*

Name of participant (please print)			
Signature		Date	

In the event that the participant's decision to withdraw is communicated verbally, the Study Researcher will need to provide a description of the circumstances below.

Declaration by Study Researcher

I have given a verbal explanation of the implications of withdrawal from the research project and I believe that the participant has understood that explanation.

Name of researcher (please print)			
Signature		Date	

Note: All parties signing the consent section must date their own signature

Appendix E

Standard Operation Procedure.

Sampling, recruitment, coding, treatment allocation and randomization procedures

Polyphenol Olive Oil Project

Standard Operating Procedure

Sampling, recruitment, coding, treatment allocation and randomization procedures

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Contents

1.1 Introduction and purpose

A randomized, crossover, controlled intervention study is any research study that allocates human participants or groups of humans to two or more treatment arms in order to evaluate the effects on specific research outcomes. Randomization is of fundamental importance in a randomized, crossover, controlled trial since random allocation of study participants to treatment arms ensures that any differences between the groups at trial entry are entirely due to chance and that each study participant will have the same likelihood of receiving each treatment.

The purpose of the current SOP is to describe the procedures for randomization of eligible study participants identified during the first screening phase in the two treatment arms that will receive high polyphenol olive oil first followed by low polyphenol olive oil (**intervention group AB)** or low polyphenol olive oil first followed by high polyphenol olive oil **(intervention group BA)**.

This SOP follows the advice of the "CONsolidated Standards Of Reporting Trials" (CONSORT) Group, which provides a standardized guidance to authors in improving the reporting of randomised clinical trials and is a prerequisite for publishing scientific articles that report the results from these trials by many scientific journals.

1.2 Sampling and recruitment procedures

The study will be conducted with healthy adults, aged 18 to 48 years old, men and women, residing in Melbourne (Australia) and are fulfilling all other eligibility criteria as described in the study protocol. The study will be advertised through printed material (i.e posters, flyers etc.), social media as well as channels moderated by La Trobe University.

At the first screening, all interested participants will be evaluated via telephone interview or face-to-face meeting, to assess their eligibility for participation to the study using the "First screening questionnaire".

This first screening will identify 50 eligible subjects that will be equally and randomly allocated to the two study groups i.e intervention group AB and intervention group BA (n=25 adults in each study group).

1.3 Randomization procedures

The block randomization method will be used to randomize study participants in the two study groups. Randomization of study participants to each of the two study groups will be based on a computer-generated sequence, which will be known only by one research team member. The size of each block will be 6 in order to ensure the equal allocation of study participants to the two study groups. An example of the block randomization procedure is presented below:

Block 1	Block size: 6
ID	Study group
01	Intervention AB
02	Intervention BA
03	Intervention BA
04	Intervention AB
05	Intervention AB
06	Intervention BA

Block 2	Block size: 6
ID	Study group
07	Intervention BA
08	Intervention AB
09	Intervention BA
10	Intervention BA
11	Intervention AB
12	Intervention AB

Block 3	Block size: 6
ID	Study group
13	Intervention AB
14	Intervention BA
15	Intervention AB
16	Intervention BA
17	Intervention AB
18	Intervention BA

Block 4	Block size: 6
ID	Study group
19	Intervention AB
20	Intervention AB
21	Intervention AB
22	Intervention BA
23	Intervention BA
24	Intervention BA

Block 5	Block size: 6
ID	Study group
25	Intervention BA
26	Intervention BA
27	Intervention AB
28	Intervention AB
29	Intervention BA
30	Intervention BA

Block 6	Block size: 6
ID	Study group
31	Intervention AB
32	Intervention AB
33	Intervention BA
34	Intervention BA
35	Intervention AB
36	Intervention AB

Block 7	Block size: 6
ID	Study group
37	Intervention BA
38	Intervention AB
39	Intervention BA
40	Intervention AB
41	Intervention BA
42	Intervention AB

Block 8	Block size: 6
ID	Study group
43	Intervention BA
44	Intervention BA
45	Intervention BA
46	Intervention AB
47	Intervention AB
48	Intervention AB

Block 9	Block size: 2
ID	Study group
49	Intervention BA
50	Intervention AB

Furthermore, in order to compensate for early withdraws and/or dropouts of study participants we included four additional randomization blocks.

Block 10	Block size: 6
ID	Study group
51	Intervention AB
52	Intervention BA
53	Intervention AB
54	Intervention AB
55	Intervention BA
56	Intervention BA

Block 11	Block size: 6
ID	Study group
57	Intervention BA
58	Intervention AB
59	Intervention AB
60	Intervention AB
61	Intervention BA
62	Intervention BA

Block 12	Block size: 6
ID	Study group AB
63	Intervention AB
64	Intervention BA
65	Intervention BA
66	Intervention BA
67	Intervention AB
68	Intervention AB

Block 13	Block size: 2
ID	Study group
69	Intervention AB
70	Intervention Ba

1.4   Coding procedures

A unique identification (ID) code will be provided to each study participant at the recruitment stage (when the individual is registered as a new participant). Further to participants' registration to the study, a 4-digit ID code will be added with each digit corresponding to specific information as indicated in **Table 1** below.

Table 1. Explanation of digits used to produce the ID code for study participants at baseline.

Digit	Indicates	Coding
1st to 2nd	Study group	30: Intervention group AB (T1) → BA (T4)
		33: Intervention group BA (T1) → AB (T4)
3rd to 4th	Study number	An ascending 2-digit study number will be provided to each eligible based on the order to the adult's entry to the study.

The following two examples indicate the coding that two adult participants will receive at baseline.

Example 1:

ID	3	0	0	4
	↓		↓	
	Study group: Intervention AB		Adult: 4	

Example 2:

ID	3	3	1	4
	↓		↓	
	Study group: Intervention BA		Adult: 14	

Important Note!
The ID needs to be consistently and correctly entered by research assistants to all CRFs and questionnaires before these are filled out at each time point of examination (i.e. baseline and each follow-up visit).

Appendix F

Certificate of Analysis

modern olives laboratory services

PO BOX 92 LARA VIC 3212 TEL: 03 5272 9570 FAX: 03 5272 9599 EMAIL: lab@modernolives.com.au

Certificate of Analysis   AU 18/0684

Company:	LaTrobe University	**Report Date:**	19/07/2018
Attention:	Jane Willcox	**Date Received:**	17/07/2018
Address:		**Sampled By:**	Submitter
	VIC	**Sample Type:**	Extra Virgin Olive Oil
Phone:	03 9479 1520		
Email:	J.Willcox@latrobe.edu.au		

Sample Description	Batch	Laboratory Reference
Moro Everyday Cooking Olive Oil Batch 18106E12		AU 18/0684/1

NATA Accreditation № 15594

Accredited for compliance with ISO/IEC 17025 - Testing.

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PO BOX 92 LARA VIC 3212 TEL: 03 5272 9570 FAX: 03 5272 9599 EMAIL: lab@modernolives.com.au

RESULTS

Biophenols profile	Units	AU 18/0684/1				Method	Uncertainty
Biophenols profile							
Hydroxytyrosol	mg/kg	5.3				IOC/T.20/No.29	
Tyrosol	mg/kg	5.1				IOC/T.20/No.29	
Vanillic acid + Caffeic acid	mg/kg	0.0				IOC/T.20/No.29	
Vanillin	mg/kg	0.6				IOC/T.20/No.29	
p-Coumaric Acid	mg/kg	1.0				IOC/T.20/No.29	
Hydroxytyrosol Acetate	mg/kg	0.0				IOC/T.20/No.29	
Ferulic acid	mg/kg	0.8				IOC/T.20/No.29	
o-Coumaric Acid	mg/kg	0.0				IOC/T.20/No.29	
Decarb. oleuro aglycone, Ox Al	mg/kg	2.7				IOC/T.20/No.29	
Oleacein	mg/kg	6.3				IOC/T.20/No.29	
Oleuropein	mg/kg	1.0				IOC/T.20/No.29	
Oleuro aglycone, Al	mg/kg	0.8				IOC/T.20/No.29	
Tyrosol Acetate	mg/kg	0.1				IOC/T.20/No.29	
Decarb. ligstr aglycone, Ox Al	mg/kg	3.8				IOC/T.20/No.29	
Oleocanthal	mg/kg	11.2				IOC/T.20/No.29	
Pinoresinol + 1 Acetoxy pinore	mg/kg	5.5				IOC/T.20/No.29	
Cinnamic Acid	mg/kg	2.0				IOC/T.20/No.29	
Ligstroside aglycone, Al	mg/kg	0.4				IOC/T.20/No.29	
Oleuro aglycone, Ox Al Hy	mg/kg	3.0				IOC/T.20/No.29	
Luteolin	mg/kg	1.8				IOC/T.20/No.29	
Oleuro aglycone, Al Hy	mg/kg	22.0				IOC/T.20/No.29	
Ligstro aglycone, Ox Al Hy	mg/kg	1.4				IOC/T.20/No.29	
Apigenin	mg/kg	1.0				IOC/T.20/No.29	
Methyl-Luteolin	mg/kg	3.0				IOC/T.20/No.29	
Ligstroside aglycone, Al Hy	mg/kg	7.9				IOC/T.20/No.29	
Total Biophenols - HPLC	ppm	86.4				IOC/T.20/No.29	18.777

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(*) Asterisk denotes tests covered under our NATA scope of accreditation, or the scope of accreditation of our designated approved subcontractors at the time this report was issued.

(^) Circumflex denotes tests subcontracted to a designated approved supplier. All subcontractors are regularly evaluated as part of our approved supplier program; however, their NATA or TGA accreditation status is not.

Australian Standard® 5264-2011 limits are only for the sample type specified. If the result is in bold and underlined, it indicates that the sample does not comply with the limits stated for the category/grade/type.

American Oil Chemists' Society (AOCS) recognition for chemical and sensory analysis is valid for this current year.

AOCS chemist approval since 2009.

International Olive Council (IOC) recognition for chemical analysis is valid for this current year.

TGA licence Nº: MI-2017-LI-01351-1

Peter Nixon
Senior Chemist

Claudia Guillaume
Laboratory Manager

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PO BOX 92 LARA VIC 3212 TEL: 03 5272 9670 FAX: 03 5272 9699 EMAIL: lab@modernolives.com.au

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Certificate of Analysis   AU 18/0351

Company:	Boundary Bend Olives Pty Ltd	**Report Date:**	1/06/2018
Attention:	Tank Farm	**Date Received:**	7/05/2018
Address:	PO BOX 92	**Sampled By:**	Submitter
	LARA VIC 3212	**Sample Type:**	Extra Virgin Olive Oil
Phone:	03 5272 9500		
Email:	tankfarm@boundarybend.com		

Sample Description	Laboratory Reference
100251180507BR01001 CE Hojiblanca 2018	AU 18/0351/1

NATA Accreditation № 15594

Accredited for compliance with ISO/IEC 17025 - Testing.

Biophenols profile	Units	AU 18/0351/1				Method	Uncertainty
Cinnamic Acid	mg/kg	3.7				IOC/T.20/No.29	
Ligstroside aglycone, Al	mg/kg	3.2				IOC/T.20/No.29	
Oleuro aglycone, Ox Al Hy	mg/kg	13.8				IOC/T.20/No.29	
Luteolin	mg/kg	13.9				IOC/T.20/No.29	
Oleuro aglycone, Al Hy	mg/kg	44.6				IOC/T.20/No.29	
Ligstro aglycone, Ox Al Hy	mg/kg	6.4				IOC/T.20/No.29	
Apigenin	mg/kg	9.3				IOC/T.20/No.29	
Methyl-Luteolin	mg/kg	5.0				IOC/T.20/No.29	
Ligstroside aglycone, Al Hy	mg/kg	9.0				IOC/T.20/No.29	
Total Biophenols - HPLC	ppm	320.3				IOC/T.20/No.29	18.777
-	-	-	-	-	-	-	-
Antioxidants	Units	AU 18/0351/1				Method	Uncertainty
☐ Tocopherols	ppm	197.0				ISO 9936	9.147
Squalene	ppm	12,101.3				MO-SM-P-13	354.809
-	-	-	-	-	-	-	-
Contaminants	Units	AU 18/0351/1				Method	Uncertainty
Benzo(a)pyrene	ug/kg	<5				AOCS Cd 21-91	0.587
Bis(2-ethylhexyl) phthalate	mg/kg	0.16				MOSMP20	0.17
-	-	-	-	-	-	-	-

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(*) Asterisk denotes tests covered under our NATA scope of accreditation, or the scope of accreditation of our designated approved subcontractors at the time this report was issued.

(^) Circumflex denotes tests subcontracted to a designated approved supplier. All subcontractors are regularly evaluated as part of our approved supplier program; however, their NATA or TGA accreditation status is not.

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AOCS chemist approval since 2009.

International Olive Council (IOC) recognition for chemical analysis is valid for this current year.

TGA licence N°: MI-2017-LI-01351-1

Claudia Guillaume
Laboratory Manager

Appendix G

Socio-demographic data questionnaire

OliveOil Trial: CRF Form	Version 5.0, 2019_28.05.2019			
Participant number		**Date**		
Chief Investigator: George Moschonis (In any case call: Katerina Sarapis , 9479 5812)				
Country of birth	**Date of birth**	**Age (years)**		
English 1st language	**Gender**			
○ Yes	○ Female			
○ No	○ Male			
	○ Other:			
Highest level of education attained	**Ethnicity**		**Country of birth**	
Total years of education:			Mother	Father
○ Primary	○ ATSI	○ African		
○ Secondary	○ Caucasian Australian	○ Asian		
○ Tertiary	○ Caucasian European	○ Islander		
○ Trade	○ Caucasian American	○ Latin American		
○ Other:		○ African American		
Prescribed medications				
Supplements				

Appendix H

Participant booklet

Dietary Intake/3-day Food Diary

Physical activity/Active Australia Survey questionnaire

COLLEGE OF SCIENCE, HEALTH AND ENGINEERING

Polyphenol-olive oil study

Participant information booklet

Participant ID _____

COLLEGE OF SCIENCE, HEALTH AND ENGINEERING

Contents

LA TROBE
UNIVERSITY

COLLEGE OF SCIENCE, HEALTH AND ENGINEERING

Introduction and overview

Dear participant,

Thank you for your participation in our research study. We have provided the following overview in case you are unsure of certain points about the trial; however, if you have any further queries, please do not hesitate to contact the research staff email: 19662040@students.latrobe.edu.au.

What do I have to do?

The trial will run over 10 weeks. To participate in this trial, you are asked to

- Consume 60ml of olive oil each day during two runs of three weeks.
- During each of the three-week periods, you are also asked to complete the sections in this booklet
- Throughout the trial, you will be asked to attend 4 meetings with the research staff. During this time, we will take a blood sample, ask you about your diet and physical activity levels, record your blood pressure and weight, and ask you to complete a cognitive performance tool.

What information should I remember to tell the research staff?

If any the following occur during your participation in the study, please tell the research staff as soon as you can.

- Become pregnant
- Prescribed new medications or there are changes in your currently prescribed medications
- Commence any form of dietary supplement
- Fall sick, are hospitalised, or require surgery

I think I am experiencing a side-effect. What do I do?

If you are experiencing what you think might be a side-effect of your participation in this trial, please tell your doctor and research staff as soon as you can. If you feel that this is a serious side-effect, please cease consuming the olive oil and contact emergency services immediately.

When and how should I take my olive oil and how much should I take per day?

- You are asked to consume 60ml of olive oil per day.
- We recommend you consume your olive oil raw as much as possible. This means avoiding using your olive oil when frying, baking, and grilling.
- To avoid gastrointestinal symptoms, we highly recommend you space the olive oil over the day and consume with meals.

What happens if I miss a day?

If you are unable to consume your olive oil on a particular day, please record this on the daily consumption checklist

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3-day Food Diary

GENERAL INSTRUCTIONS

- We would like you to keep this diary of everything you eat and drink for 3 complete days, <u>2 weekdays and 1 weekend day</u>. Try to choose days that represent your typical eating patterns, if you are unable to choose typical days, there is space on the diary in the notes/comments section for you to let us know for e.g. if you went out to dinner or were on holidays.

- This is a **VERY** important part of the study and will greatly add to the information you have already provided us.

- It is very important that you **DO NOT** change what you eat and drink just because you are keeping a record.

- Write any food or drink down as soon as you consume them.

- If you don't eat a particular meal or snack, please draw a line through it to let us know that you haven't eaten anything.

- Please use a pen and write clearly.
- If you have any queries, please telephone (03) 9479 5812.

Thank you for your efforts in keeping this food diary!

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INSTRUCTIONS FOR RECORDING FOODS

Please keep an accurate record of everything that you eat and drink. Start with the first thing you eat and drink (when you wake up) and end with the last thing that you eat and drink at bedtime (before you go to bed)

Please keep the following things in mind when you are filling out your food diary;

- **Describe the form** the food is in (canned, powdered, dried, fresh etc).

- Record the **brand name** of the food or any special characteristics e.g. colour of packaging.

- **Describe the food in detail**. Do not simply write peaches, bread or yoghurt...

    E.g. SPC tinned peaches in syrup
    E.g. TIP TOP (toast thick) sliced white bread
    E.g. Yoplait 97% fat free strawberry yoghurt

- Include the **quantity** for all foods and beverages. To describe amounts use common household measures (teaspoon, tablespoon, cup), standard serving sizes (a slice of bread, biscuit), metric measurements (cm, grams, mL) or the ruler and circles to measure circumference and thickness **(see page 4)**.

- Explain the **cooking method** e.g. Roasted potato or steamed fish.

- Don't forget to **include the fat/oil** that you use to cook with *e.g. 2 tablespoons Olive oil.*

- For **foods with multiple ingredients** e.g. pasta, sandwich, soup, stir-fry, curry etc., please list all ingredients in the dish, including quantities where possible.

- There is space at the back of the food diary to **record recipes**. Feel free to send along a recipe or food label to improve accuracy of your analysis. *Recipes which we have provided you with do not need to be written down.*

- Include **items you add at the table** *e.g. 1 tablespoon Heinz salt reduced tomato sauce, ½ tsp pepper.*

- Many **packet foods have weights** printed on them, so please use these to record how much you ate. If possible, please include the food label.

- When you are **eating out**, and are unable to use household measures use your body for comparison,
    o **Fist** = 1 cup of fruit or 1 medium whole, raw fruit
    o **Thumb** = 30g of cheese or meat
    o **Tip of Thumb** = Approximately 1 teaspoon

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La Trobe University
Victoria 3086 Australia

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- **One Cupped Hand** = 30-60g of dry goods (nuts, cereal, pretzels etc.)

Ways to Size Up Your Servings

85 grams of meat is about the size and thickness of a deck of playing cards. **85g** =
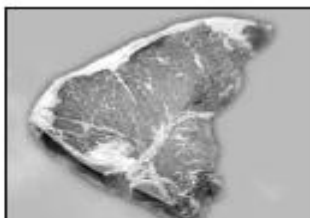
30 grams of cheese is about the size of 4 stacked dice. **30g** =

½ cup of ice cream is about the size of a tennis ball **½ Cup** =

1 cup of mashed potatoes or broccoli is about the size of your fist. **1 Cup** =

1 teaspoon of butter or peanut butter is about the size of the tip of your thumb. **1 tsp.** =

Photos courtesy of: National Dairy Council, USA

Use comparisons- for describing portion sizes, it may be easier to describe portion sizes by comparing to objects eg. Potato the size of a hen's egg, cheese-the size of a matchbox.

Weight on packaging- may also be useful in determining the amount eaten- eg. One buddy size coca cola= 600ml.

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MEASURING PAGE
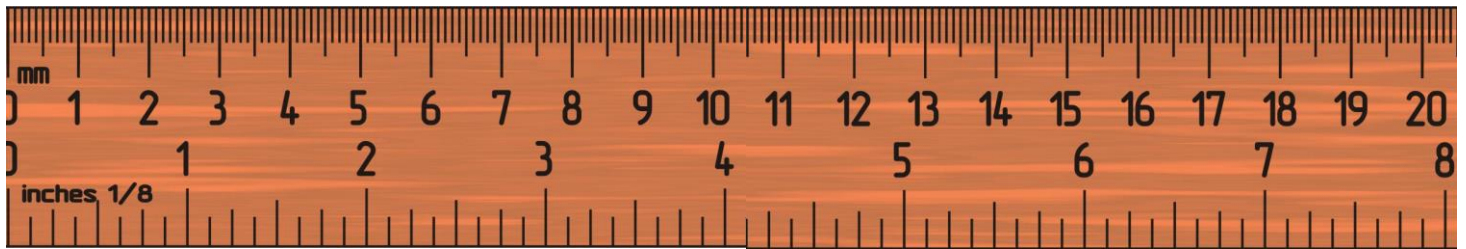
How long is it

Please note if level, rounded or heaped, teaspoons or tablespoons.

Level   Rounded   Heaped

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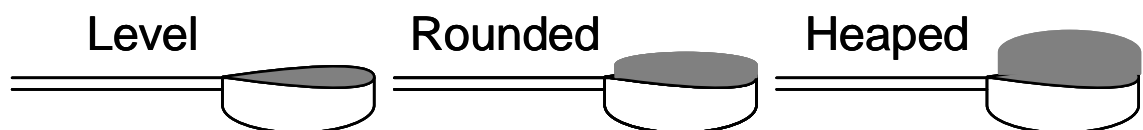
FOOD RECORD – EXAMPLE

Day of the week Thursday   **Date** 01 / 09 / 2013

MEAL/SNACK + Time	FOOD and DRINKS consumed (with type and brand)	QUANTITY (cups, spoons, no.)	Was Olive oil consumed? How much?	COOKING METHOD	WHERE/ WHO?
EARLY MORNING 7.00am	Coles low-fat Milk Nestle Milo	1 glass 1 heaped tspn	X		At home, kitchen table, alone
BREAKFAST 9.00am	Sultana Bran Coles low-fat Milk Tip Top White Bread (Toast sliced) Flora light margarine Black earl grey tea	1 cup ½ cup 2 slices 1 heaped tbspn 1 cup	X	Toasted	At home, kitchen table, with wife
MORNING TEA 11.00am	Banana Nescafe instant coffee Coles Full Cream Milk Sugar- white	1 med- 15cm long 1 heaped tsp 2 level tbspn 1 level tsp	X	Raw	At work, desk, alone
LUNCH 1.00pm	White bread roll - Bakers Delight Virginian Ham Coon Tasty Cheese Tomato Coca Cola Grapes	1 roll- Circle F 3 large slices- 18 cm long, a thick 2 slices (30g) 1 slice- Circle G, b thick 1 Can (375mL) 10 grapes	Yes, 1 tablespoon	Raw Raw	At work, tea room, with colleagues
AFTERNOON TEA					
DINNER 7.00pm	Chicken stir fry **see recipe 1**. White wine - Riesling	1 serve 2 wine glasses	Yes, 1 tablespoon		At home, kitchen table, with wife
SUPPER 9.00pm	Chocolate self saucing pudding-white wings Peters vanilla ice cream Natural Almonds	1 large serve- slice 10cm long 5 cm wide 6 cm thick ½ cup ½ cupped hand		Baked	At home, in front of TV, alone
OTHER SNACKS	Tim-Tams Allens snake lollies	3 biscuits hand full			At work with collegues.

Do you feel that today was typical of your usual diet?   Yes   No   Somewhat

Notes/Comments: There was a party at work today and I had the Tim-Tams and lollies

DON'T FORGET TO INCLUDE DRINKS: (Tea, Coffee, Water, Juice, Milk, Alcohol, Soft Drink ETC and QUANTITY- 1 cup/ mug/ 100ml etc) and of you add sugar to coffee tea i.e. 2 sugars, or 1 equal etc.)

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DAY 1

Day of the week _____   **Date** _____/_____/_____

MEAL/SNACK	FOOD and DRINKS consumed (type and brand)	QUANTITY (cups, spoons, no. etc.)	Was Olive oil consumed? How much?	COOKING METHOD	WHERE/ WHO?
EARLY MORNING Time:					
BREAKFAST Time:					
MORNING TEA Time:					
LUNCH Time:					
AFTERNOON TEA Time:					
DINNER Time:					
SUPPER Time:					
OTHER SNACKS					

Do you feel that today was typical of your usual diet?   Yes   No   Somewhat

Notes/Comments:

How do you incorporate 60mL of raw olive oil in your daily diet:

DON'T FORGET TO INCLUDE DRINKS: (Tea, Coffee, Water, Juice, Milk, Alcohol, Soft Drink ETC and QUANTITY- 1 cup/ mug/ 100ml etc) and of you add sugar to coffee tea i.e. 2 sugars, or 1 equal etc.)

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DAY 2

Day of the week _____   **Date** _____/_____/_____

MEAL/SNACK	FOOD and DRINKS consumed (type and brand)	QUANTITY (cups, spoons, no. etc.)	Was Olive oil consumed? How much?	COOKING METHOD	WHERE/ WHO?
EARLY MORNING Time:					
BREAKFAST Time:					
MORNING TEA Time:					
LUNCH Time:					
AFTERNOON TEA Time:					
DINNER Time:					
SUPPER Time:					
OTHER SNACKS					

Do you feel that today was typical of your usual diet?   Yes   No   Somewhat

Notes/Comments:

How do you incorporate 60mL of raw olive oil in your daily diet:

DON'T FORGET TO INCLUDE DRINKS: (Tea, Coffee, Water, Juice, Milk, Alcohol, Soft Drink ETC and QUANTITY- 1 cup/ mug/ 100ml etc) and of you add sugar to coffee tea i.e. 2 sugars, or 1 equal etc.)

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DAY 3

Day of the week _____   **Date** _____/_____/_____

MEAL/SNACK	FOOD and DRINKS consumed (type and brand)	QUANTITY (cups, spoons, no. etc.)	Was Olive oil consumed? How much?	COOKING METHOD	WHERE/ WHO?
EARLY MORNING Time:					
BREAKFAST Time:					
MORNING TEA Time:					
LUNCH Time:					
AFTERNOON TEA Time:					
DINNER Time:					
SUPPER Time:					
OTHER SNACKS					

Do you feel that today was typical of your usual diet?   Yes   No   Somewhat
Notes/Comments:

How do you incorporate 60mL of raw olive oil in your daily diet:

DON'T FORGET TO INCLUDE DRINKS: (Tea, Coffee, Water, Juice, Milk, Alcohol, Soft Drink ETC and QUANTITY- 1 cup/ mug/ 100ml etc) and of you add sugar to coffee tea i.e. 2 sugars, or 1 equal etc.)

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RECIPES

EXAMPLE

Recipe: <u>Chicken Stir-fry</u>

Number of servings this recipe makes: 4   **Number of servings you ate:**   1

INGREDIENTS	AMOUNT	COOKING METHOD
Chicken thighs	500g	Chicken and vegetables fried in oil. Sauce added and peanuts sprinkled on top. Rice cooked using rice cooker
Canola oil	2 tablespoons	
Carrot	1 med – 18cm long	
Red capsicum	1 med – circle H, 11cm high	
Snow peas	100g	
Peanuts (salted)	1 handful	
Kantong Sweet n Sour sauce	1 jar (300g)	
Basmati rice	2 cup uncooked	

Recipe: _____

Number of servings this recipe makes:_____ Number of servings you ate:_____

INGREDIENTS	AMOUNT	COOKING METHOD

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Recipe: _____

Number of servings this recipe makes:_____ Number of servings you ate:_____

INGREDIENTS	AMOUNT	COOKING METHOD

Recipe: _____

Number of servings this recipe makes:_____ Number of servings you ate:_____

INGREDIENTS	AMOUNT	COOKING METHOD

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Recipe: _____

Number of servings this recipe makes:_____ Number of servings you ate:_____

INGREDIENTS	AMOUNT	COOKING METHOD

Recipe: _____

Number of servings this recipe makes:_____ Number of servings you ate:_____

INGREDIENTS	AMOUNT	COOKING METHOD

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Daily consumption checklist and adverse events log

Day	Did I consume 60mL of olive oil today?		Did I have any adverse effects today? Please list anything that comes to mind
1	○ Yes	○ No	
2	○ Yes	○ No	
3	○ Yes	○ No	
4	○ Yes	○ No	
5	○ Yes	○ No	
6	○ Yes	○ No	
7	○ Yes	○ No	
8	○ Yes	○ No	
9	○ Yes	○ No	
10	○ Yes	○ No	
11	○ Yes	○ No	
12	○ Yes	○ No	
13	○ Yes	○ No	
14	○ Yes	○ No	
15	○ Yes	○ No	
16	○ Yes	○ No	
17	○ Yes	○ No	
18	○ Yes	○ No	
19	○ Yes	○ No	
20	○ Yes	○ No	
21	○ Yes	○ No	

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Active Australia questionnaire

The following questionnaire consists of eight questions to assess participation in various types of physical activities that you may have done in the last week. Please answer all questions.

1. In the last week, how many times have you walked continuously, for at least 10 minutes, for recreation, exercise or to get to or from places?

times

2. What do you estimate was the total time that you spent walking in this way in the last week?

In hours and/or minutes

minutes

hours

3. In the last week, how many times did you do any vigorous gardening or heavy work around the yard, which made you breathe harder or puff and pant?

times

4. What do you estimate was the total time that you spent doing vigorous gardening or heavy work around the yard in the last week?

In hours and/or minutes

minutes

hours

The next questions exclude household chores, gardening or yardwork:

5. In the last week, how many times did you do any vigorous physical activity which made you breathe harder or puff and pant? (e.g. jogging, cycling, aerobics, competitive tennis)

		times

6. What do you estimate was the total time that you spent doing this vigorous physical activity in the last week?

In hours and/or minutes

			minutes

		hours

7. In the last week, how many times did you do any other more moderate physical activities that you have not already mentioned? (e.g. gentle swimming, social tennis, golf)

		times

8. What do you estimate was the total time that you spent doing these activities in the last week?

In hours and/or minutes

			minutes

		hours

Study contact information

If you would like to know more about the trial at any stage, feel free to contact Katerina Sarapis (PhD candidate, research staff). If you are experiencing a severe adverse reaction, please contact emergency services immediately.

Contact details

Katerina Sarapis

Telephone: +610394795812

Email: 19662040@students.latrobe.edu.au

This research has been approved by the La Trobe University Human Research Ethics Committee (approval number HEC17-067). If you have any complaints or concerns about your participation in the study that the researcher has not been able to answer to your satisfaction, you may contact the Senior Human Ethics Officer, Ethics and Integrity, Research Office, La Trobe University, Victoria, 3086 (P: 03 9479 1443, E: humanethics@latrobe.edu.au). Please quote the application reference number HEC17-067

Appendix I

Anthropometric Assessment of Body Size

OLIVAUS study

Anthropometric assessment of body size

(Katerina Sarapis-PhD candidate)

1.1 Introduction and purpose

Anthropometry is the measurement of the human body in terms of the dimensions of bone, muscle, and adipose (fat) tissue. Measures of subcutaneous adipose tissue are important as individuals with high values are reported to be at increased risk to develop chronic diseases. Combined with the dietary and related questionnaire data, and the biochemical determinations, anthropometry is essential and provides critical information that will assist in describing and interpreting the data collected from the individuals of the OLIVAUS study sample.

The purpose of the current SOP is to provide step-by-step instructions on the exact procedures that the researcher (KS) will have to follow for conducting anthropometric measurements at baseline and follow-up examination. As such, participant's weight, height and waist circumference will be measured using standard techniques (International Standards for Anthropometric Assessment, 2011), in duplicates during four timepoints.

1.2 Height

1.2.1 Introduction

Height measurement provides information on the size and proportions of the human body. When taken in conjunction with other anthropometric measures it is an indicator of, and can predict the nutritional status, health and survival of a population.

1.2.2 Equipment

- Stadiometer (SE206, Seven Hills, NSW, Australia)
- Antibacterial surface wipes
- Step Stool

1.2.3 Procedure

1. Ensure that participants have removed outdoor clothing, shoes and any hair ornaments (e.g. large hair grips; head bands, pony tail holders etc) that will interfere with the measurement. Do not ask participants to remove headscarves, turbans or hairstyles that are worn for religious or cultural reasons.

2. Instruct the study subject to step under the headboard, standing as straight as possible with their feet together flat on the centre of the base plate. Their weight should be evenly distributed through both legs. Their back, shoulders, head, buttocks and calves should be positioned against the backboard of the stadiometer. (Figure 1)

3. Position the participant's head in the horizontal Frankfurt plane. The Frankfurt Plane is an imaginary line passing through the upper margin of the external ear canal and across the top of the lower bone of the eye socket, under the eye. This position is important if an accurate reading is to be obtained.

4. Once the Frankfurt plane position is achieved, instruct the subject to keep their eyes focused on a point straight ahead and without moving their head position, to take a deep breath and stand tall aiming to straighten their spine. Shoulders should be relaxed.

5. The moveable headboard is then gently lowered until it touches the crown of the head.

6. The height measurement is taken at maximum inspiration, with the examiners eye's level with the headboard. (Use the step stool to stand on, so that you are in an appropriate position to be able to read the height measurements taken).

7. Height measurement is recorded (to the nearest 0.1 cm) in participant's Case Report Form (CRF).

8. Repeat the measurement and record the second height value. If the two height measurements differ by more than 0.4 cm, then a third reading needs to be taken.

9. Wipe the headboard with the antibacterial surface wipes after use.
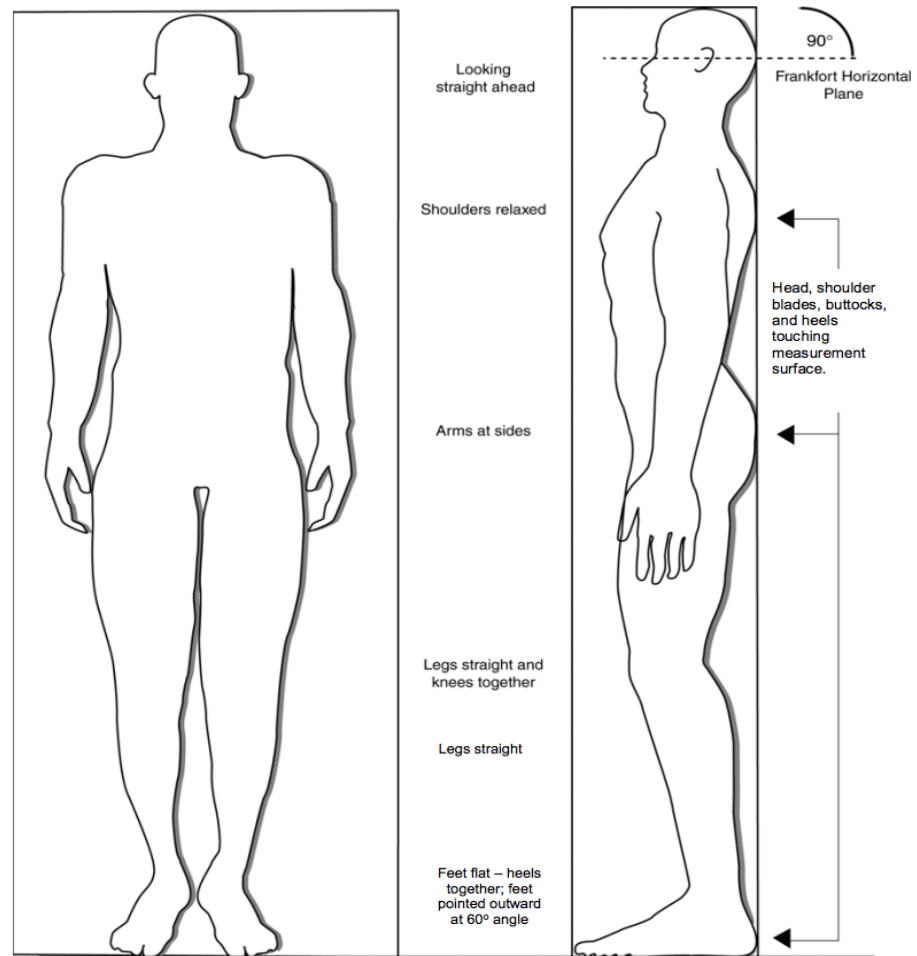
Figure 1: Positioning of study subject for height measurement

1.3 Weight

1.3.1 Introduction

Weight measurement is an indicator of and can predict the nutritional status and health of a population. In conjunction with the height measurement it can be used to derive the Body Mass Index (BMI).

1.3.2 Equipment

- Digital scales (WM203, Willawong, QLD, Australia)
- Antibacterial surface wipes

1.3.3 Procedure

1. Check that scales are on a level floor, away from any objects. Do not use on carpeted surfaces. Ensure that the mains power supply is turned on (Figure 2).
2. Ask the participant to remove shoes, heavy outer garments such as jackets and cardigans, heavy jewellery, and to empty their pockets of all items.
3. Switch the scale on.
4. When the display reads 0.00 ask the participant to stand on the scale with his/her feet together in the centre. Study subject must keep still, head facing forward and should be hanging loosely with their hands at their sides.

Figure 2: Digital scale their arms

5. Read the result of the digital display. If the study participant moves excessively while the scales are stabilising you may get a false reading. If you think this is the case reweigh the study participant.
6. Record the measurement (to the nearest 0.01kg) in the participaant's CRF.
7. Repeat the measurement and record the second weight value.

1.4 Waist circumference

1.4.1 Introduction

Central obesity is associated with clustering of cardiovascular risk factors. People with central obesity are known to be at higher risk of developing chronic disease. To measure central obesity, waist circumference (WC) appears to be a better indicator than BMI and waist-to-hip ratio. WC measurement is convenient, and it is more strongly correlated with intra-abdominal fat content and cardiovascular risk factors.

1.4.2 Equipment

- Flexible steel tape calibrated in cm with mm graduations (Luftkin W606PM, Maryland, USA).
- Antibacterial surface wipes

1.4.3 Procedure

1. Measurement of WC should not be over clothing unless it is very light and close fitting such as a swimming costume or bodysuit. Explain to the participant the importance of this measurement and that clothing can substantially affect the reading. As such, it is compulsory to remove all outer layers of clothing, such as jackets, heavy or baggy jumpers, cardigans and waistcoats, belts, tight garments that intend to alter the shape of the body (i.e corsets, lycra body suits and support tights/underwear). Other garments can be undone or lifted up/down so that the waist can be seen and measured. Some participants may be wearing religious or other symbols which they cannot remove and which may

affect the measurement. Do not offend the participant by asking them to remove such items. Make a note if the measurement is likely to be affected by this.

2. Instruct the participant to stand erect with their abdomen relaxed, arms at the sides, feet together and their weight equally divided over both legs.

3. Ask the participant to identify their umbilicus and then using your index and middle finger locate the position (Figure 3).

4. Pass the tape around the participant's waist.

5. Once the tape is in position, stand to the side of the participant to take the measurement.

6. Ask the participant to breathe out gently to prevent them from contracting their muscles or from holding their breath. At the end of normal expiration, measure and record the waist circumference with the tape horizontal and in contact with the skin without compressing the tissue.

7. Record the measurement to the nearest 0.1 cm in the participant's CRF.

8. Repeat the procedure to obtain a second measurement and record the value.

9. Wipe the tape with the antibacterial surface wipes after use.
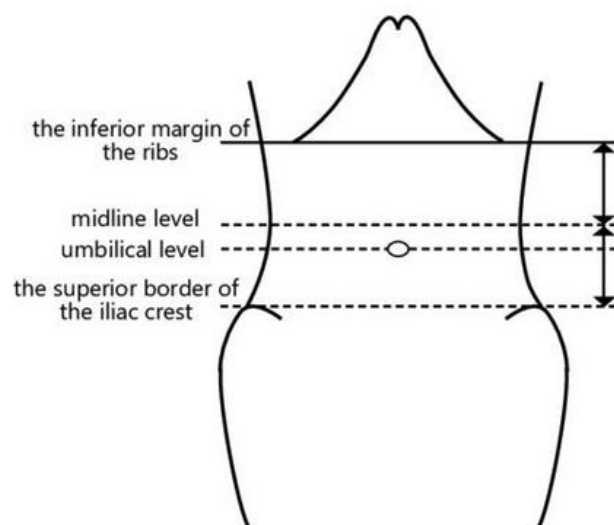
Figure 3: Measurement of waist circumference at umbilical level

Appendix J

Standard Operating Procedure

Blood collection, processing, handling, and storage procedures

Standard Operating Procedure

Blood collection, processing, handling, and storage procedures

Katerina Sarapis (PhD candidate)

1.1 Purpose

The purpose of the current SOP is to provide step-by-step instructions on the exact procedures that the researcher (Katerina Sarapis) will have to follow for conducting venous blood collection for biochemical analysis at baseline and follow-up examination.

1.2 General procedures for venous blood collection

Early-morning venous blood samples will be obtained from each participant for biochemical screening tests following a 12-hour overnight fast, at baseline (T1), with follow up tests at the end of first intervention phase (T3), commencement of second intervention phase (T4) and endpoint (T6). (figure 1)
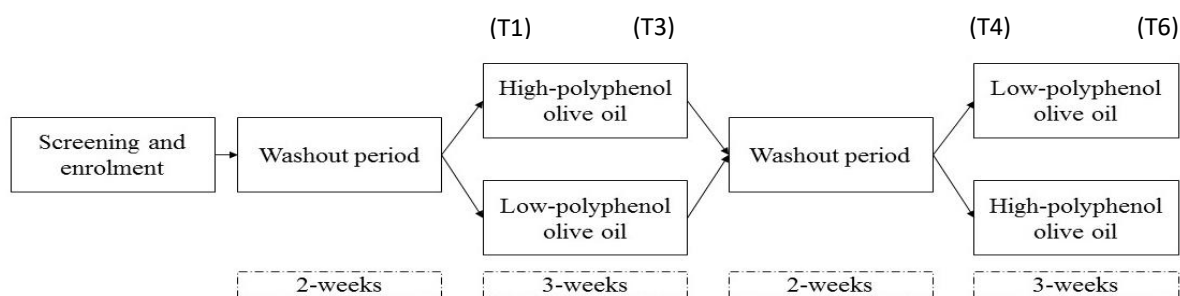
Figure 1: Study design

The researcher will perform venepuncture to obtain 25 mL of blood (or more provided that there is enough justification for this and that we will have adequate storage capacity in -80^{0}C. (figure 2)
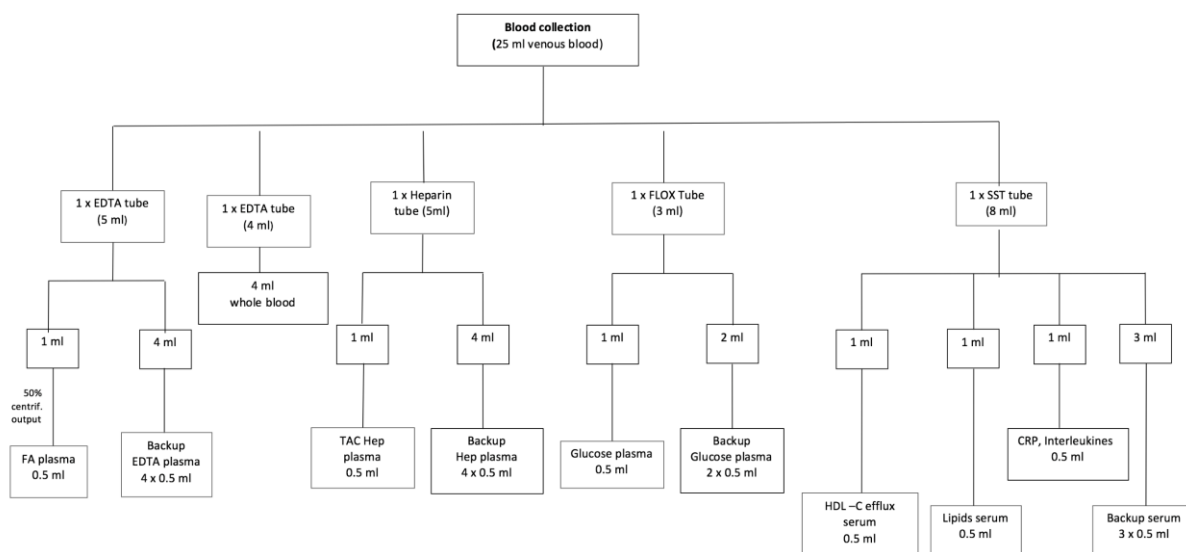
Figure 2: Flow diagram of the blood processing procedure

1.2.1 Consumables and supplies required for performing venepuncture

The consumables and supplies that will be used for performing the venepuncture in the study are the following:

- Disposable Latex gloves will have to be worn by the researcher and by anyone else who may assist with the blood collection.
- Alcohol preps will be used for cleaning the venepuncture site.
- Winged steel needles appropriate for adults with an extension tube (a butterfly) will be used. Use a butterfly with either a syringe or an evacuated tube with an adaptor; a butterfly can provide easier access and movement, but movement of the attached syringe may make it difficult to draw blood.
- Sterile gauze pads.
- Adhesive hypo allergic bandages (plasters or Band-Aids) will be applied to the puncture site to minimize the risk of infection.
- Plastic Bag for Waste will be used to dispose all of the biohazardous waste generated during the day as well as a sharps biocan to dispose of all needles.

1.2.2 Steps in obtaining venous blood from the participant

The steps for obtaining venous blood samples from the study participants are provided below:

Step 1: Complete general preparation.

- Find an indoor site to encourage privacy during blood collection. The site should have a table or other   piece of furniture with a flat surface where you can lay out all consumables/ supplies. An examination bed should be readily available if the respondent feels faint and needs to lie down.
- Introduce yourself and identify the client. Participant identification is crucial to insuring that the blood specimen is being drawn from the individual designated on the request form.

- Ensure that each subject has completed a 12-hour fast.
- Wash and dry your hands, put on gloves before initiating blood collection from the participant.
- Take out a clean absorbent paper sheet and spread it over a flat surface where you will lay out your consumable and supplies. You will want to have all general materials in easy reach when you begin collecting blood samples from the participants

Step 2: Prepare the participant for the venepuncture.

- The individual should be seated comfortably in a chair with arm extended on the slanting armrest to form a straight line from the shoulder to the wrist. The arm and elbow should be supported firmly by the armrest and should not be bent at the elbow.
- Ask each volunteer if they have a history of fainting. If so, ensure that the blood sample is only drawn whilst the subject is lying down on a bed.
- Describe to the participant exactly what will be done during the collection of the blood sample.

Step 3: Prepare the venepuncture site.

- Apply (tighten) tourniquet.
- Ask the participant to close his/her hand so that the veins will become more prominent and thus easier to enter. Vigorous hand exercise or "pumping" should be avoided.
- Select the vein site. Palpate and trace the path of veins several times with the index finger. If superficial veins are not readily apparent, blood can be forced into the vein by gently massaging the arm from wrist to elbow. Several sharp taps at the vein site with index and second finger will cause the vein to dilate.
- Loosen tourniquet.
- The venepuncture site must be cleansed once with an alcohol swab to prevent any chemical or microbiologic contamination of either the patient or the specimen.
- Check equipment, tube selection and thread needle (or butterfly) securely onto tube holder (barrel).
- Re apply the tourniquet. A tourniquet allows the veins to fill with blood, thus making the veins more prominent and easier to enter. Do not leave the tourniquet on for longer than 1 minute otherwise it may result in either hemoconcentration or variation in blood test values.
- Remove needle cover and check bevel uppermost.

Step 4: Blood drawing

- Puncture the skin 3–5 mm away from the vein; this allows good access without pushing the vein away.
- If the needle enters alongside the vein rather than into it, withdraw the needle slightly without removing it completely, and angle it into the vessel.
- Insert the tube into the holder and commence filling the tubes.
- Draw blood slowly and steadily.
- Release the tourniquet as soon as blood flow is established. Tourniquet release allows the blood circulation to return to normal and also reduces bleeding at the venipuncture site.
- Remove the tube from the holder and invert (x number of times) to mix the blood with tube additives. Place blood samples on ice if required..
- Place a cotton wool above the venepuncture site, withdraw the needle and apply pressure.
- Dispose of needle in a sharps container.
- Check site and apply an adhesive bandage.
- Label all tubes immediately.

Figure 3 graphically presents all steps in obtaining venous blood from the study subject.

Put on well-fitting, non-sterile gloves.
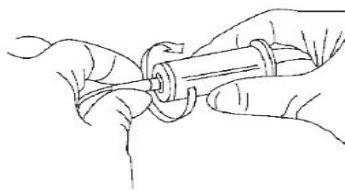
Attach the end of the winged infusion set to the end of the vacuum tube and insert the collection tube into the holder until the tube reaches the needle.
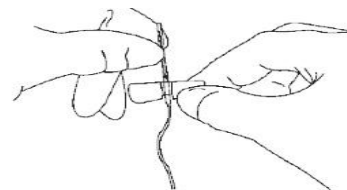
Remove the plastic sleeve from the end of the butterfly.
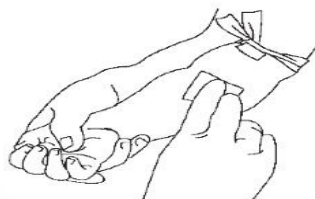
Disinfect the collection site and allow to dry.

Use a thumb to draw the skin tight, about two finger widths below the venepuncture site.

Push the vacuum tube completely onto the needle.

Place dry gauze over the venepuncture site and slowly withdraw the needle.

Remove the butterfly from the vacuum tube holder.

Dispose of the butterfly in a sharps container.

Properly dispose of all contaminated supplies.
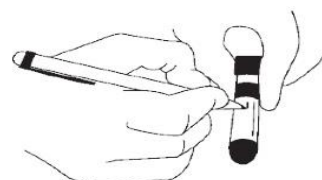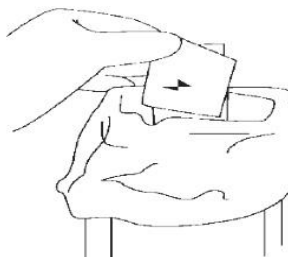
Label the tube with the patient identification number and date.
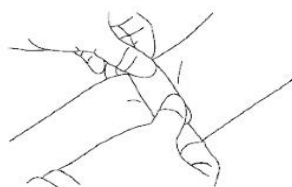
Put an adhesive bandage on the patient if necessary.
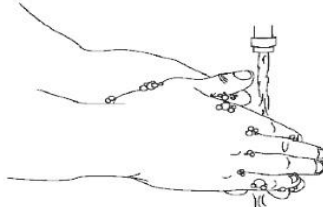
Remove gloves, dispose of them appropriately and perform hand hygiene (if using soap and water, dry hands with single-use towels).

1.3 Blood processing and handling

1.3.1 Centrifuge procedure

Collected venous blood will be centrifuged and the extracted plasma and/or serum will be pipetted into aliquots according to the blood collection protocol.

- The Thermo scientific Heraeus megafuge 8 centrifuge will be used to centrifuge the human blood. (Figure 4)
- Set up in a well-ventilated environment, on a horizontally levelled and rigid surface with adequate load-bearing capacity.
- As safety zone maintain a clear radius of at least 30 cm around the centrifuge. Do not place any dangerous substances within this security zone.
- Open the centrifuge door by pressing the open key.
- Place the remaining tubes containing blood into appropriate sized adapters.
- Place the tubes containing water in opposite adapters, where they should mirror the placement of the tubes holding blood.
- Never place both tubes housing water and blood into the same adapters but should be placed in different adapters for even weight distribution.
- Place the adapters carefully and gently into the rotor buckets
- Seal the buckets with the lids and close the centrifuge.
- Use only with rotors which have been loaded properly.
- Make sure the rotor is locked properly into place before operating the centrifuge.
- Never overload the rotor.
- Never start the centrifuge when the centrifuge door is open.

Figure 4: Centrifuge

- Do not lean on the centrifuge.
- Do not place anything on top of the centrifuge during a run.
- Gently close the centrifuge door. The centrifuge door mechanism will click and lock in place.
- Turn on the centrifuge by pressing the start key.
- Select the required speed and time from preprogramed setting or manually using the arrow keys (1300xg for 10 mins for each tube).

Once the centrifuge has completely stopped spinning wait for an audible sound and then open the centrifuge. Remove the tubes from the centrifuge and place them in a tube rack.

1.3.2 Handling of collected blood

Five different types of test tubes will be used per study participant to collect venous blood. The collected blood will be designated for plasma and serum separation. As far as plasma separation is concerned, four blood collection tubes with added anticoagulant will be used. As such, there will be one EDTA containing tube of 5 ml for plasma extraction, one EDTA tube of 4 ml for whole blood storage, one heparin containing tube of 5 ml and one FLOX tube containing a glucosidase inhibitor of ~3 ml. As far as serum separation is concerned, one SST blood collection tube of 8ml will be used. All information regarding blood collection tubes are presented in Table 1.

Table 1. Volume of blood in different test tubes

Test tube	Blood volume	Designated for:
EDTA tube	5 ml	EDTA plasma extraction
EDTA tube	4 ml	Whole blood
Heparin gel tube	5 ml	Heparin plasma extraction
FLOX tube	3 ml	Fasting plasma glucose

SST tube	8 ml	Serum extraction
Total blood:	25 ml	

- The blood (5 ml) collected in the EDTA tube will be centrifuged at 2350 rpm for 10 min and the extracted plasma will be pipetted into 5 aliquots of 500 μl (considering a 50% efficiency of centrifugation in plasma extraction). One aliquot of 500 μl will be used for determining oxidized LDL, while the 4 aliquots of 500 μl each will be stored at -80ºC, as indicated in Table 2.

Table 2. Volumes and use of EDTA plasma aliquots.

Plasma aliquot no.	EDTA plasma volume	Designated for the analysis of:
1	500 μl	Oxidized LDL
		Designated for:
2	500 μl	Storage at -80ºC
3	500 μl	Storage at -80ºC
4	500 μl	Storage at -80ºC
5	500 μl	Storage at -80ºC

- The blood (5ml) collected in the heparin gel tube will be centrifuged at 2350 rpm for 10 min and the extracted (heparin) plasma will be pipetted into 5 aliquots of 500 μl (considering a 50% efficiency of centrifugation in plasma extraction). One aliquot of 500 μl will be used for determining the total antioxidant capacity (TAC), while the 4 aliquots of 500 μl each will be stored at -80ºC, as indicated in Table 3.

Table 3. Volumes and use of heparin plasma aliquots.

Plasma aliquot no.	Heparin plasma volume	Designated for the analysis of:
1	500 μl	Total Antioxidant Capacity (TAC)
		Designated for:
2	500 μl	Storage at -80ºC
3	500 μl	Storage at -80ºC
4	500 μl	Storage at -80ºC
5	500 μl	Storage at -80ºC

- The blood (3 ml) collected in the FLOX tube will be centrifuged at 2350 rpm for 10 min and the extracted plasma will be pipetted into 3 aliquots of 500 μl (considering a 50% efficiency of centrifugation in plasma extraction). One aliquot of 500 μl will be used for determining the concentrations of plasma glucose while the 2 aliquots of 500 μl each will be stored at -80ºC, as indicated in Table 4.

Table 4. Volumes and use of glucose plasma aliquots.

Plasma aliquot no.	Glucose plasma volume	Designated for the analysis of:
1	500 μl	Plasma glucose
		Designated for:
2	500 μl	Storage at -80ºC
3	500 μl	Storage at -80ºC

- The blood (8ml) collected in the SST tube will be kept at room temperature for ~30 min where it will be allowed to clot. Clotted blood will be centrifuged at 2350 rpm for 10 min In a refrigerated centrifuge.

Following centrifugation, it is important to immediately transfer the liquid component (serum) into a clean polypropylene tube using a Pasteur pipette. The serum should be apportioned into 6 aliquots of 500 μl each. Three of these aliquots will be used for biochemical analyses of HDL cholesterol efflux, inflammatory markers (i.e CRP, interleukines) and lipids (TC, LDL,HDL,TG) while the 3 aliquots of 500 μl each will be stored at -80ºC, as summarized in Table 5.

Table 5. Volumes and use of serum aliquots.

Serum aliquot no.	Serum volume	Designated for the analysis of:
1	500 μl	HDL cholesterol efflux
2	500 μl	TC, LDL, HDL, TG
3	500 μl	CRP, Intereukines
		Designated for:
4	500 μl	Storage at -80ºC
5	500 μl	Storage at -80ºC
6	500 μl	Storage at -80ºC

NOTE: It is essential that ONLY NON-HAZARDOUS waste be placed in the wastepaper/ general rubbish bins. Pipette tips should be disposed in sharps containers, whereas laboratory and associated waste directly involved in specimen processing (i.e blood collection tubes, gloves etc) must be disposed in biological waste bags.

1.4 Blood storage

Eppendorf tubes or screw cap tubes must be clearly labelled with identification, media used and date, placed in a freezer well rack and should not be stored for long periods on a bench, but must be transferred with an ice esky box to a dedicated storage area (i.e. refrigerator, cold room or cupboard) as soon as possible.

Laboratory coats must be removed and hung up before leaving laboratory areas and should be laundered once a week. Hands must be washed with an antibacterial agent BEFORE leaving laboratory (Hibiclens/Microshield or equivalent, followed by extensive rinsing).

Appendix K

Extra Virgin Olive Oil high in polyphenols improves antioxidant status in adults. A double-blind, randomized, controlled, cross-over study (OLIVAUS)

ORIGINAL CONTRIBUTION

Extra virgin olive oil high in polyphenols improves antioxidant status in adults: a double-blind, randomized, controlled, cross-over study (OLIVAUS)

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Abstract

Purpose Olive oil polyphenols have been associated with cardiovascular health benefits. This study examined the antioxidant and anti-inflammatory effect of extra-virgin high polyphenol olive oil (HPOO) vs. low polyphenol olive oil (LPOO) in healthy Australian adults.

Methods In a double-blind cross-over trial, 50 participants (aged 38.5 ± 13.9 years, 66% females) were randomized to consume 60 mL/day of HPOO (320 mg/kg polyphenols) or LPOO (86 mg/kg polyphenols) for three weeks. Following a 2-week wash-out period, participants crossed-over to the alternate treatment. Plasma oxidized low-density lipoprotein (ox-LDL), total antioxidant capacity (TAC), high-sensitivity C-reactive protein (hs-CRP) and anthropometrics were measured at baseline and follow-up.

Results Fourty-three participants completed the study. Although there were no significant differences between treatments in the total sample, plasma ox-LDL decreased by 6.5 mU/mL (95%CI − 12.4 to − 0.5) and TAC increased by 0.03 mM (95% CI 0.006–0.05) only in the HPOO arm. Stratified analyses were also performed by cardiovascular disease risk status defined by abdominal obesity (WC > 94 cm in males, > 80 cm in females) or inflammation (hs-CRP > 1 mg/L). In the subgroup with abdominal obesity, ox-LDL decreased by 13.5 mU/mL (95% CI − 23.5 to − 3.6) and TAC increased by 0.04 mM (95% CI 0.006–0.07) only after HPOO consumption. In the subgroup with inflammation, hs-CRP decreased by 1.9 mg/L (95% CI − 3.7 to −0.1) only in the HPOO arm.

Conclusions Although there were no significant differences between treatments, the changes observed after HPOO consumption demonstrate the antioxidant and anti-inflammatory effect of this oil, which is more pronounced in adults with high cardiometabolic risk (Clinical Trial Registration: ACTRN12618000706279).

Keywords Olive oil · Extra virgin olive oil · Polyphenols · Cardiovascular disease · Antioxidant · Inflammation

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, accounting for 21% of deaths in 2017 [1]. According to the latest Australian data, a similarly high proportion of mortality (26% of all deaths) was attributed to CVD, in 2017–2018 [2]. Factors such as oxidative stress and chronic vascular inflammation are closely related

to endothelial dysfunction, which is a determinant in the development of atherosclerosis and an early predictor for cardiovascular events [3]. Both oxidative stress and inflammation may cause injury to endothelial cells, promoting a pro-inflammatory response, as evidenced by the increased expression of endothelial dysfunction markers such as adhesion molecules and cytokines (e.g., Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)), with the latter being responsible for the secretion of C-reactive protein (CRP) [4]. A large body of evidence has established that oxidized low density lipoprotein (ox-LDL) is a highly immunogenic particle which plays a major role in the initiation and progression of atheromatic plaque formation within the arterial wall, and

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it is therefore considered as a hallmark in the pathogenesis of atherosclerosis [5–7]. However, the oxidative damage process can be interrupted by the presence of various antioxidants (endogenous/exogenous and antioxidant enzymes) [8]. In this regard, plasma total antioxidant capacity (TAC), which reflects the overall antioxidant status in vivo, has been shown to be inversely associated with risks of chronic diseases including CVD [9].

Extensive evidence indicates that certain dietary patterns are cardioprotective [10]. One of the most globally researched and evidence-based dietary approaches for the prevention and management of chronic diseases is the traditional Mediterranean diet (MedDiet), which has been shown to improve cardiovascular risk factors, including markers of oxidative stress and inflammation [11–13]. The Mediterranean dietary pattern is a plant-based diet, rich in unsaturated fatty acids and antioxidants present in a variety of staple foods, such as fruits, vegetables, nuts, legumes, wholegrain cereals and extra virgin olive oil (EVOO) [11, 14]. Olive oil (OO) is the principal source of dietary fat and a unique culinary component of the MedDiet. OO contains variable concentrations of polyphenols (also referred to as biophenols), that can be influenced by factors such as olive cultivar, region and soil, ripeness of the fruit, as well as the extraction procedure [15]. Notably, virgin OOs (VOOs), obtained by mechanical extraction methods (i.e. direct-press or centrifugation), preserve high phenolic content, whereas refined OOs (ROOs), subjected to both physical and/ or chemical processing, have a lower phenolic content [16]

Olive oil polyphenols have been associated with several cardiovascular health benefits, mainly due to their antioxidant and anti-inflammatory properties [16–18]. In this context, the EUROLIVE Study, a European multicenter study, demonstrated that daily consumption of polyphenol-rich OO (366 mg/kg, phenolic content; 25 mL, daily dose) significantly reduced blood levels of ox-LDL [19]. Two recent meta-analyses, one conducted by our own group, synthesized the available evidence from intervention studies examining high phenolic OO (HPOO) versus low phenolic OO (LPOO) consumption on various cardiovascular risk markers, and reported a beneficial effect of HPOO on reducing markers related to oxidative stress and inflammation (i.e., ox-LDL and CRP, respectively) compared to the LPOO treatment arm [17, 20]. In 2019, a network meta-analysis reported a dose–response relationship between higher intakes of OO phenolic compounds and lower ox-LDL values [21]. However, *most of the intervention studies included in the above-mentioned meta-analyses have been conducted in Mediterranean populations that are accustomed to high OO intake, an observation that highlights the need for additional research in multiethnic populations with different habitual food consumption.* Moreover, *despite the evidence on the unique cardioprotective properties of polyphenols in EVOO, these are*

not currently recognized by CVD guidelines, thus underlining the need for additional evidence of higher methodological quality. Hence, the present study *aimed to investigate the effect of daily consumption of (60 ml) raw extra virgin HPOO, compared to LPOO, for 3 weeks, on secondary outcome measures such as oxidative status and inflammation biomarkers in Australian adults with no previously diagnosed medical conditions.*

Materials and methods

Study population

The "OLIVAUS study" [22] was conducted according to the Guidelines for Good Clinical Practice (GCP), the guidelines laid down in the Declaration of Helsinki and the CONSORT reporting guidelines. All procedures involving human subjects were approved by the Human Research Ethics Committee of La Trobe University (HEC17-067) and written informed consent was obtained from all volunteers. The trial protocol has been registered with the Australia New Zealand Clinical Trials Registry ACTRN12618000706279.

Participants were recruited in Melbourne, Australia via social media, La Trobe University staff email database advertising, word of mouth and study posters on display at the campus. A standardized screening procedure was followed to identify eligible participants, who were required to be within the age range of 18–75 years and a body mass index (BMI) 18.5–40 kg/m^2. Exclusion criteria included non-English speaking individuals, pregnant or lactating women, smokers, individuals on a special type of diet for medical reasons (e.g., gluten free for coeliac disease) and/ or with a high habitual OO intake (> 1 tablespoon/day). Exclusion also applied if individuals were taking vitamins or antioxidant supplements as part of a regular regime and were unable to discontinue their use for the duration of the trial (except for iron, calcium and Vitamin D). Finally, study subjects taking prescribed medication (e.g., antihypertensive agents, lipid-lowering drugs, nonsteroidal anti-inflammatory drugs) and those with diagnosed chronic diseases (diabetes, hyperlipidaemia, hypertension, inflammatory conditions), gut-related diseases or any other condition that could impair adherence, were also excluded. Additional details on the procedures followed to identify eligible study participants is provided elsewhere [22].

Study design and procedure

The OLIVAUS study was a double-blind, cross-over, randomised controlled trial (RCT) aiming to evaluate the effect of extra virgin HPOO compared to a commercially available LPOO on both novel i.e., high density lipoprotein

cholesterol (HDL-c) efflux (primary outcome) and routinely measured CVD risk markers, including oxidative status and inflammation. The primary outcome (HDL-c efflux) will be reported elsewhere as this is not within the scope of this paper. Prior to the main study, a pilot study was conducted to test the feasibility of the study protocol and the data collection tools [23]. Enrolled participants were randomly assigned in a 1:1 ratio, to one of the two treatment arms, i.e., extra virgin HPOO or LPOO. The randomization into one of the two treatment arms was performed in blocks of six using a computerized random number generator in excel software. An independent senior researcher not otherwise involved in the study generated the sequence.

Study participants were requested to consume *a daily dose of 60 mL of either type of raw OO over two intervention periods of 3 weeks each, in conjunction with their habitual diet*. The two types of OO varied only in their *phenolic content*, (*320 mg/kg in HPOO vs. 86 mg/kg in LPOO*) but did not differ with respect to their other nutrient composition, including fatty acid profile. Two washout periods, of 2 weeks each, during which study participants were instructed to avoid olives and OO consumption, preceded the first and the second intervention periods of OO administration. We chose a 2-week washout period as this was sufficient to eliminate the carry-over effect of OO polyphenols between interventions, considering the short half-life of OO's phenolic compounds [24]. In addition, the intervention in the present study was designed with a daily dose of 60 mL OO, which reflects the habitual intake in Mediterranean populations where the cardioprotective benefits of virgin OO have previously been reported [17, 20, 21].

Participants were provided with OO at the beginning of each intervention period. The OO was supplied in dark coloured glass containers to minimise phenolic content loss due to sunlight. To ensure blinding of the researchers to the OO type, each bottle was assigned a different code number that was concealed from study participants and research team members. The code was disclosed only after the completion of the statistical analyses. To assess the level of adherence to the intervention, participants were instructed to return the containers at the end of each intervention period so that the daily amount of unconsumed OO could be measured and recorded. Study participants were also instructed to keep a written record of daily OO consumed during each intervention period using a provided log sheet. This information was collected by research team members after the end of each intervention period. Full details of the study protocol, including a comparison of the concentrations of total polyphenols and polyphenol subclasses in each of the two types of OOs, are provided elsewhere [22].

Measurements

Socio-demographics, use of medication and dietary supplements

Socio-demographic data were collected from eligible participants during a scheduled interview at our trial clinic room located at La Trobe University. Trained researchers conducted all interviews using a standardized questionnaire. Specifically, the socio-demographic data collected during this interview included age, gender, language(s) spoken at home, level of education, ethnicity and parental country of birth. Any medications and dietary supplements taken by the study participants were also recorded.

Dietary intake

A 3-day food diary was used to collect information on the dietary intake of study participants during two weekdays and one weekend day (preferably non-consecutive) at baseline and follow-up of each 3-week intervention period. Specifically, study participants were instructed to record details on their intake of food and beverages, including information on the quantity, type/brand and cooking methods of the consumed items. The level of detail required to be recorded in the diary, as well as additional strategies on how to incorporate raw, uncooked OO in their habitual diet was provided to study participants at a pre-baseline meeting by a trained nutritionist. The completed food diaries were returned and checked by the research team members for potential wrong or missing entries during the scheduled interviews with the study participants. FoodWorks®9 software (Xyris Software Pty Ltd, Queensland, Australia) was used for assessing dietary intake and extracting data on energy, micro- and macronutrients as well as the consumption of food groups and individual food items.

Anthropometric measurements

Anthropometric measurements were conducted four times during the study, i.e. at baseline and follow up of each intervention period. Body weight and standing height were measured with study participants in light clothing and barefoot, using a digital scale (WM203, Willawong QLD, Australia) to the closest 0.1 kg and a wall-mounted stadiometer (SE206, Seven Hills, NSW, Australia) to the nearest 0.1 cm, respectively. Waist circumference (WC) was measured to the nearest 0.1 cm, using a flexible steel tape calibrated in cm with mm graduations (Luftkin W606PM, Maryland, USA) directly over the skin at the umbilicus level. Body mass index (BMI) was calculated using Quetelet's equation (weight (kg)/height (m)^2). Using World Health Organization (WHO) cut-off points for BMI, study participants

were classified as underweight (BMI < 18.5 kg/m^2), normal weight (BMI 18.5–24.9 kg/m^2), overweight (BMI 25.0–29.9 kg/m^2) or obese (BMI ≥ 30 kg/m^2) [25]. Furthermore, gender-specific WC cut-off points proposed by WHO were also used to categorise study participants for CVD risk: normal (WC < 94 cm in men and < 80 cm in women), High CVD risk (WC 94–102 cm in men and 80–88 cm in women) and very high CVD risk (WC > 102 cm in men and 88 cm in women) [26].

Biochemical analyses

At baseline and follow up of each intervention period (in the morning following a 10-h overnight fast), venous blood was collected from participants by a trained researcher at La Trobe University using standard venipuncture. Using the Centers for Disease Control/American Heart Association (CDC/AHA) cut-off points for CRP, study participants were classified as low cardiometabolic risk (CRP < 1 mg/L), intermediate (CRP, 1–3 mg/L) and high cardiometabolic risk (CRP > 3 mg/L) [27].

Collected venous blood was centrifuged (Hettic Rotina 420r, Massachusetts, USA) at 2350 rpm for 10 min at 4^{0}C and the extracted plasma and/or serum was apportioned into aliquots of 500 μl each and stored at -80^{0}C until analysis. The TAC in serum plasma (collected in lithium heparin tubes) was measured using the Cell Biolabs, Inc. OxiSelect Total Antioxidant Capacity (TAC) Assay Kit (San Diego, CA, USA). This colorimetric assay, performed in 96 well microtiter plates, is based on the reduction of copper (II) to copper (I) by antioxidants in the plasma, such as uric acid. Upon reduction, the copper (I) ion reacts with a coupling chromogenic reagent. Absorbance was measured at 490 nm using the Omega Fluostar Plate Reader. Absorbance values are proportional to the sample's total reductive capacity. Antioxidant capacity was determined by comparison to uric acid standards. All samples and standards were measured in duplicate. Results were expressed as mM Uric Acid Equivalents (UAE). The detection limit was 0.0039 mM. Intra-assay coefficient of variation (CV%) was 2.3% (range 0.1–7.7%). Laboratory analysis for TAC assay was conducted by AL, KP and OF. Plasma ox-LDL concentrations (collected in K3 EDTA tubes) were measured using the Mercodia oxidized-LDL ELISA kit (Mercodia AB, Uppsala, Sweden). This ELISA assay is a solid phase two-site enzyme immunoassay in which 2 monoclonal antibodies are directed against separate antigenic determinants on the oxidized apoB molecule. The detection limit was 0.6 mU/L. Intra-assay CV was 6.3% (range 5.5–7.3%). Finally, the Alinity c CRP Vario assay (SENTINEL CH, Milano, Italy) was used for the quantitative immunoturbidimetric determination of hs-CRP in human serum. The detection limit was 0.4 mg/L (intra-assay

CV was 0.8%). Laboratory analysis for ox-LDL assay was performed by MG.

Sample size calculation

Power calculations indicated that a sample size of 40 was adequate to provide sufficient statistical power to detect a statistically significant between-group difference of 5% and a standard deviation (SD) of 11% in HDL-c efflux levels, which was the primary outcome of the OLIVAUS study, with power 80% and 5% level of significance [28]. The total sample size was set at 50 study participants, to account for an attrition rate of 20%. A post hoc power calculation that was based on the available 50 participants, showed that this sample size provided more than 90% statistical power to detect significant between-group differences both in TAC and ox-LDL. However, the sample size of 50 participants did not provide enough statistical power in the case of hs-CRP, as the calculated statistical power was 31%.

Statistical analysis

All statistical analyses were conducted using the SPSS statistical software for Windows (IBM, version 24.0; IBM, Armonk, NY, USA). For all continuous variables, the Kolmogorov–Smirnov test was performed to examine the normality of their distribution. A general linear model, i.e. repeated-measures ANOVA (analysis of variance) was used to examine the between-group differences (treatment effect; i.e., extra virgin HPOO *vs* LPOO) of mean values at each time point of measurement, the within-group changes (time effect) from baseline to follow-up in each intervention arm, and the differences in the changes from baseline to follow-up between the two intervention arms (Treatment × Time interaction effect). Both per protocol (PP) and intention–to-treat (ITT) analyses were performed. The PP analyses were conducted in study participants who had full data from baseline to follow-up in the first or the second intervention period. For the ITT analyses, multiple imputations were conducted to compensate for all missing values. Considering that the PP and the ITT analyses provided similar results, (i.e. mean values, mean changes and statistical significance), the results arising from the latter are presented in this article. In all statistical analyses, adjustments were made for gender and age. Statistical analyses were conducted on the total sample, but stratified analyses were also performed to examine the effect of the implemented treatments in participants with high CM risk, defined based on the WC and hs-CRP levels. Data is presented either as mean ± SD, as estimated marginal means and standard errors (SE) or as mean change and 95% Confidence Interval of change (CI) for continuous variables and as frequency (n) and percentage (%) for categorical ones. All

reported *P* values are two-tailed, and the level of statistical significance is set at $P < 0.05$.

Results

Fifty volunteers ($n = 33$ females, and $n = 17$ males) were eligible and enrolled in the study from July 2018 through to October 2019. Seven participants discontinued the intervention, due to inability to comply ($n = 4$) and for personal reasons ($n = 3$) and therefore, there was complete data for 43 participants. Figure 1 provides the study participant flow diagram.

Baseline characteristics of study participants

Table 1 presents the descriptive characteristics of study participants in terms of their socio-demographics, anthropometric and biochemical indices in the total sample ($n = 50$) and by treatment arm. Study participants had a mean age of 38.5 ± 13.9 years (total range 20 to 70 years) and mean education of 17.3 ± 3.5 years. In addition, most study participants were females (66%) and born in Australia (70%). The mean BMI was 24.7 ± 3.5 kg/m^2 and mean WC was 86.9 ± 11.2 cm. In addition, 44% of study participants were overweight and 4% were obese. Based on their WC measurements, 16% of study participants had a high cardiometabolic risk and 24% had very high cardiometabolic risk. Regarding the oxidative status profile of study participants, their mean TAC and mean ox-LDL was 0.5 ± 0.1 mM and 75.4 ± 21.2 mU/mL, respectively. Furthermore, mean circulating hs-CRP for the cohort was 1.7 ± 2.8 mg/L, while 70% of study participants were categorised as low risk (hs-CRP < 1 mg/L), 12% intermediate risk (hs-CRP, 1–3 mg/L) and 18% high risk (hs-CRP > 3 mg/L) based on their inflammation status. Finally, there were no significant differences between treatment arms in any of the abovementioned baseline characteristics, thus indicating homogeneity at baseline.

Effect of LPOO and HPOO on dietary intake

The changes observed in dietary energy and macronutrient intake from baseline to follow-up after each 3-week intervention, as well as the differences between treatment arms are presented elsewhere [16]. In brief, the changes from baseline to follow-up were not significantly different between the two treatment arms. However, dietary energy intake increased significantly in participants following LPOO (by 1806.1 kJ/day, 95% CI 1075.4–2536.8) and HPOO (by 1766.6 kJ/day, 95% CI 1035.9–2497.3). Consumption of LPOO and HPOO also significantly increased intake of total fat (by 49.3 g/day, 95% CI 41.1–57.4 and 46.0 g/day, 95% CI 37.8–54.1, respectively), SFA (by 7.4 g/day, 95% CI 4.0–10.8 and 6.5 g/

day, 95% CI 3.1–9.9, respectively), MUFA (by 36.8 g/day, 95% CI 33.2–40.3 and by 35.1 g/day, 95% CI 31.6–38.6, respectively) and PUFA (by 3.1 g/day, 95% CI 1.0–5.1 and by 3.0 g/day, 95% CI 1.0–5.1, respectively). Table 2 demonstrates the changes observed in micronutrient intake from baseline to follow-up and the relevant differences between the two intervention arms. The changes were not significant between-groups, but there was a significant increase within groups in α-tocopherol intake (by 7.5 mg/day, 95% CI 5.7–9.3 and by 7.0 mg/day, 95% CI 5.2–8.8) and vitamin E intake (by 7.9 mg/day, 95% CI 2.1–13.8 and by 12.4 mg/day, 95% CI 6.6–18.2) after LPOO and HPOO consumption, respectively. No significant within-group changes or between-group differences were observed in the other examined macronutrients (protein, carbohydrates and dietary fibre) or micronutrients (β-carotene, vitamin C, selenium and zinc). The changes in intake of other food groups that are high in phenolic content (i.e., whole grain cereals, fruits, vegetables, legumes, nuts/seeds, soy products, oils, fruit juices, alcoholic drinks, tea and coffee) as determined by phenol explorer [29] were also examined. No significant within-group changes or between-group differences were observed in the daily intake of the abovementioned food and beverage items over the intervention period (data not shown).

Effect of LPOO and HPOO on anthropometrics

The changes observed in anthropometric indices from baseline to follow-up after each 3-week intervention, as well as the differences between the two treatment arms are presented elsewhere [16]. In brief, no significant between-group differences were observed in any of the examined outcomes. A small but significant increase in body weight by 0.4 kg (95% CI 0.2–0.7) was observed only in the LPOO treatment arm. No within-group changes were observed in any of the other outcomes (i.e., BMI and WC).

Effect of LPOO and HPOO on markers of oxidative status and inflammation

The effect of the two OO interventions on oxidative status markers is illustrated in Figs. 2 and 3. The changes in ox-LDL and TAC from baseline to follow up were not significantly different between the two treatment arms. However, for the total sample following HPOO consumption, compared to baseline, plasma ox-LDL decreased significantly by 6.5 mU/mL (95% CI: -12.4 to -0.5) (Fig. 2), and TAC increased by 0.03 mM (95% CI: 0.006 to 0.05) (Fig. 3). In addition, stratified analyses of study participants with higher WC measures (> 94 cm in males, > 80 cm in females), thus indicating cardiometabolic risk, exhibited a total decrease in ox-LDL by 13.5 mU/mL (95% CI − 23.5 to − 3.6) (Fig. 2) and an increase in TAC by
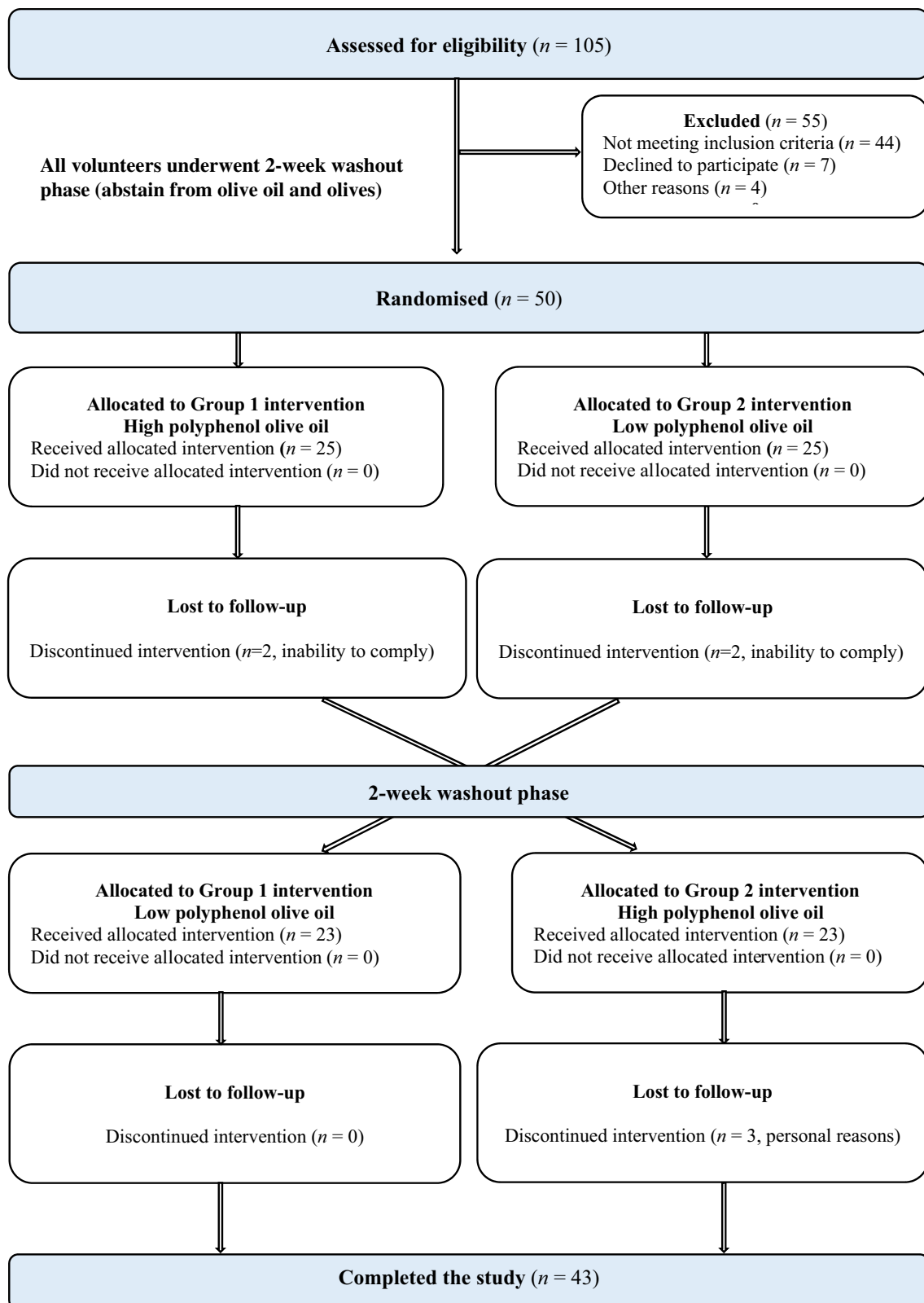
Fig. 1 Study participant flow diagram

Table 1 Baseline descriptive characteristics of study participants

	Total sample ($n=50$)	Low Polyphenol OO ($n=25$)	High Polyphenol OO ($n=25$)	P value*
Socio-demographics	Mean (SD)	Mean (SD)	Mean (SD)	
Age (years)	38.5 (13.9)	38.1 (14.8)	39.0 (13.2)	0.818
Education (years)	17.3 (3.5)	17.3 (4.2)	17.2 (2.8)	0.968
Gender	(%)	(%)	(%)	
Females	66.0	64	68	0.765
Males	34.0	36	32	
Country of Birth	(%)	(%)	(%)	
Australia, NZ, Pacific Islanders	70.0	68.0	72.0	0.321
Europe	10.0	4.0	16.0	
South America	8.0	12.0	4.0	
Middle East & Asia	12.0	16.0	8.0	
Anthropometrics	Mean (SD)	Mean (SD)	Mean (SD)	
Height (cm)	168.9 (9.6)	170.6 (10.4)	167.2 (8.6)	0.220
Weight (kg)	70.7 (12.8)	72.7 (13.7)	68.5 (11.8)	0.249
BMI (kg/m^2)	24.7 (3.5)	24.9 (3.7)	24.4 (3.2)	0.617
Waist circumference (cm)	86.9 (11.2)	88.2 (11.9)	85.6 (10.6)	0.434
Weight status categories[†]	(%)	(%)	(%)	
Underweight	2.0	0.0	4.0	0.252
Normal weight	50.0	56.0	44.0	
Overweight	44.0	36.0	52.0	
Obese	4.0	8.0	0.0	
Waist circumference categories[‡]	(%)	(%)	(%)	
Normal	50.0	44.0	56.0	0.632
High risk	16.0	20.0	12.0	
Very high risk	34.0	36.0	32.0	
Biochemical indices	Mean (SD)	Mean (SD)	Mean (SD)	
TAC (mM)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	0.826
ox-LDL (mU/mL)	75.4 (21.2)	70.5 (16.4)	80.4 (24.4)	0.099
hs-CRP (mg/L)	1.7 (2.8)	1.9 (2.6)	1.6 (3.1)	0.663
hs-CRP categories[§]	(%)	(%)	(%)	
Low risk	70.0	64.0	76.0	0.596
Intermediate risk	12.0	16.0	8.0	
High risk	18.0	20.0	16.0	

SD standard deviation, *OO* olive oil, *BMI* body mass index, *TAC* total antioxidant capacity, *ox-LDL* oxidized low density lipoprotein, *hs-CRP* high sensitivity C-reactive protein

[*]*p* values for testing between-group differences in continuous variables were derived from the independent samples *t* test. *P* values for examining associations between categorical variables were derived from the Chi-square test

[†]Weight status categories: Underweight, BMI < 18.5 kg/m^2; Normal weight, $18.5 \leq$ BMI < 25 kg/m^2; Overweight, $25 \leq$ BMI < 30 kg/m^2; Obese, BMI $\geq$ 30 kg/m^2

[‡]Waist circumference categories: Normal, WC < 80 cm in women & < 94 cm in men; High risk, 80–88 cm in women & 94–102 cm in men; Very high risk: WC > 88 cm in women and > 102 cm in men

[§]hs-CRP categories: Low risk, hs-CRP < 1 mg/L; Intermediate risk, 1–3 mg/L; High risk, hs-CRP > 3 mg/L

0.04 mM (95% CI 0.006–0.07) (Fig. 3) after HPOO intake. No within-group changes or between-group differences were observed in circulating hs-CRP of the total sample from baseline to follow up (Fig. 4). However, stratified analyses of participants with intermediate-high cardiometabolic risk based on their inflammation status (hs-CRP > 1 mg/L), demonstrated a significant decrease in hs-CRP by 1.9 mg/L (95% CI − 3.7 to − 0.1) after HPOO consumption (Fig. 4), but this change was not found to differ compared to the non-significant change observed in the LPOO treatment arm. No within-group

Table 2 Effect of low polyphenol OO vs. high polyphenol OO on mean changes in micronutrient intake

	Baseline Mean (SEM)	Follow-up Mean (SEM)	Mean change (95% CI) (Time effect)	p value (Treatment*time effect)
β-carotene intake (ug/day)				
Low Polyphenol OO (n = 50)	4379.1 (552.0)	4733.9 (686.8)	354.8 (− 828.1 to 1537.8)	0.368
High Polyphenol OO (n = 50)	3869.4 (552.0)	4986.6 (686.8)	1117.2 (− 65.8 to 2300.1)	
p value (Treatment effect)	0.515	0.795		
Vitamin C intake (mg/day)				
Low Polyphenol OO (n = 50)	122.3 (9.4)	114.6 (22.6)	− 7.7 (− 53.7 to 38.3)	0.196
High Polyphenol OO (n = 50)	112.9 (9.4)	147.8 (22.6)	34.9 (− 11.1 to 80.9)	
p value (Treatment effect)	0.479	0.301		
α-tocopherol intake (mg/day)				
Low Polyphenol OO (n = 50)	12.5 (0.9)	20.0 (0.8)	**7.5 (5.7–9.3)**	0.693
High Polyphenol OO (n = 50)	13.2 (0.9)	20.2(0.8)	**7.0 (5.2–8.8)**	
p value (Treatment effect)	0.580	0.864		
Vitamin E (mg/day)				
Low Polyphenol OO (n = 50)	17.2 (2.0)	25.1 (2.9)	**7.9 (2.1–13.8)**	0.282
High Polyphenol OO (n = 50)	16.0 (2.0)	28.4 (2.9)	**12.4 (6.6–18.2)**	
p value (Treatment effect)	0.677	0.416		
Selenium intake (ug/day)				
Low Polyphenol OO (n = 50)	87.9 (5.1)	90.2 (6.5)	2.2 (− 9.0 to 13.4)	0.409
High Polyphenol OO (n = 50)	88.2 (5.1)	97.1 (6.5)	8.8 (− 2.4 to 20.1)	
p value (Treatment effect)	0.966	0.453		
Zinc intake (mg/day)				
Low Polyphenol OO (n = 50)	10.7 (0.5)	11.5 (0.6)	0.821 (− 0.3 to 2.0)	0.327
High Polyphenol OO (n = 50)	11.5 (0.5)	11.6 (0.6)	0.018 (− 1.1 to 1.2)	
p value (Treatment effect)	0.243	0.981		

All statistical analyses were adjusted for gender and age. Results in bold indicate statistical significance ($p < 0.05$)

OO olive oil, *SEM* standard error of the mean, *CI* confidence interval

changes or between-group differences were observed in hs-CRP levels after stratified analyses of study participants with higher WC measures (Fig. 4).

Adherence to treatment

Adherence to treatment was overall high in the study cohort and did not differ significantly between the two treatment arms as shown in Supplementary Table (Online resource 1). Based on the OO volume returned by participants after each intervention period, adherence was found to be 92% for both the LPOO and HPOO treatment arm after the first intervention period and 92% for the LPOO and 90% for the HPOO treatment arm after the second intervention period.

Discussion

The present double blind, cross-over, randomized controlled trial investigated the effect of daily consumption of 60 mL raw extra virgin HPOO (320 mg/kg, phenolic content) in comparison with LPOO (86 mg/kg, phenolic content), each for 3 weeks, on circulating biomarkers of oxidative status and inflammation in Australian adults with no previously diagnosed medical condition. No between-group differences were observed in any of the examined biomarkers between the two treatment arms. However, HPOO consumption induced a significant increase in TAC and a reduction in plasma ox-LDL in the total cohort.
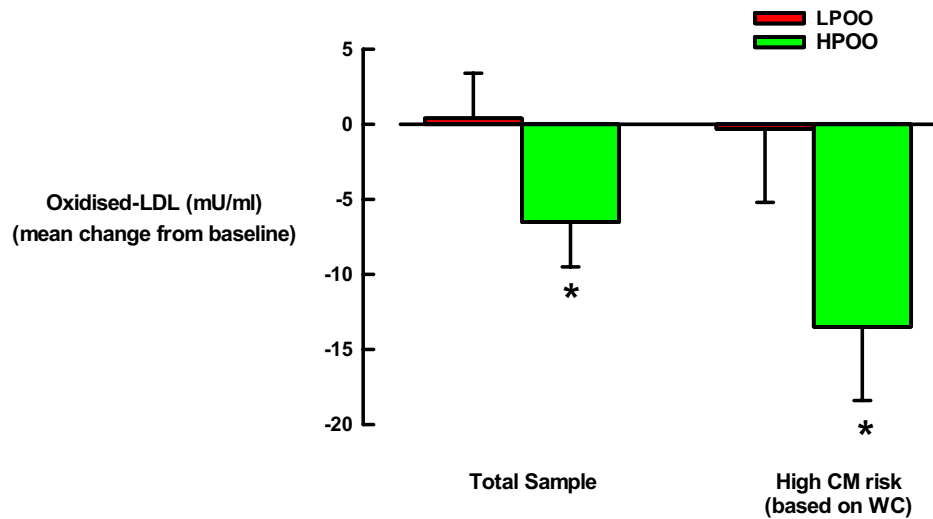
Fig. 2 Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil (HPOO) and low polyphenol olive oil (LPOO) on oxidised-LDL (ox-LDL). Results are presented as mean changes ± standard errors from baseline to follow-up for the total sample ($n = 50$) and for participants with high cardiometabolic risk (CM) ($n = 25$, LPOO group; $n = 25$, HPOO group) based on waist circumference (WC) measures (i.e., > 94 cm in males, > 80 cm in females). HPOO, 320 mg/kg polyphenols; LPOO, 86 mg/kg polyphenols. *$p < 0.05$, significant within-group change from baseline to follow-up
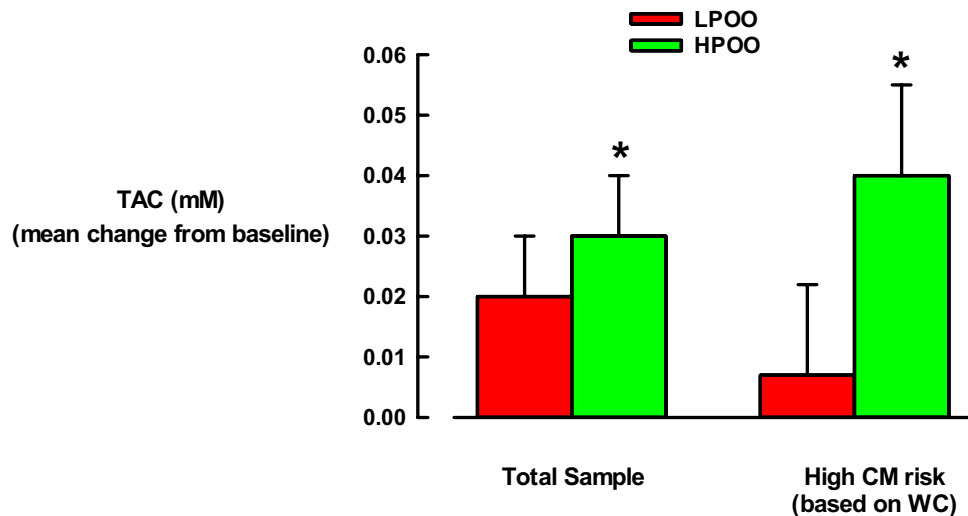
Fig. 3 Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil (HPOO) and low polyphenol olive oil (LPOO) on total antioxidant capacity (TAC). Results are presented as mean change ± standard errors from baseline to follow-up for the total sample ($n = 48$, LPOO group; $n = 46$, HPOO group) and for par- ticipants with high cardiometabolic risk (CM) ($n = 23$, LPOO group; $n = 22$, HPOO group) based on waist circumference (WC) measures (i.e., > 94 cm in males, > 80 cm in females). HPOO, 320 mg/kg polyphenols; LPOO; 86 mg/kg polyphenols. *$p < 0.05$, significant within- group change from baseline to follow-up

Furthermore, when the statistical analyses were stratified to include only participants with a high cardiometabolic risk based on their WC measures, HPOO consumption induced an even more pronounced increase in TAC and a greater reduction in plasma ox-LDL. A significant decrease was also observed in circulating hs-CRP for those participants with intermediate-high cardiometabolic risk

based on their inflammation status. No significant within-group changes were observed after LPOO consumption.

The changes observed in ox-LDL (by 6.5 mU/mL and 13.5 mU/mL in the total sample and high cardiometabolic risk subsample based on WC measures, respectively) in the present study are comparable to the changes reported in previous clinical trials which have examined the effect of VOO

Fig. 4 Effect of 3-weeks daily consumption of extra virgin high poly-phenol olive oil (HPOO) and low polyphenol olive oil (LPOO) on high-sensitivity C-reactive protein (hs-CRP). Results are presented as mean change ± standard errors from baseline to follow-up for the total sample ($n = 50$), participants with high cardiometabolic risk (CM) ($n = 25$, LPOO group; $n = 25$, HPOO group) based on waist circum-ference (WC) measures (i.e., > 94 cm in males, > 80 cm in females;) and for participants with intermediate-high CM risk ($n = 16$, LPOO group; $n = 14$, HPOO group) based on hs-CRP levels (i.e. > 1 mg/L). HPOO, 320 mg/kg polyphenols; LPOO; 86 mg/kg polyphenols. *$p < 0.05$ significant within-group change from baseline to follow-up

intake on oxidative status markers. In this context, Marrugat et al. [30] demonstrated that a 3-week intervention of three OOs (daily dose of 25 mL) that differed only in their phe-nolic content (ROO, 0 mg/kg phenolic content; common OO, 68 mg/kg phenolic content; VOO, 150 mg/kg phenolic content) demonstrated a significant decrease in ox-LDL by 14.5 mU/mL after VOO consumption in healthy adults. In another RCT, Hernaez et al. [31] reported that the daily con-sumption of 25 mL raw HPOO (366 mg/kg, phenolic con-tent) for 3-weeks led to a significant decrease in ox-LDL by 3.2 mU/mL compared to LPOO (2.7 mg/kg, phenolic con-tent) in healthy men. Furthermore, Moreno-Luna et al. [32] found that daily consumption of 60 mL HPOO (564 mg/kg, phenolic content) for 8 weeks induced a significant reduction in ox-LDL compared to ROO (polyphenol free), in young women with mild hypertension.

The differences in the daily doses of the treatment OOs, their phenolic content and the duration of the intervention between the aforementioned clinical trials and our study pro-vides an explanation of the different reported effects [21, 33]. However, in most of these previous clinical trials, OO was consumed as part of the MedDiet, further suggesting that many of the OO attributed benefits might be also related to other foods in this diet that are considered good sources of nutrients with antioxidant properties. In this context, a recent network meta-analysis reported that consumption of OO with a moderate polyphenol concentration (~ 60 mg/kg) as part of the MedDiet is sufficient to induce antioxidant

effects [33]. In the present study, however, this was not the case, since OO was not consumed as part of the Mediter-ranean dietary pattern. This in conjunction with the cross-over study design enabled us to isolate the effects of a single ingredient in the MedDiet, i.e., EVOO.

The mechanisms by which OO polyphenols can protect LDL from oxidation may be reflected in their ability to coun-teract both metal- and radical-dependent oxidation and to act as chain-breaking antioxidants for peroxidation [34]. In addition to the potential direct antioxidant properties of OO polyphenols, these have been found to preserve the activity of other antioxidants present in OO (i.e. tocopherols), thus increasing the resistance of LDL against oxidation [35, 36]. Considering that antioxidants exert their effect against oxi-dation synergistically, no single antioxidant could represent overall antioxidant status in vivo. For this reason, several biomarkers have been used to reflect the human body's anti-oxidant status, including plasma TAC [9]. Previous studies have found low plasma levels of TAC in individuals with coronary heart disease [37], thus providing some initial indi-cations on the link between overall antioxidant status and CVD risk [38]. In the present study, plasma TAC increased significantly after HPOO consumption by 0.03 mM in the total sample and by 0.04 mM in participants with high car-diometabolic risk based on their WC measures. This finding provides further evidence of the ability of OO polyphenols to enhance endogenous antioxidant status. Similar to our results, a single-arm clinical trial with healthy adults found

that daily consumption of 50 mL EVOO rich in phenolic compounds (510 mg/kg, phenolic content) for 4 weeks increased plasma TAC levels by 0.3 mM [39]. Furthermore, the VOLOS study showed that 7 weeks of daily consumption of 40 mL EVOO containing 166 mg/kg polyphenols, increased TAC significantly by 0.08 mM compared to LPOO (2 mg/kg, phenolic content,) in adults with mild dyslipidemia [40].

In addition to oxidation, increased blood levels of inflammatory molecules, such as CRP and various inflammatory cytokines, have been closely linked to endothelial dysfunction and serve as additional risk predictors for CVD [41]. It is recognized that foods rich in phenolic compounds, including EVOO, have cardioprotective effects due to their anti-inflammatory properties [17, 21, 33]. The mechanisms by which polyphenols can exert their anti-inflammatory effect seems to be mediated via their regulatory role in the production and secretion of a wide variety of pro-inflammatory molecules [42]. In the present study, HPOO consumption was found to significantly reduce hs-CRP levels by 1.9 mg/L in adults classified as intermediate-high cardiometabolic risk based on their hs-CRP levels (i.e., > 1 mg/L). In another study, Moreno-Luna et al., [32] demonstrated that women with mild hypertension had reduced CRP concentrations (also by 1.9 mg/L) after daily intake of 60 mL HPOO (564 mg/kg, phenolic content) for 8 weeks compared to ROO (polyphenol free). Furthermore, Fito et al., [43] reported reduced CRP levels by 1.1 mg/L in stable coronary disease patients after 3-weeks of 50 ml VOO (161 mg/kg, phenolic content) consumption in comparison with LPOO (14.67 mg/kg, phenolic content). On the other hand, a 4-week intervention that compared the daily intake of 25 mL EVOO (577 mg/kg, phenolic content) with ROO (polyphenols not detectable) did not produce any changes in CRP, IL-6 or TNF-α in individuals with type 2 diabetes and overweight [44].

In terms of dietary intake changes, in the current study, both LPOO and HPOO treatment equally increased intake of specific micro-nutrients (i.e., vitamin E, α-tocopherol, selenium, zinc) that have established antioxidant and/or anti-inflammatory properties [9]. This finding was expected due to the similar nutrient composition of the two treatment OOs, with the only exception being their phenolic content. The implication of these dietary intake changes is that the only dietary factor that can explain the significant and favorable within-group changes observed after extra virgin HPOO consumption was polyphenols, since their intake was 4-folds higher in the HPOO compared to the LPOO treatment arm (320 mg/kg vs. 86 mg/kg). Furthermore, the non-significant between-group differences with regards to the changes in ox-LDL, TAC and CRP can be explained by the phenolic content of the LPOO examined in the present study. In this regard, although the concentration of polyphenols in the LPOO was lower compared to extra virgin HPOO, it was still high enough to produce some clinically significant health benefits, also in synergy to the intake of the other important bioactive nutrients that are present in the intervention OOs. Moreover, the increased energy intake and body weight after the addition of 60 mL of the intervention oils in participants' daily diet, would be expected to lead to a non-beneficial effect of the test oils on the examined outcomes. However, this was not the case in our study, indicating a potential counterbalancing effect of OO polyphenols on weight gain, whilst confirming their antioxidant and anti-inflammatory activity.

The findings of the present study should be interpreted taking into consideration its strengths and limitations. The main strength is the study's randomized, double-blind cross-over design, which allows each participant to act as their own control by consuming both intervention OOs, thus reducing inter-individual variability. This design also enabled us to isolate the effects of OO polyphenols on the examined outcomes, since it allowed us to control for potential confounding effect caused by differences in the intake of other nutrients with antioxidant and/or anti-inflammatory properties. Another strength is that study participants remained in line with their habitual diet, hence allowing to directly assess the benefits of OO consumption. One of the limitations of the current study is that although participant adherence to the intervention was overall high, measurements of compliance relied on self-reporting and were therefore subjective. Another limitation is that the present study was not adequately powered to detect significant changes in hs-CRP, considering that the study sample size was originally calculated on the basis of the expected differences only in the primary outcome (i.e., HDL-efflux). Lastly, despite the inclusion of a washout period before the initiation of the intervention and between the intervention periods, there is no guarantee that any potential carry-over effect on the examined biomarkers was completely avoided. However, pairwise comparisons that examined potential carry-over effects were insignificant for all biomarkers.

Conclusions

The OLIVAUS study examined the effect of OO polyphenols on oxidative status and inflammation biomarkers in Australian adults. There were no significant differences between the two OO treatment arms in any of the examined outcomes. There was however a significant within-group reduction in plasma ox-LDL and hs-CRP and an increase in plasma TAC after the daily consumption of 60 mL of extra virgin HPOO for 3-weeks, which was more pronounced in individuals with increased WC measures and hs-CRP levels, thus indicating elevated metabolic risk, at pre-intervention. Considering the strong link of oxidative stress and inflammation with CVD

risk, *these findings highlight the preventive potential of extra virgin HPOO in healthy individuals and* especially *in those with a higher risk for CVD and therefore most in need for primary prevention initiatives.* Finally, *considering that all previous intervention studies were conducted in Mediterranean populations, the results of our study provide new evidence for a potentially widely accessible dietary intervention that can be also incorporated in the multicultural Australian context as a means for the primary prevention of CVD.*

Declarations

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Appendix L

The Effect of High Polyphenol Extra Virgin Olive Oil on Blood Pressure and Arterial Stiffness in Healthy Australian Adults (OLIVAUS): a randomized, controlled, cross-over study

Article

The Effect of High Polyphenol Extra Virgin Olive Oil on Blood Pressure and Arterial Stiffness in Healthy Australian Adults: A Randomized, Controlled, Cross-Over Study

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Abstract: Extra virgin olive oil (EVOO) is suggested to be cardioprotective, partly due to its high phenolic content. We investigated the effect of extra virgin high polyphenol olive oil (HPOO) versus low polyphenol olive oil (LPOO) on blood pressure (BP) and arterial stiffness in healthy Australian adults. In a double-blind, randomized, controlled cross-over trial, 50 participants (age 38.5 ± 13.9 years, 66% female) were randomized to consume 60 mL/day of either HPOO (360 mg/kg polyphenols) or LPOO (86 mg/kg polyphenols) for three weeks. Following a two-week washout period, participants crossed over to consume the alternate oil. Anthropometric data, peripheral BP, central BP and arterial stiffness were measured at baseline and follow up. No significant differences were observed in the changes from baseline to follow up between the two treatments. However, a significant decrease in peripheral and central systolic BP (SBP) by 2.5 mmHg (95% CI: −4.7 to −0.3) and 2.7 mmHg (95% CI: −4.7 to −0.6), respectively, was observed after HPOO consumption. Neither olive oil changed diastolic BP (DBP) or measures of arterial stiffness. The reductions in SBP after HPOO consumption provide evidence for a potentially widely accessible dietary intervention to prevent cardiovascular disease in a multiethnic population. Longer intervention studies and/or higher doses of EVOO polyphenols are warranted to elucidate the potential effect on DBP and arterial stiffness.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity worldwide. Established risk factors such as hypertension, dyslipidaemia, diabetes and obesity contribute to 9.7 million annual deaths related to CVD globally [1]. The most recent Australian data, collected between 2017 and 2018, indicates 27% of all deaths (43,477 deaths) were attributed to CVD [2]. A similarly high proportion of Australian adults (~34%) have diagnosed hypertension [3]. Previous studies have indicated that changes in peripheral and central hemodynamics such as in peripheral (brachial) and central (aortic) BP and pressure wave reflections contribute to the development of adverse cardiovascular events [4]. Moreover, stiffening of the central elastic arteries, such as the aorta and the pulmonary arteries, is an accepted independent predictor of CVD risk and is positively associated with systolic hypertension [5,6]. Several surrogate markers reflecting vascular health are used in clinical practice. Pulse wave velocity (PWV), estimated by non-invasive applanation tonometry and pulse wave analysis, is considered the gold-standard marker of arterial stiffness [7]. Furthermore, systemic arterial wave reflections, as measured by the augmentation index (AIx), provide additional clinical information on CVD, while assessment of central BP and pulse pressure (PP) provides further predictive value beyond the corresponding brachial BP [6,8,9].

Extensive evidence indicates that certain dietary patterns are cardioprotective [10]. The traditional Mediterranean diet (MedDiet), has been shown to improve CVD risk factors including lipidemic and glycaemic profile, markers of inflammation and oxidative stress [11,12]. The MedDiet is plant rich, with staple foods consisting of wholegrain cereals, vegetables, fresh fruit, seafood, legumes, nuts and red wine [12,13]. Previous studies have demonstrated that MedDiet food components improve vascular health. Moderate consumption of red wine has been found to reduce BP and improve arterial stiffness in healthy individuals [14] and patients with coronary artery disease [15,16]. Furthermore, regular consumption of olive oil (OO), which is the principal source of dietary fat in the MedDiet, has demonstrated BP-lowering effects [17,18].

The reported cardioprotective benefits of OO have been mostly attributed to the presence of variable concentrations of bioactive compounds, including polyphenols (also referred to as biophenols), mainly known for their anti-inflammatory and antioxidant properties [12,19,20]. One of the determinants of final OO polyphenol concentration is the oil extraction procedure [20–22]. In particular, extra virgin olive oil (EVOO) is obtained by mechanical extraction techniques under conditions that preserve high polyphenol concentrations, whereas refined OO (ROO) is subject to both physical and/or chemical processing, which significantly lowers the phenolic content [22]. Although there is some evidence linking dietary polyphenol intake, including those in virgin OOs, with decreased CVD risk [23,24], this favourable effect of polyphenols is not currently taken into consideration by dietary guidelines, thus indicating a need for further relevant evidence.

Our research team recently conducted a meta-analysis of the published literature to determine the effects of HPOO consumption, compared with LPOO, on cardiovascular markers [19]. This meta-analysis indicated that HPOO can improve outcomes related to cholesterol (total and high-density lipoprotein (HDL)) and oxidative stress (oxidized low-density lipoprotein (LDL) and malondialdehyde) compared to the LPOO intervention arm. However, no significant changes were observed with respect to SBP and DBP after either OO consumption (daily dose ranged between 25 and 75 mL), while none of the included studies reported measures of arterial stiffness. In 2019, a network meta-analysis reported that EVOO may reduce oxidized LDL (ox-LDL) and LDL cholesterol compared to ROO and LPOO, respectively, while a dose–response relationship was observed between higher intakes of OO phenolic compounds and lower SBP and ox-LDL values [25]. As also stated

in another recent review, most intervention studies investigating the effect of HPOO on CVD risk markers have been conducted in Mediterranean populations that have high habitual OO intake [26], thus highlighting the need for additional research on multiethnic populations with different habitual food cultures. Hence, the aim of the current study was to examine the effect of daily consumption of (60 mL) raw extra virgin HPOO, compared to LPOO, for 3 weeks, on peripheral and central BP and arterial stiffness in Australian adults with no previously diagnosed medical condition.

2. Materials and Methods

2.1. Study Population

The OLIVAUS study [20] was conducted according to the Guidelines for Good Clinical Practice (GCP), the guidelines laid down in the Declaration of Helsinki and the CONSORT reporting guidelines. All procedures involving human subjects were approved by the Human Research Ethics Committee of La Trobe University (HEC17-067) and written informed consent was obtained from all volunteers. The trial protocol has been registered with the Australia New Zealand Clinical Trials Registry ACTRN12618000706279.

All participants were recruited in Melbourne, Australia, via social media and La Trobe University email database advertising, word of mouth and posters on campus. A standardized screening procedure was followed in order to identify eligible participants, who were required to be within the age range of 18–75 years and a body mass index (BMI) of 18.5–40 kg/m^2. Exclusion criteria included non-English-speaking individuals, pregnant or lactating women, smokers, individuals on a special type of diet for medical reasons (e.g., gluten free for coeliac disease) and/or with a high habitual OO intake (>1 tablespoon/day). Exclusion also applied if individuals were taking vitamins or antioxidant supplements as part of a regular regime and were unable to discontinue their use for the duration of the trial (with the exception of iron, calcium and Vitamin D). Finally, study subjects taking prescribed medication (e.g., antihypertensive agents, lipid-lowering drugs, non-steroidal ant-inflammatory drugs) and those with diagnosed chronic diseases (diabetes, hyperlipidaemia, hypertension, and inflammatory conditions), gut-related diseases or any other condition that could impair adherence were also excluded.

2.2. Study Design and Procedure

The OLIVAUS study was a double-blind, cross-over, randomized controlled trial (RCT) aiming to evaluate the effect of extra virgin HPOO consumption on CVD risk markers in comparison with a commercially available OO which was low in polyphenols (LPOO). Prior to the main study, a pilot study was conducted with five study participants in order to test the feasibility of the study protocol and the data collection tools [27]. Enrolled participants were randomly assigned, in a 1:1 ratio, to one of two treatment arms, i.e., Group (1) extra virgin HPOO/LPOO or Group (2) LPOO/extra virgin HPOO, using the block-randomization method of a software program for sequence. Blocks of 6 participants were generated by a senior researcher, who was not directly involved in the participant recruitment or data collection phase. Allocation of each participant was emailed to the research team at the commencement of the study by a researcher who was not involved in any participant contact.

Study participants were requested to consume a daily dose of 60 mL of either type of raw OO over 2 intervention periods of 3 weeks each, in conjunction with their habitual diet. The two types of OO varied only in their phenolic content (i.e., 360 mg/kg in HPOO vs. 86 mg/kg in LPOO) but did not differ with respect to the rest of their nutrient composition, including their fatty acid profile. Two washout periods, of 2 weeks each, during which study participants were instructed to avoid olives and OO consumption, preceded the first and the second intervention periods of OO administration. The intervention in the present study was designed with a daily dose of 60 mL OO, which reflects the habitual intake in populations where the cardioprotective benefits of virgin OO have been previously reported [19,25,26].

Participants were provided with OO bottles at the beginning of each intervention period. The OOs were supplied in dark coloured glass containers to minimise phenolic content loss due to sunlight. To ensure blinding of the researchers to the OO type, each bottle was assigned a different code number that was concealed from study participants and research team members. This was disclosed only after the completion of the statistical analyses. To assess the level of adherence to the intervention, participants were instructed to return the containers at the end of each intervention period so that the daily amount of unconsumed OO could be measured and recorded. Study participants were also instructed to keep a written record of daily OO consumed during each intervention period using a checklist provided to them. This information was recorded by research team members after the end of each intervention period. Full details of the study protocol, including a comparison of the concentrations of total polyphenols and polyphenol subclasses in each of the two types of OOs, are provided elsewhere [20].

2.3. Measurements

2.3.1. Socio-Demographics, Use of Medication and Dietary Supplements

Socio-demographic data were collected from eligible participants during a scheduled interview at our trial clinic room located at La Trobe University. Trained researchers conducted all interviews using a standardized questionnaire. Specifically, the socio-demographic data collected during this interview included age, gender, language(s) spoken at home, level of education, ethnicity and parental country of birth. Any medications and dietary supplements taken by the study participants were also recorded.

2.3.2. Dietary Intake

A 3-day food diary was used to collect information on the dietary intake of study participants during two weekdays and one weekend day (preferably non-consecutive) at baseline and follow up of each intervention period. Specifically, study participants were instructed to record details on their intake of food and beverages, including information on the quantity, type/brand and cooking methods of the consumed items. The level of detail required to be recorded in the diary as well as additional strategies on how to incorporate raw, uncooked OO in their habitual diet were provided to study participants at a pre-baseline meeting by a trained nutritionist. The completed food diaries were returned and checked by the research team members for potential wrong or missing entries during the scheduled interviews with the study participants. All dietary intake data were analyzed for energy, macro- and micronutrient content using FoodWorks®9 software (Xyris Software Pty Ltd., Brisbane, Queensland, Australia).

2.3.3. Physical Activity

Physical activity (PA) was assessed using the Active Australia Survey (AAS) questionnaire [3], a tool that has been validated in the Australian population. This questionnaire is designed to assess participation in a range of leisure-time physical activities of light, moderate and vigorous intensity. The questionnaire consists of eight questions, which assess the number of sessions and total weekly time (hours and/or minutes) spent for each activity type. Study participants were required to complete and submit the AAS questionnaire during the week preceding the interviews at the first baseline and at the last follow-up meeting. The amount of time (in minutes per day) that study participants were engaged in physical activity of different intensity was calculated and used for data analysis.

2.3.4. Anthropometric Measurements

Anthropometric measurements were conducted four times during the study, i.e., at baseline and follow up of each intervention period. Body weight and standing height were measured with study participants in light clothing and barefoot, using a digital scale (WM203, Willawong QLD, Australia), to the closest 0.1 kg and a wall-mounted stadiometer (SE206, Seven Hills, NSW, Australia) to the

nearest 0.1 cm, respectively. Waist circumference (WC) was measured to the nearest 0.1 cm, using a flexible steel tape calibrated in cm with mm graduations (Luftkin W606PM, Sparks, MD, USA) directly over the skin at the umbilicus level. Body mass index (BMI) was calculated using Quetelet's equation (weight (kg)/height (m)^2). Using World Health Organization (WHO) cut-off points for BMI, study participants were classified as underweight (BMI < 18.5 kg/m^2), normal weight (BMI 18.5–24.9 kg/m^2), overweight (BMI 25.0–29.9 kg/m^2) or obese (BMI ≥ 30 kg/m^2) [28]. Furthermore, gender-specific WC cut-off points proposed by the WHO were also used to categorise study participants for CVD risk: normal (WC < 94 cm in men and <80 cm in women), high CVD risk (WC 94–102 cm in men and 80–88 cm in women) and very high CVD risk (WC > 102 cm in men and 88 cm in women) [29].

2.3.5. Hemodynamic Indices

Blood Pressure

Peripheral (brachial) and central (aortic) blood pressure (BP) were measured using applanation tonometry with a SphygmoCor XCEL device (Model XCEL, AtCor Medical, Sydney, Australia), at baseline and follow-up examinations at each intervention period. Following a minimum of 5 min rest in the supine position, peripheral brachial systolic BP (SBP) and diastolic BP (DBP) was measured using a blood pressure cuff affixed to the upper left arm. Three consecutive BP recordings were made and the average of the last two recordings was used for data analysis. In addition, central SBP and DBP, as well as PP measures were automatically derived via the brachial BP cuff. The BP categories recommended by the American College of Cardiology (ACC)/American Heart Association (AHA) were used to classify study participants into those with Normal BP (SBP/DBP < 120/80 mmHg), Elevated BP (SBP 120–129 mmHg and DBP < 80 mmHg), Hypertension Stage I (SBP 130–139 mmHg or DBP 80–89 mmHg) and Hypertension Stage II (SBP ≥ 140 mmHg or DBP ≥ 90 mmHg) [9].

Arterial Stiffness

Measures of peripheral and central arterial stiffness, using pulse wave analysis (PWA) and pulse wave velocity (PWV), were obtained non-invasively with the SphygmoCor XCEL device (Model XCEL, AtCor Medical, Sydney, Australia). This was carried out using the standard procedure as outlined in our previous paper [8]. PWA is a non-invasive, valid and reliable technique to investigate mechanical properties of the arterial tree, using central blood pressures and analysis of systemic arterial wave reflection. Peripheral arterial stiffness indices of augmentation pressure (AP) and the augmentation index (AIx) were derived automatically by the device as part of the standard BP measurement procedure. The AP was calculated as the difference between the first and second systolic peak, while the AIx was calculated as the percentage contribution that the AP makes to the overall PP ($AIx = AP/PP \times 100$). PWV was measured using a tonometer to capture the carotid waveform, while a femoral cuff was placed high on the left thigh in order to capture the femoral waveform. The PWV was then calculated by dividing the distance between the carotid and femoral measurement sites by the transit time. This method is considered the gold standard technique for assessing central arterial stiffness.

2.4. Sample Size Calculation

Power calculations showed that a sample size of 40 was adequate to provide sufficient statistical power to detect a statistically significant between-group difference of 5% and a standard deviation (SD) of 11 in HDL-C efflux levels (i.e., the primary outcome of the OLIVAUS study), with 80% power and 5% level of significance [30]. The total sample size was set at 50 study participants, in order to also account for an attrition rate of 20%. Although, the selected sample was adequate for the examination of HDL-C efflux, this might not be the case for the secondary outcomes of the OLIVAUS study, including BP and measures of arterial stiffness.

2.5. Statistical Analysis

All statistical analyses were conducted using the SPSS statistical software for Windows (IBM, version 24.0; IBM, Armonk, NY, USA). For all continuous variables, the Kolmogorov–Smirnov test was performed to examine the normality of their distribution. A general linear model, i.e., repeated-measures ANOVA (analysis of variance), was used to examine the between-group differences (treatment effect, i.e., extra virgin high vs. low polyphenol OO) of mean values at each time point of measurement, the within-group changes (time effect) from baseline to follow up in each intervention arm, and the differences in the changes from baseline to follow up between the two intervention arms (treatment × time interaction effect). Both per protocol (PP) and intention-to-treat (ITT) analyses were performed. The PP analyses were conducted in study participants who had full data from baseline to follow up in the first or the second intervention period. For the ITT analyses, multiple imputations were conducted in order to compensate for all missing values. Five imputed models derived from this process. Considering that the PP and the ITT analyses provided similar results (i.e., mean values, mean changes and statistical significance), the results coming from the latter are presented in this article. In all statistical analyses, adjustments were made for gender and age. Data are presented either as the mean ± SD, as estimated marginal means and standard errors (SE) or as the mean change and 95% confidence interval of change (CI) for continuous variables and as frequency (*n*) and percentage (%) for categorical ones. All reported *p* values are two tailed, and the level of statistical significance is set at $p < 0.05$.

3. Results

Fifty volunteers (*n* = 33 females, and *n* = 17 males), from 105 interested individuals who agreed to be screened, were eligible and enrolled in the study from July 2018 through to October 2019. Seven participants discontinued the intervention, due to inability to comply (*n* = 4) and for personal reasons, (*n* = 3) and therefore 43 participants completed the study. Figure 1 provides the study participant flow diagram. Minor adverse events were recorded after the consumption of both intervention OOs, with nausea and heart burn being the most common symptoms. The proportion of participants that experienced symptoms of nausea (24%) and heart burn (6%) was comparable between the HPOO and LPOO group.

3.1. Baseline Characteristics of Study Participants

Table 1 presents the descriptive characteristics of study participant socio-demographics, anthropometrics and hemodynamic indices in the total sample (*n* = 50) and by gender. Study participants had a mean age of 38.5 ± 13.9 years (the age range was between 20 and 70 years) and their mean years of education was 17.3 ± 3.5. In addition, the majority of study participants were females (66%), had a tertiary education (86%) and were born in Australia (70%). No significant gender differences were observed in any of these socio-demographic characteristics. The mean BMI and WC was 24.7 ± 3.5 kg/m^2 and 86.9 ± 11.2 cm, respectively, with no significant differences between genders. In addition, 44% of study participants were overweight and 4% were obese. Based on their WC measurements, 16% had a high cardiometabolic risk and 24% had very high risk. Although there were no significant differences between genders observed in BMI and WC, compared to females, male study participants were taller (179.3 ± 6.8 cm vs. 163.6 ± 5.8 cm, $p < 0.001$) and had a higher body weight (79.6 ± 9.6 kg vs. 66.1 ± 11.9 kg, $p < 0.001$). At baseline, the mean peripheral SBP and DBP for the cohort was 120.0 ± 13.4 and 69.9 ± 8.4 mmHg, respectively, while 18% of study participants were categorised as having elevated BP, 20% had Stage 1 Hypertension and 8% had Stage 2 Hypertension. Mean central SBP and DBP was 106.8 ± 13.3 and 70.6 ± 8.7 mmHg, respectively, mean heart rate was 61.5 ± 10.2 bpm and PWV was 9.5 ± 1.4 m/s. There were no significant differences between genders in any of these hemodynamic indices.
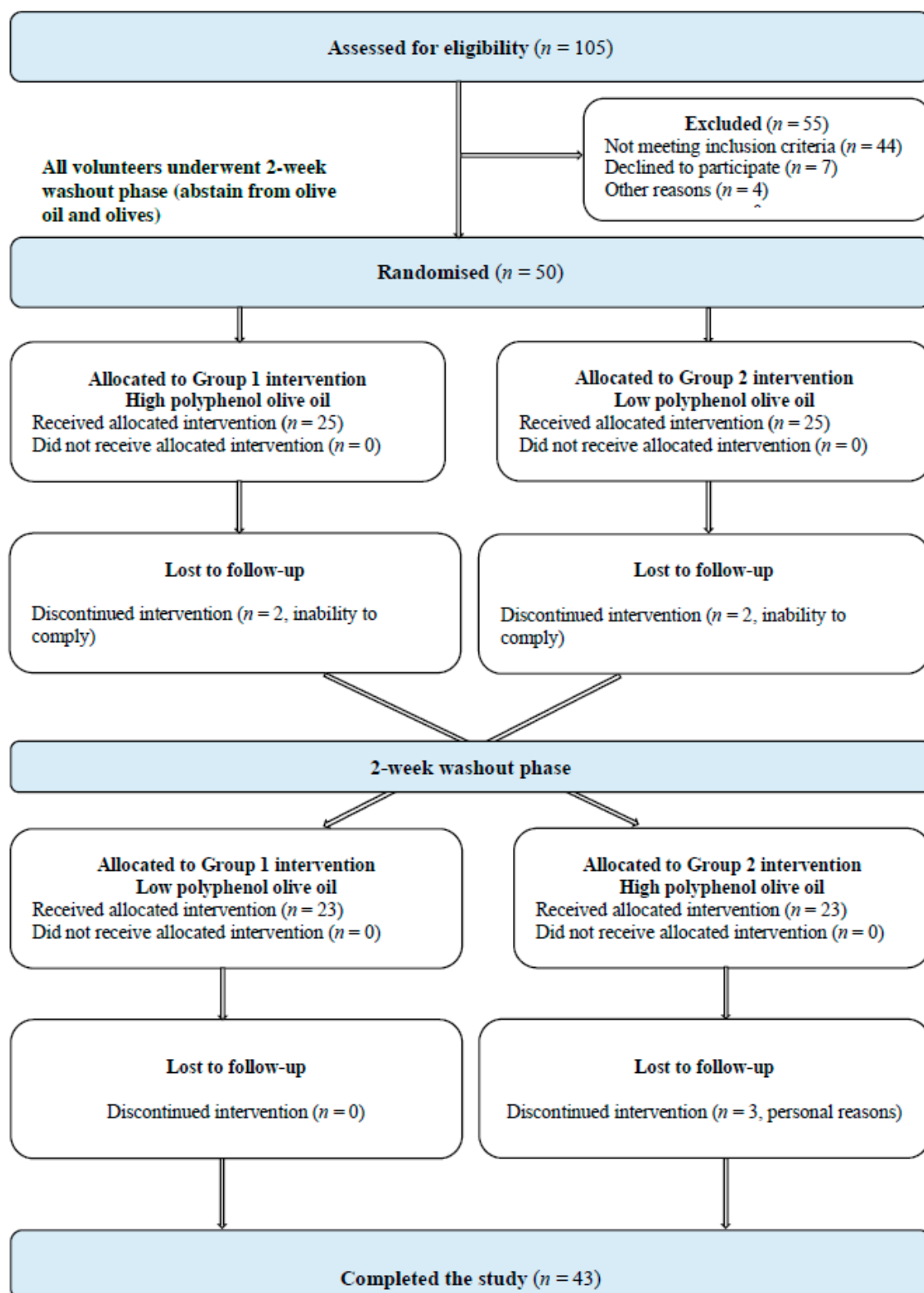
Figure 1. Study participant flow diagram.

Table 1. Descriptive characteristics of study participants.

Socio-Demographics	Total Sample (*n* = 50) Mean (SD)	Males (*n* = 17) Mean (SD)	Females (*n* = 33) Mean (SD)	*p*-Value *
Age (years)	38.5 (13.9)	33.4 (11.6)	41.2 (14.4)	0.058
Education (years)	17.3 (3.5)	17.4 (3.7)	17.2 (3.5)	0.895
Highest Level of Education	*n* (%)	*n* (%)	*n* (%)	0.241
Secondary School	2 (4.0)	0 (0.0)	2 (6.1)	
Tertiary	43 (86.0)	17 (100.0)	26 (78.8)	
Trade	2 (4.0)	0 (0.0)	2 (6.1)	
Other	3 (6.0)	0 (0.0)	3 (9.1)	
Country of Birth				0.798
Australia, NZ, Pacific Islanders	35 (70.0)	11 (64.7)	24 (72.7)	
Europe	5 (10.0)	2 (11.8)	3 (9.1)	
South America	4 (8.0)	1 (5.9)	3 (9.1)	
Middle East and Asia	6 (12.0)	3 (17.6)	3 (9.1)	
Anthropometrics	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Height (cm)	168.9 (9.6)	179.3 (6.8)	163.6 (5.8)	**<0.001**
Weight (Kg)	70.7 (12.8)	79.6 (9.6)	66.1 (11.9)	**<0.001**
BMI (kg/m^2)	24.7 (3.5)	24.7 (2.4)	24.6 (3.9)	0.915
Waist Circumference (cm)	86.9 (11.2)	88.9 (8.7)	85.9 (12.3)	0.364
Weight Status Categories [†]	*n* (%)	*n* (%)	*n* (%)	
Underweight	1 (2.0)	1 (3.0)	0 (0)	0.649
Normal Weight	25 (50.0)	16 (48.5)	9 (52.9)	
Overweight	22 (44.0)	14 (42.4)	8 (47.1)	
Obese	2 (4.0)	2 (6.1)	0 (0)	
Waist Circumference Categories [‡]				
Normal	25 (50.0)	13 (39.4)	12 (70.6)	0.105
High Risk	8 (16.0)	6 (18.2)	2 (11.8)	
Very High Risk	17 (34.0)	14 (42.4)	3 (17.6)	
Hemodynamic Indices				
Peripheral Blood Pressure	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Peripheral SBP (mmHg)	120.0 (13.4)	121.7 (9.1)	119.1 (15.2)	0.454
Peripheral DBP (mmHg)	69.9 (8.4)	69.7 (8.9)	70.0 (8.3)	0.904
Peripheral Blood Pressure Categories [§]	*n* (%)	*n* (%)	*n* (%)	
Normal Blood Pressure	27 (54.0)	20 (60.6)	7 (41.2)	0.399
Elevated Blood Pressure	9 (18.0)	4 (12.1)	5 (29.4)	
Hypertension Stage 1	10 (20.0)	6 (18.2)	4 (23.5)	
Hypertension Stage 2	4 (8.0)	3 (9.1)	1 (5.9)	
Central Blood Pressure	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Central Aortic SBP (mmHg)	106.8 (13.3)	106.9 (8.5)	106.8 (15.3)	0.971
Central Aortic DBP (mmHg)	70.6 (8.7)	70.3 (9.1)	70.8 (9.7)	0.843
Pulse Pressure (mmHg)	36.0 (8.9)	36.2 (7.9)	35.9 (9.5)	0.930
Heart Rate (bpm)	61.5 (10.2)	58.1 (8.9)	63.2 (10.4)	0.092
Systemic Arterial Stiffness				
Augmented Pressure (mmHg)	6.8 (6.8)	4.8 (4.2)	7.8 (7.6)	0.077
Augmented Index (%)	16.6 (14.9)	12.2 (9.4)	18.9 (16.8)	0.077
Pulse Wave Velocity (m/s)	9.5 (1.4)	9.5 (1.3)	9.5 (1.5)	0.933

* *p*-values were derived from the Student's *t*-test for continuous variables and from the chi-square test for categorical variables. Results in bold indicate *p* < 0.05, and are therefore statistically significant. BMI, body mass index; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; [†]: Weight status categories: Underweight, BMI < 18.5 kg/m^2; Normal weight, 18.5 ≤ BMI < 25 kg/m^2; Overweight, 25 ≤ BMI < 30 kg/m^2; Obese, BMI ≥ 30 kg/m^2. [‡]: Waist circumference categories: Normal, WC < 80 cm in women and <94 cm in men; High risk, 80–88 cm in women and 94–102 cm in men; Very high risk: WC > 88 cm in women and >102 cm in men. [§]: Peripheral BP categories: Normal BP, SBP < 120 and DBP < 80 mmHg; Elevated BP, SBP > 120–129.9 and DBP < 80 mmHg; Hypertension Stage 1, SBP 130–139.9 or DBP 80–89.9 mmHg; Hypertension Stage 2, SBP > = 140 or DBP > = 90.

3.2. Effect of LPOO and HPOO on Dietary Intake and Physical Activity

The changes observed in dietary energy, macro- and micronutrient intake from baseline to follow up, as well as the differences between treatment arms are summarized in Table 2. The changes from baseline to follow up were not significantly different between the two treatment arms. However, dietary energy intake increased significantly in participants following LPOO (by 1806.1 kJ/day, 95% CI: 1075.4 to 2536.8) and HPOO (by 1766.6 kJ/day, 95% CI: 1035.9 to 2497.3). Consumption of LPOO and HPOO also significantly increased intake of total fat (by 49.3 g/day, 95% CI: 41.1 to 57.4 and 46.0 g/day, 95% CI: 37.8 to 54.1, respectively), SFA (by 7.4 g/day, 95% CI: 4.0 to 10.8 and 6.5 g/day, 95% CI: 3.1 to 9.9, respectively), MUFA (by 36.8 g/day, 95% CI: 33.2 to 40.3 and by 35.1 g/day, 95% CI: 31.6 to 38.6,

respectively) and PUFA (by 3.1 g/day, 95% CI: 1.0 to 5.1 and by 3.0 g/day, 95% CI: 1.0 to 5.1, respectively). In addition, no significant within-group changes or between-group differences were observed in the other examined macronutrients (protein, carbohydrates and dietary fibre), nor in micronutrients such as sodium, potassium, magnesium and calcium, including caffeine. Regarding physical activity, no within-group changes or between-group differences were observed in the time study participants were engaged in physical activities of moderate–vigorous intensity over the intervention period (data not shown).

Table 2. Effect of low polyphenol OO vs. high polyphenol OO on mean changes in dietary energy, macro- and micronutrient intake.

	Baseline Mean (SEM)	Follow Up Mean (SEM)	Mean Change (95% CI) (Time Effect)	*p*-Value (Treatment * Time Effect)
Energy intake (KJ/day)				
Low Polyphenol OO (*n* = 50)	8712.8 (328.3)	10518.9 (344.4)	**1806.1 (1075.4 to 2536.8)**	0.940
High Polyphenol OO (*n* = 50)	8892.6 (328.3)	10659.2 (344.4)	**1766.6 (1035.9 to 2497.3)**	
p-value (Treatment effect)	0.700	0.774		
Protein intake (g/day)				
Low Polyphenol OO (*n* = 50)	102.0 (5.5)	100.7 (5.2)	−1.3 (−14.3 to 11.8)	0.924
High Polyphenol OO (*n* = 50)	97.4 (5.5)	97.0 (5.3)	−0.4 (−13.4 to 12.7)	
p-value (Treatment effect)	0.558	0.619		
Carbohydrates (g/day)				
Low Polyphenol OO (*n* = 50)	214.8 (10.1)	213.4 (11.1)	−1.5 (−23.5 to 20.6)	0.972
High Polyphenol OO (*n* = 50)	219.9 (10.1)	217.8 (11.1)	−2.0 (−24.0 to 20.0)	
p-value (Treatment effect)	0.726	0.776		
Total fat intake (g/day)				
Low Polyphenol OO (*n* = 50)	79.9 (4.0)	129.2 (4.5)	**49.3 (41.1 to 57.4)**	0.571
High Polyphenol OO (*n* = 50)	84.3 (4.0)	130.3 (4.5)	**46.0 (37.8 to 54.1)**	
p-value (Treatment effect)	0.441	0.870		
SFA intake (g/day)				
Low Polyphenol OO (*n* = 50)	27.7 (1.5)	35.1 (1.9)	**7.4 (4.0 to 10.8)**	0.707
High Polyphenol OO (*n* = 50)	28.8 (1.5)	35.3 (1.9)	**6.5 (3.1 to 9.9)**	
p-value (Treatment effect)	0.620	0.953		
MUFA intake (g/day)				
Low Polyphenol OO (*n* = 50)	30.6 (1.7)	67.3 (1.9)	**36.8 (33.2 to 40.3)**	0.514
High Polyphenol OO (*n* = 50)	31.8 (1.7)	67.0 (1.9)	**35.1 (31.6 to 38.6)**	
p-value (Treatment effect)	0.605	0.877		
PUFA intake (g/day)				
Low Polyphenol OO (*n* = 50)	14.6 (1.0)	17.7 (1.0)	**3.1 (1.0 to 5.1)**	0.971
High Polyphenol OO (*n* = 50)	15.7 (1.0)	18.7 (1.0)	**3.0 (1.0 to 5.1)**	
p-value (Treatment effect)	0.483	0.469		
Fibre intake (g/day)				
Low Polyphenol OO (*n* = 50)	29.7 (1.7)	30.7 (1.8)	0.9 (−3.1 to 4.9)	0.314
High Polyphenol OO (*n* = 50)	29.6 (1.7)	33.5 (1.8)	3.8 (−0.2 to 7.8)	
p-value (Treatment effect)	0.963	0.268		
Sodium intake (mg/day)				
Low Polyphenol OO (*n* = 50)	2611.1 (269.5)	2287.1 (168.9)	−324.0 (−878.2 to 230.3)	0.994
High Polyphenol OO (*n* = 50)	3096.7 (269.5)	2775.5 (168.9)	−321.2 (−875.5 to 233.1)	
p-value (Treatment effect)	0.206	**0.044**		
Potassium intake (mg/day)				
Low Polyphenol OO (*n* = 50)	3486,7 (227.0)	3389.3 (170.5)	−97.3 (−631.1 to 436.4)	0.488
High Polyphenol OO (*n* = 50)	3334.4 (227.0)	3501.9 (170.5)	167.5 (−366.2 to 701.3)	
p-value (Treatment effect)	0.636	0.642		
Magnesium intake (mg/day)				
Low Polyphenol OO (*n* = 50)	574.2 (92.9)	446.0 (18.9)	−128.2 (−308.4 to 52.0)	0.271
High Polyphenol OO (*n* = 50)	433.6 (92.9)	447.6 (18.9)	14.0 (−166.2 to 194.2)	
p-value (Treatment effect)	0.287	0.953		
Calcium intake (mg/day)				
Low Polyphenol OO (*n* = 50)	1005.0 (92.3)	1056.2 (95.6)	51.1 (−205.1 to 307.3)	0.916
High Polyphenol OO (*n* = 50)	977.3 (92.3)	1009.2 (95.6)	31.9 (−224.3 to 288.1)	
p-value (Treatment effect)	0.832	0.729		
Caffeine intake (mg/day)				
Low Polyphenol OO (*n* = 46)	199.4 (54.1)	182.0 (35.6)	−17.4 (−122.3 to 87.6)	0.612
High Polyphenol OO (*n* = 43)	242.8 (56.0)	186.7 (36.8)	−56.1 (−164.6 to 52.5)	
p-value (Treatment effect)	0.578	0.926		

All statistical analyses were adjusted for gender and age. Results in bold indicate statistical significance (*p* < 0.05). OO, olive oil; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SEM, standard error of the mean; CI, confidence interval.

3.3. Effect of LPOO and HPOO on Anthropometrics

Table 3 summarizes the changes observed in anthropometric indices from baseline to follow up and the relevant differences between the two intervention arms. There was a small but significant increase in body weight by 0.4 kg (95% CI: 0.2 to 0.7) following the LPOO intervention, but this change was not found to differ compared to the non-significant change observed in HPOO group. No within-group changes or between-group differences were observed in BMI and WC after the daily consumption of the two intervention oils.

Table 3. Effect of low polyphenol OO vs. high polyphenol OO on mean changes in anthropometric indices.

	Baseline Mean (SEM)	Follow Up Mean (SEM)	Mean Change (95% CI) (Time Effect)	*p*-Value (Treatment * Time Effect)
Weight (kg)				
Low Polyphenol OO (*n* = 50)	70.8 (1.5)	71.2 (1.5)	**0.4 (0.2 to 0.7)**	0.163
High Polyphenol OO (*n* = 50)	70.7 (1.5)	70.9 (1.5)	0.2 (−0.1 to 0.4)	
p-value (Treatment * effect)	0.993	0.902		
Height (cm)				
Low Polyphenol OO (*n* = 50)	168.9 (0.9)	169.0 (0.9)	0.1 (−0.2 to 0.4)	0.890
High Polyphenol OO (*n* = 50)	168.9 (0.9)	169.0 (0.9)	0.1 (−0.1 to 0.4)	
p-value (Treatment effect)	0.974	0.992		
BMI (kg/m^2)				
Low Polyphenol OO (*n* = 50)	24.7 (0.4)	24.8 (0.4)	0.1 (−0.01 to 0.2)	0.305
High Polyphenol OO (*n* = 50)	24.7 (0.4)	24.7 (0.4)	0.02 (−0.1 to 0.1)	
p-value (Treatment effect)	0.993	0.897		
Waist circumference (cm)				
Low Polyphenol OO (*n* = 50)	87.1 (1.3)	87.4 (1.2)	0.3 (−0.1 to 0.7)	0.501
High Polyphenol OO (*n* = 50)	87.1 (1.3)	87.3 (1.2)	0.1 (−0.2 to 0.5)	
p-value (Treatment effect)	1.000	0.919		

All statistical analyses were adjusted for gender and age. Results in bold indicate statistical significance ($p < 0.05$). OO, olive oil; SEM, standard error of the mean; CI, confidence interval.

3.4. Effect of LPOO and HPOO on Peripheral BP, Central BP and Arterial Stiffness

The effect of the two intervention OOs on peripheral and central BP are illustrated in Figure 2. The changes from baseline to follow up were not significantly different between the two treatment arms. However, compared to baseline, peripheral (brachial) and central (aortic) SBP was significantly reduced after HPOO by 2.5 mmHg (95% CI: −4.7 to −0.3) and by 2.7 mmHg (95% CI: −4.7 to −0.6), respectively. No other significant within-group changes or between-group differences were observed in peripheral and central DBP, as well as in the rest of the examined hemodynamic (i.e., PP and HR) and arterial stiffness indices (i.e., AP, AIx and PWV) (Table 4).

Table 4. Effect of low polyphenol OO vs. high polyphenol OO on mean changes in hemodynamic and arterial stiffness indices.

	Baseline Mean (SEM)	Follow Up Mean (SEM)	Mean Change (95% CI) (Time Effect)	*p*-Value (Treatment * Time Effect)
Pulse pressure (mmHg)				
Low Polyphenol OO (*n* = 50)	35.7 (1.0)	36.4 (1.0)	0.7 (−0.8 to 2.1)	0.296
High Polyphenol OO (*n* = 50)	36.3 (1.0)	35.9 (1.0)	−0.4 (−1.9 to 1.1)	
p-value (Treatment effect)	0.653	0.723		
Pulse rate (bpm)				
Low Polyphenol OO (*n* = 50)	61.1 (1.3)	59.4 (1.4)	−1.7 (−3.9 to 0.4)	0.403
High Polyphenol OO (*n* = 50)	61.0 (1.3)	60.6 (1.4)	−0.4 (−2.6 to 1.7)	
p-value (Treatment effect)	0.954	0.553		
Augmented pressure (mmHg)				
Low Polyphenol OO (*n* = 50)	6.5 (0.7)	6.0 (0.7)	−0.5 (−1.6 to 0.6)	0.987
High Polyphenol OO (*n* = 50)	6.9 (0.7)	6.3 (0.7)	−0.5 (−1.6 to 0.5)	
p-value (Treatment effect)	0.692	0.714		
Augmented index (%)				
Low Polyphenol OO (*n* = 50)	16.2 (1.7)	14.6 (1.8)	−1.7 (−4.2 to 0.8)	0.807
High Polyphenol OO (*n* = 50)	16.6 (1.7)	15.4 (1.8)	−1.2 (−3.7 to 1.3)	
p-value (Treatment effect)				
Pulse wave velocity (m/s)				
Low Polyphenol OO (*n* = 50)	9.6 (0.1)	9.5 (0.1)	−0.03 (−0.3 to 0.2)	0.926
High Polyphenol OO (*n* = 50)	9.5 (0.1)	9.4 (0.1)	−0.05 (−0.3 to 0.2)	
p-value (Treatment effect)	0.679	0.608		

All statistical analyses were adjusted for gender and age. OO, olive oil; SEM, standard error of the mean; CI, confidence interval.
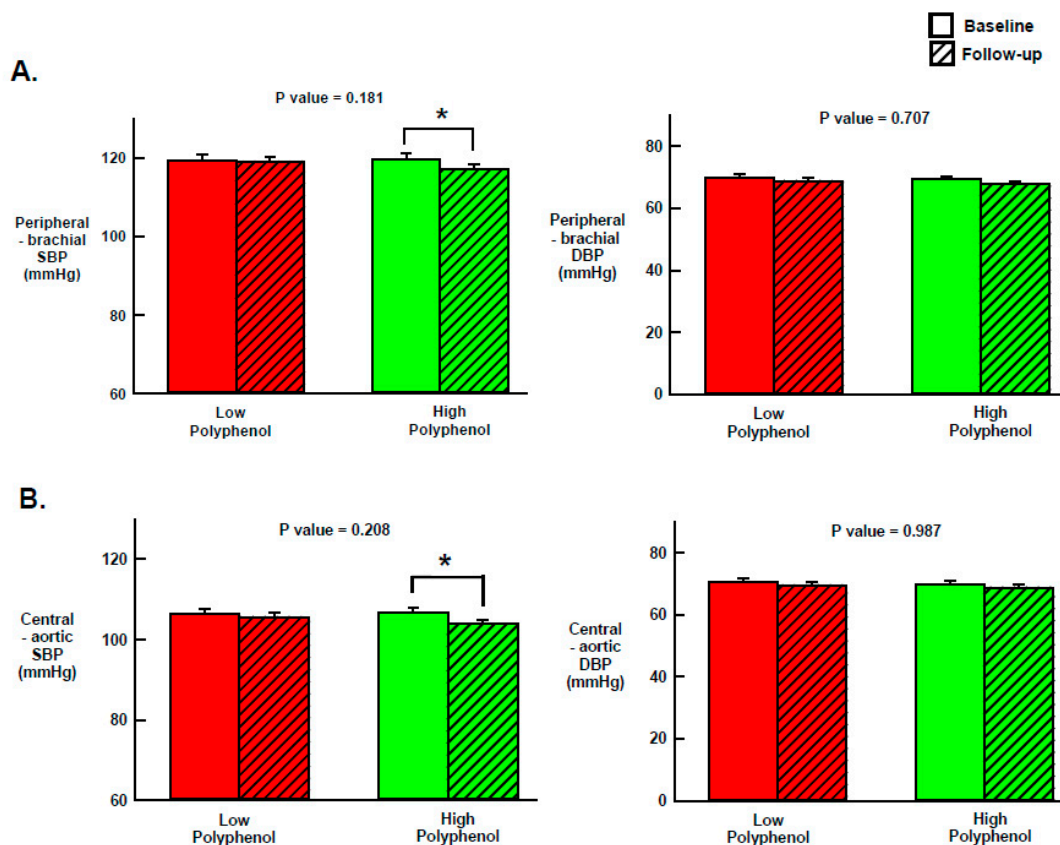
Figure 2. Effect of 3-weeks daily consumption of extra virgin high polyphenol olive oil (HPOO; 360 mg/kg polyphenols) and low polyphenol OO (LPOO; 86 mg/kg polyphenols) on mean peripheral (**A**) and central (**B**) blood pressure. *N* = 50 participants. SBP, systolic blood pressure; DBP, diastolic blood pressure. Data are mean±standard deviation. The P values in the Figure indicate the between-group differences in the changes from baseline to follow-up (treatment*time effect) for each blood pressure measure. The asterisk (*) indicates significance (*p* < 0.05) of within-group changes from baseline to follow-up.

3.5. Compliance to Treatment

Compliance to treatment was high, as reflected in the OO volume returned by participants after each intervention period. Based on the measured actual remaining OO, compliance was found to be 92% for both the LPOO and HPOO group after the first intervention period, while 92% for the LPOO group and 90% for the HPOO group after the second intervention period. Nevertheless, compliance was not found to differ significantly between the two groups (Supplementary Table S1).

4. Discussion

The present double-blind, cross-over, randomized controlled trial investigated the effect of daily consumption of 60 mL raw extra virgin HPOO in comparison with LPOO, each for 3 weeks, on BP and arterial stiffness in Australian adults. The key finding was that peripheral and central SBP decreased significantly by 2.5 and 2.7 mmHg, respectively, after extra virgin HPOO (phenolic content 360 mg/kg) consumption. However, no significant differences were observed between the two interventions with regards to the changes in peripheral and central SBP. No significant within-group changes or between-group differences were either observed on diastolic BP, and measures of arterial stiffness.

The significant decrease reported from our study in peripheral SBP is consistent with the limited number of RCTs that have examined the effect of HPOO consumption on peripheral BP, but after providing different doses of OO, different phenolic content of the administered oil, and varying intervention duration. In this regard, Moreno-Luna et al. [24] described that daily consumption of 60 mL HPOO with the highest phenolic content reported in the published literature (i.e., 564 mg/kg) for 8 weeks, significantly reduced peripheral SBP and DBP (by 7.9 and 6.6 mmHg, respectively) compared to ROO with no polyphenols, in young women with mild hypertension. Using an OO with high polyphenol concentration comparable to our study, Bondia-Pons et al. [31] reported that 9 weeks daily consumption of 25 mL of OO (366 mg/kg of polyphenols) significantly decreased peripheral SBP (~2.4 to 4.4 mmHg) in healthy non-Mediterranean men living in Europe. Other authors have described that 3 weeks daily consumption of 25 mL of HPOO (366 mg/kg of polyphenols) induced a significant reduction in peripheral SBP by 4.2 mmHg, through modulating the expression of genes that are related to the renin–angiotensin–aldosterone system (RAAS) [32]. In agreement with our results, the two aforementioned studies did not show any significant changes in peripheral DBP. These findings are supported by a recent meta-analysis, reporting that consumption of OOs with at least 150 mg/kg polyphenols significantly reduces peripheral SBP but not peripheral DBP [26], although there is evidence coming from one clinical trial indicating that OO with less phenolic content might also exert SBP-lowering effects. In this context, the NUTRAOLEUM study showed that daily consumption of 30 mL of virgin OO (phenolic content, 124 mg/kg) for 3 weeks significantly reduced peripheral SBP by 2.0 mmHg but not peripheral DBP, in healthy adults [33].

It is noteworthy, that other clinical trials also examining the effect of HPOO on peripheral SBP and DBP reported either significant results only for peripheral DBP or no significant findings on BP. In this regard, the EUROLIVE study demonstrated that 3 weeks of daily consumption of 25 mL EVOO, containing 366 mg/kg of polyphenols, significantly reduced peripheral DBP, but had no effect on SBP in healthy men [34], while another recent meta-analysis showed no significant pooled effect of the consumption of HPOO (150 to 800 mg/kg phenolic content) on peripheral SBP and DBP [19].

To the best of our knowledge, this is the first study reporting a significant reduction in central SBP after consumption of HPOO. This is of importance, considering that raised central BP has been positively associated with cardiovascular risk and mortality [16,35]. The effect of different bioactive nutrients (e.g., omega 3 fish oils, Vitamin C, and Vitamin E) on central hemodynamic markers (i.e., central SBP and DBP), either in the acute postprandial state or after long-term use, has been previously reported [36]. However, there is currently no evidence stemming from long-term RCTs regarding the effects of OO polyphenols alone, on these markers. Considering the scarcity of evidence and although not directly comparable with our study, Papamichael et al. [16] reported significant postprandial reductions in both central SBP and DBP, ranging from 3 to 5 mmHg, after the consumption of meals

combining OO and red wine by healthy study participants. However, the combined meal design makes it difficult to give attribution to the OO and/or wine for the favourable effects observed on central BP. The study's authors proposed that certain nutrients may exert a decrease in peripheral resistance, and consequently wave reflections and left ventricular afterload, thus resulting in a decrease in central SBP [16]. However, the mechanisms by which virgin OO minor compounds might exert their beneficial effects on central hemodynamic markers remain unclear. Therefore, further studies are warranted to reach final conclusions about the effect of OO polyphenols on central SBP and/or DBP.

Dietary intake and body weight changes observed in our study deserve comment in the context of the favourable effect of HPOO on peripheral and central SBP. In this regard, the addition of 60 mL of OO in participants' habitual diet resulted in significant increases in caloric intake leading to weight gain in both intervention groups. Previous cross-sectional and prospective studies have reported that body weight gain is directly associated with increases in arterial BP in normotensive subjects [37], with a 1 kg increase in body weight predicting a 0.63 and 0.42 mmHg increase in SBP and DBP, respectively [38]. Based on the above, the increases in body weight of 0.2 and 0.4 kg observed in the HPOO and LPOO group, respectively, would be expected to lead to corresponding increases in BP. However, that was not the case in our study, indicating a potential counterbalancing effect of OO polyphenols on weight gain. In this regard, the phenolic content of 360 mg/Kg in HPOO, which was much higher compared with the effective threshold of 150 mg/kg reported by a recent meta-analysis [26], could provide a basis for interpreting the significant reduction in peripheral and central SBP observed in this group, despite the non-significant weight gain of 0.2 kg.

Further to dietary energy intake, our study also recorded the intake of macro- (i.e., protein and dietary fibre) and micronutrients (i.e., sodium, potassium, magnesium and calcium) that have established effects on BP levels [39–42]. In this regard, there were no differences between the two intervention arms, indicating that the only dietary factor which could account for the observed favourable effect to reduce SBP, was the higher phenolic content in HPOO compared to LPOO. However, it is not clear why the higher phenolic content in HPOO has a significant lowering effect only on SBP but not on DBP. It could be speculated that both quantity (i.e., dose) and quality (i.e., chemical structure) of polyphenols in EVOO may exert differential effects on the vascular system [43,44]. Nevertheless, further clinical trials are required to examine the effect of OOs with different phenolic profile on BP, arterial stiffness and other cardiometabolic risk markers.

To the best of our knowledge, the OLIVAUS study is the first human clinical trial to investigate the effect of OO polyphenols on measures of arterial stiffness through applanation tonometry. Stiffening of the arterial wall in the larger central arterial system represents an important CVD risk marker [6] and the early detection of such abnormalities can inform relevant preventive or treatment initiatives. However, the present study did not detect within-group changes or between-group differences in any measures of arterial stiffness after either OO intervention. The absence of significant findings may be partly attributed to our study being adequately powered for its primary outcome, while this might not be the case for the secondary outcomes, including measures of arterial stiffness. Despite the scarcity of evidence in this field, there is a large body of published literature documenting the effect of polyphenols on biochemical markers of endothelial function, which also represent other surrogate measures of arterial stiffness. In this context, Sanchez-Rodriquez et al. [33] investigated the effect of three virgin OOs enriched with polyphenols (124, 490 and 487 mg/kg) and triterpenes (86 ppm, 86 ppm and 389 ppm, respectively) on endothelial function biomarkers in healthy adults. These investigators reported significant reductions in plasma levels of the vasoconstrictor hormone endothelin-1 at the end of the three interventions and regardless of triterpene content. In another clinical trial in women with mild hypertension, daily consumption of extra virgin HPOO (564 mg/kg polyphenols) for 8 weeks significantly decreased plasma levels of asymmetrical dimethylarginine (ADMA), which is a surrogate marker of poor endothelial function [45]. The participants also had significantly increased concentrations of vasodilating nitric oxide (NO) molecule after the intervention, supporting a beneficial effect of high polyphenol OO on endothelial function [24,45].

The findings reported in our study should be interpreted in light of its strengths and limitations. The main strength of the present study is its randomized, double-blind, cross-over design that reduces interindividual variability and increases the external validity of the study findings. The use of applanation tonometry represents another strength, since it is a state-of-the-art, non-invasive method to measure BP and arterial stiffness. In addition, in a multiethnic population that is not accustomed to a high consumption of OO, our participants' compliance was overall high throughout both intervention periods. On the other hand, one of the limitations of the present study is that the sample size was calculated on the basis of the expected differences only in its primary outcome (i.e., HDL-efflux). Another limitation could be the potential effect of seasonality on the examined outcomes, due to the fact that the participants were enrolled in the study gradually (i.e., from July 2018 through to October 2019). Lastly, despite the inclusion of a washout period before the initiation of the intervention and between the intervention periods, there is no guarantee that any potential carry-over effect on the examined hemodynamic markers was completely avoided. However, pairwise comparisons that examined potential carry-over effects were insignificant for all hemodynamic markers.

5. Conclusions

To our knowledge, the OLIVAUS study is the first to examine the effect of OO polyphenols on peripheral and central SBP and DBP as well as on measures of arterial stiffness in Australian adults. Although there were no significant differences between OO treatments in any of the examined outcomes, there was a significant reduction in peripheral and central SBP after daily consumption of extra virgin HPOO for 3 weeks. This provides evidence for a potentially widely accessible dietary intervention that can reduce CVD risk in a multicultural context, such as in Australia. However, additional clinical trials of longer duration and use of EVOO with different phenolic content and profile are required to shed more light on the potential effect of OO polyphenols on other CVD risk markers, including DBP and arterial stiffness.

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Appendix P

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Figure 1.2 Schematic representation of the progressive development of atherosclerosis.

Figure 1.3 Renin-Angiotensin-Aldosterone System (RAAS)

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Figure 1.4 Schema of the link between endothelial dysfunction markers, inflammatory markers and oxidative stress with hypertension

Figure 1.5 Representation of HDL metabolic pathways

Figure 1.6 The Mediterranean Diet Food Pyramid

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Figure 1.7 Schema of the effect of dietary antioxidants on oxidative stress and inflammation on a vascular level