IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.

Lorena Calderón Pérez

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

UNIVERSITAT ROVIRA i VIRGILI

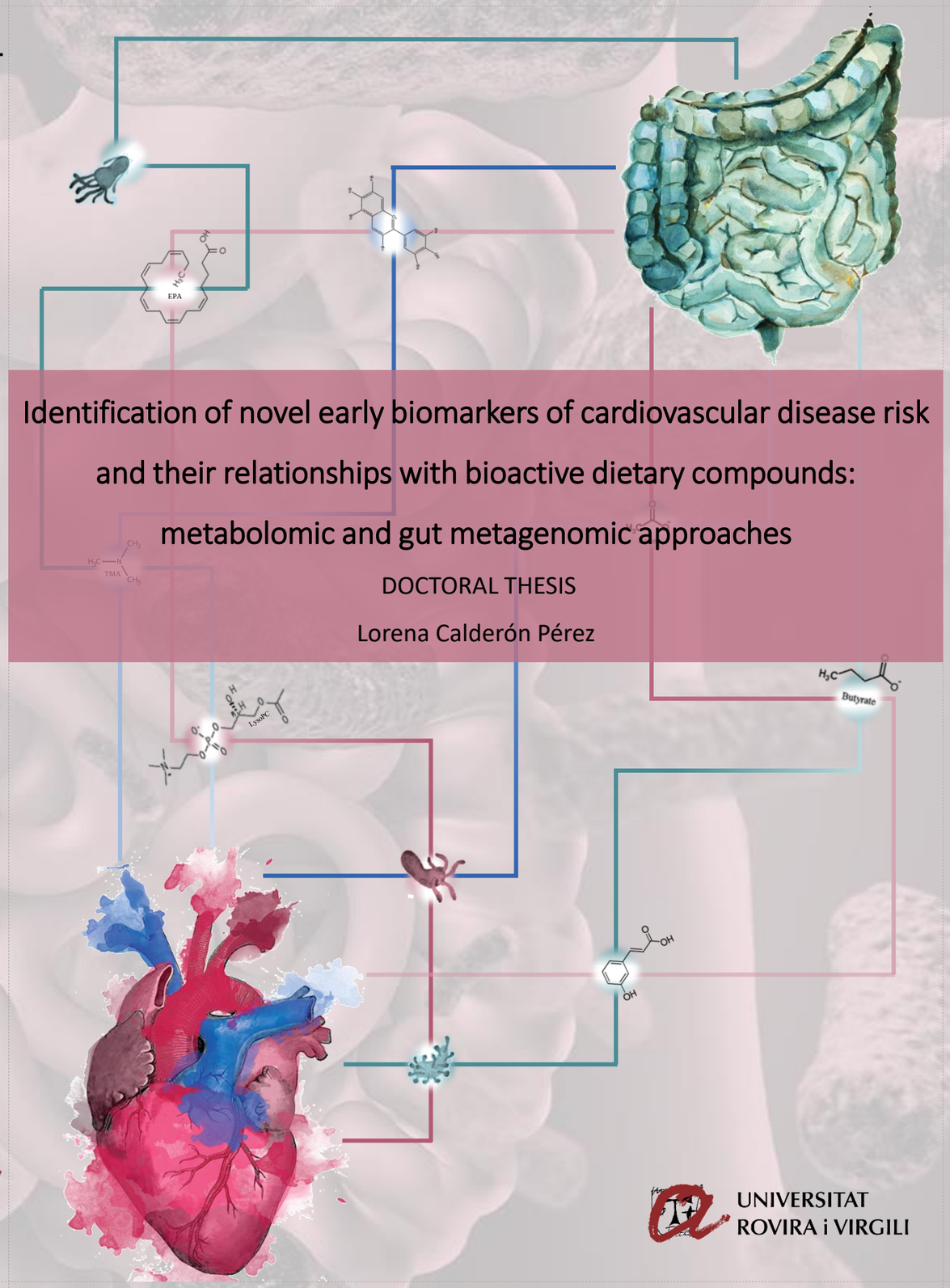
eurecat
Centre Tecnològic de Catalunya

DOCTORAL THESIS

Identification of novel early biomarkers of cardiovascular disease risk and their relationships with bioactive dietary compounds: metabolomic and gut metagenomic approaches

Lorena Calderón Pérez

2021



Identification of novel early biomarkers of cardiovascular disease risk and their relationships with bioactive dietary compounds: metabolomic and gut metagenomic approaches

DOCTORAL THESIS

Lorena Calderón Pérez

UNIVERSITAT ROVIRA i VIRGILI

Lorena Calderón Pérez

IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES

INTERNATIONAL DOCTORAL THESIS

Supervised by:

Ph.D. Rosa Maria Valls Zamora

Ph.D. Anna Pedret Figuerola

Ph.D. Laura Rubió Piqué

Rovira i Virgili University, Medicine and Surgery Department, Functional Nutrition, Oxidation and Cardiovascular Disease Research Group (NFOC-Salut); and Eurecat, Technological Center of Catalonia, Nutrition and Health Technology Unit.

Reus, Tarragona, Spain

2021

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

UNIVERSITAT ROVIRA I VIRGILI

FAIG CONSTAR que aquest treball, titulat *"Identification of novel early biomarkers of cardiovascular disease risk and their relationships with bioactive dietary compounds: metabolomic and gut metagenomic approaches"*, que presenta Lorena Calderón Pérez per a l'obtenció del títol de Doctor amb menció internacional, ha estat realitzat sota la meva direcció al Departament de Medicina i Cirurgia d'aquesta Universitat.

HAGO CONSTAR que el presente trabajo, titulado *"Identification of novel early biomarkers of cardiovascular disease risk and their relationships with bioactive dietary compounds: metabolomic and gut metagenomic approaches"*, que presenta Lorena Calderón Pérez para la obtención del título de Doctor con mención internacional, ha sido realizado bajo mi dirección en el Departamento de Medicina y Cirugía de esta universidad.

I STATE that the present study, entitled *"Identification of novel early biomarkers of cardiovascular disease risk and their relationships with bioactive dietary compounds: metabolomic and gut metagenomic approaches"*, presented by Lorena Calderón Pérez for the award of the degree of Doctor with international mention, has been carried out under my supervision at the Department of Medicine and Surgery of this university.

Reus, 16 de Desembre de 2021/Reus, 16 de Diciembre de 2021/Reus, December 16 2021

La/les directora/es de la tesi doctoral
La/s directora/s de la tesis doctoral
Doctoral Thesis Supervisor/s

Ph.D. Rosa Maria Valls Zamora

Ph.D. Anna Pedret Figuerola

Ph.D. Laura Rubió Piqué

International thesis for the award of the degree of Biomedicine Doctor with International Mention at the Universitat Rovira i Virgili, defensed by Lorena Calderón Pérez.

Funding sources: This work was supported by the Centre for the Development of Industrial Technology (CDTI) of the Spanish Ministry of Science and Innovation under grant agreement: TECNOMIFOOD project (CER-20191010), and by the Ministry of Economy and Competitiveness within the Sara Borrell postdoctoral contract awarded to Ph.D. Laura Rubió (CD14/00275).

This work was also supported by Eurecat Foundation, and the Universitat Rovira i Virgili, with the International Mobility Grant for PhD students of the Biomedicine Program 2019.

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

Acknowledgements

Me gustaría agradecer a todas las personas que de manera directa o indirecta han estado presentes durante el transcurso de ésta tesis. Primeramente, quiero agradecer al *Grupo de Investigación en Nutrición Funcional, Oxidación y Enfermedades Cardiovasculares de la Universitat Rovira i Virgili* por ofrecerme la oportunidad de colaborar en sus apasionantes proyectos, principalmente en la línea de estudios clínicos. A la Dra. Rosa Solà, por abrirme al camino en la investigación y por sacar lo mejor de cada trabajo. Gracias a tu gran experiencia y conocimiento he aprendido muchísimo, y con ello me llevo valiosas lecciones. A mis directoras de tesis, en particular a la Dra. Rosa Maria Valls y la Dra. Anna Pedret, por guiarme durante todo el proceso y por esclarecer los momentos más oscuros. Me habéis ayudado a ligar ideas y a dar sentido a un trabajo inicialmente difuso. Vuestro constante soporte ha hecho posible que la presente tesis madure y finalmente cobre sentido.

Por otro lado, agradecer a la Dra. Laura Rubió, por codirigir el trabajo, y por transmitirme su gran pasión por la microbiota intestinal a través del proyecto *Cardiogut*. Éste proyecto abrió las puertas de la presente tesis, y gracias a tu soporte han aflorado interesantes 'links' entre microbiota-hipertensión-dieta. También, agradecer al *Grupo de investigación Antioxidantes de la Universidad de Lleida (ETSEA)*, por acogerme en su lab de Antioxidantes. En especial a la Dra. Iziar A. Ludwig, por las horas dedicadas en enseñarme acerca de la determinación de TMAO por UPLC-MS/MS.

También quiero dar las gracias a la *Unidad de Genómica y Salud (FISABIO-Valencia)*, en particular a la Dra. Maria José Gosalbes, por su gran

contribución en el análisis metagenómico por secuenciación de ARN. La información de la composición y función de la comunidad microbiana intestinal es un apasionante mundo, y la Dra. Maria José ha ampliado mis conocimientos en ésta compleja área. Por otro lado, agradecer al *Grupo de Investigación en Nutrigenómica de la Universitat Rovira i Virgili*, concretamente al Dr. Manuel Suárez por su gran aportación en lipidómica, y por enriquecer los resultados de la segunda parte de ésta tesis.

Seguidamente, quiero agradecer a todo el equipo de la *Unidad Tecnológica de Nutrición y Salud (Eurecat)* por la confianza depositada en mí. Especialmente al 'NutriTeam' por su continuo apoyo, y a Judit Companys, compañera de despacho, con quién he compartido de manera paralela todo el proceso de la tesis. Agradecer todos tus ánimos y comprensión, especialmente en la última fase.

I also would like to truly thank all the lab and research core at the *Centre for Translational Microbiome Research (Karolinska Institutet, Stockholm)*, specially thank to PhD. Juan Du for receiving me in her research team. Thank you for introducing me to the fascinating PlaMi project. I learnt a lot on the study and analysis of the microbiota in reproductive health. I never imagined that I could grow 200 placenta cultures! It was a great challenge and an excellent learning experience. Also, my heartiest thanks to the rest of PhD and PhDs Shengru Wu, Liquin Cheng, Qin Liu, Sebastian-Alexandru Radu, Unnur Guðnadóttir, among others, for making the dark days in Stockholm more enjoyable. I will always miss the *Fika* time!

También a mis amigos, a los de siempre, por su empatía, y por aceptar mis

ausencias inesperadas… ¡Estoy segura que tendremos muchos más momentos por compartir!

Por último, y no menos importantes, mi familia. Agradecer a mis padres Jose Luis y Merche por su incondicional apoyo en mi carrera académica durante éstos años. Siempre habéis estado ahí en los mejores y en los peores momentos, y el logro de escribir una tesis ha sido en gran parte gracias a vosotros. A mi hermana Natalia, por sus infinitos ánimos y consejos. A mis abuelos, en especial a mi abuelo José, por su continua preocupación e interés. Y finalmente, quiero dar gracias a Richard, por su gran paciencia y comprensión especialmente en los momentos más duros. Eres quien más de cerca ha vivido todo éste proceso, y me has ayudado a hacer frente a los obstáculos y a restar importancia a mis mayores 'cargas'. Éste trabajo va dedicado a todos vosotros.

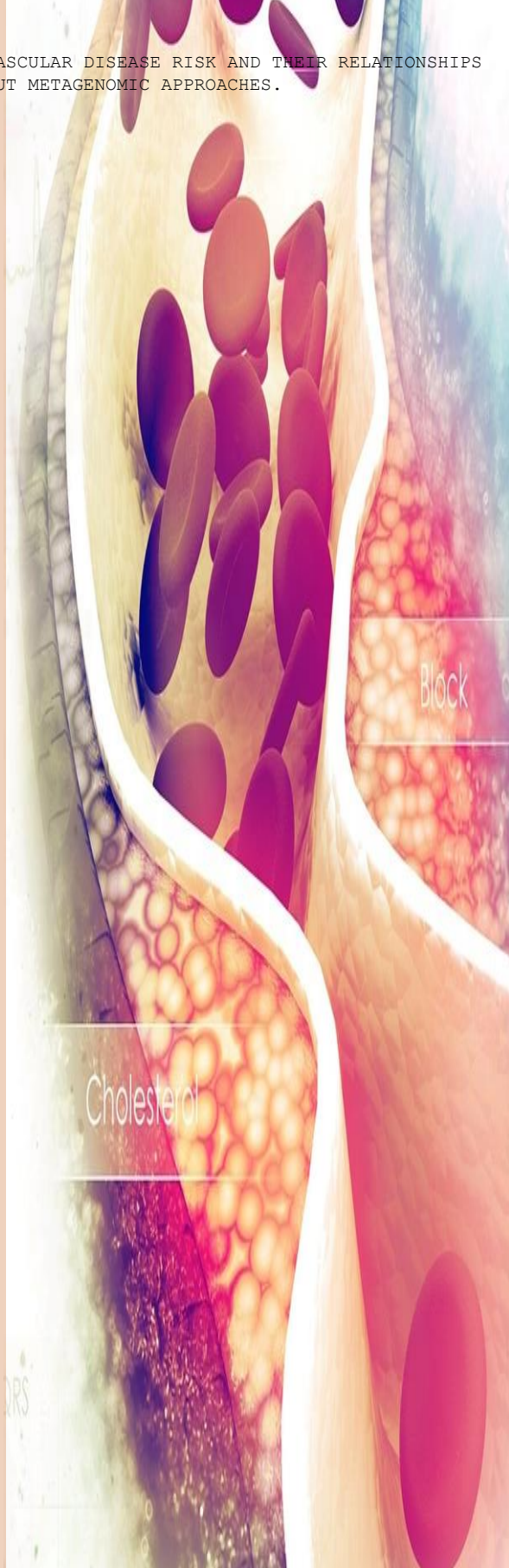
UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

"There are known knowns; there are things we know we know. We also know there are known unknowns; that is to say we know there are some things we do not know. But there are also unknown unknowns – the ones we don't know we don't know…, it is the latter category that tend to be the difficult ones"

Donald Rumsfeld

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

CONTENTS



UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

CONTENTS

CONTENTS

INDEX OF TABLES AND FIGURES BY SECTIONS

JUSTIFICATION

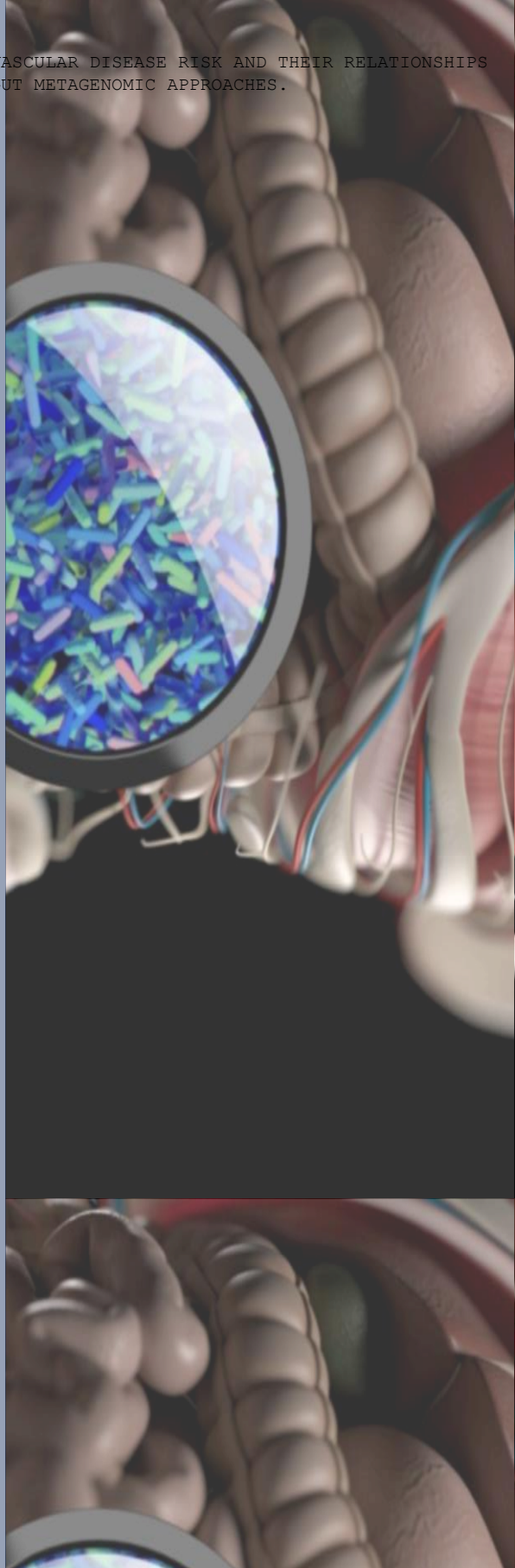
INTRODUCTION

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

SUMMARY



UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

SUMMARY

INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, reaching 18.6 million deaths in 2019. Hypertension and hypercholesterolemia are the primary causal and modifiable metabolic risk factors that directly contribute to CVD risk. These risk factors have traditionally led to the development of risk assessment models. However, they are restricted by their limited predictive value because of their delayed response once the disease progresses and pharmacological treatment is required. Therefore, it is essential to identify novel risk biomarkers that assist in recognizing the preliminary stages of the CVD pathophysiological processes. The integration of 'multiomics' approaches, such as metabolomics, lipidomics, and gut metagenomics, allows for the combination of different molecular biomarkers, including gut microbiota- and metabolite-based biomarkers, that may help to improve the accuracy in risk detection. Moreover, knowledge of early biomarker interactions with dietary compounds would benefit the implementation of preventive nutritional strategies to avoid the continuous advancement of the main causal and modifiable CVD risk factors.

OBJECTIVES

The present doctoral thesis was aimed to identify novel early gut microbiota- and metabolite-based CVD risk biomarkers suitable for the preliminary stages of human hypertension, hypercholesterolemia and other main causal and modifiable CVD risk factors through the integration of multiomics approaches and to assess their relationships with bioactive dietary compounds to speculate about the involved pathways and their possible effects in CVD development.

METHODS AND RESULTS

Two cross-sectional studies involving healthy, grade 1 hypertensive (HT) (the *Cardiogut* study), and moderate to high hypercholesterolemic (the *Bioclaims* study) individuals were performed.

In the *Cardiogut* study (***Article 1***), faecal microbiota composition and bacterial functions were determined by 16S rRNA gene sequencing and metagenomic analysis in 29 nontreated HT and 32 normotensive (NT) individuals (systolic blood pressure (SBP) ≥ 140-159 mmHg and < 120 mmHg, respectively). Moreover, microbial metabolites, including faecal and plasma short-chain fatty acids (SCFAs) and plasma trimethylamine-N-oxide (TMAO), were detected by targeted metabolomics. The results showed that a set of bacterial taxa, mainly *Bacteroides* spp., *Intestimonas* and genera of Lachnospiraceae, were enriched in HT individuals, whereas *Faecalibacterium* spp., *Roseburia* spp., and other genera of Ruminococcaceae and Christensenellaceae families were depleted. These findings suggested a strong association with hypertension. Additionally, in HT individuals, lower plasma SCFA levels and increased faecal SCFA levels suggested less efficient intestinal absorption, which is probably due to imbalanced host-microbiome crosstalk. Furthermore, in ***Article 2***, the relationships between dietary phenolic compounds (PCs) and targeted bacterial taxa in HT and NT individuals were examined. A multiple-way interaction was found for coffee PCs with SBP, diastolic blood pressure (DBP), faecal SCFAs and HT-enriched *Bacteroides* spp. However, inverse associations were noted for these PCs with NT-enriched bacterial taxa, indicating a negative impact of coffee PCs on the gut microbiota preceding blood pressure (BP) alteration. In NT individuals, olive fruit PCs were positively associated with SCFA-producing

bacteria, suggesting a protective role against the onset of hypertension. In the *Bioclaims* study (***Article 3***), plasma TMAO and serum lysophosphatidylcholines (lyso-PC) and lysophosphatidylethanolamines (lyso-PE) were identified through targeted metabolomics and lipidomics approaches in 70 individuals with low LDL cholesterol (L-LDL-c) and 48 with moderate to high (MH-LDL-c) LDL cholesterol (LDL cholesterol ≤ 115 mg/dL and 116-190 mg/dL, respectively). Predictive models were applied to discern accurate biomarkers for the onset of hypercholesterolemia. The results revealed that lyso-PC 15:0 and lyso-PE 18:2 were suitable susceptibility/risk biomarkers of hypercholesterolemia progression. In addition, in MH-LDL-c subjects, a positive correlation was found between lyso-PE 18:2, liver GGT and dietary omega (n)-polyunsaturated fatty acids (PUFAs). Then, the human lipidomic results were validated in an *in vivo* study involving 8 high-fat diet (HFD)- and 8 low-fat diet (LFD)-fed hamsters. In the HFD-fed group, the intrahepatic accumulation of polyunsaturated lyso-PLs, particularly lyso-PE 18:2, revealed a possible hepatic adaptive mechanism to counteract hypercholesterolemia progression in humans.

Finally, a systematic review and meta-analysis of randomized clinical trials was performed to clarify the effects of dietary fatty acids (FAs) on circulating bioactive lipidome and the enzymatic precursor lipoprotein-associated phospholipase A2 (Lp-PLA2) mass in healthy subjects, with CVD and with CVD risk factors (***Article 4***). The PRISMA 2020 guidelines and PICOS criteria were followed for study selection. A total of 27 randomized clinical trials were included in the systematic review, and 10 controlled trials were selected for meta-analysis. As a result, from the meta-analysis, marine omega-3 (n-3) PUFAs, provided as supplements, increased plasma pro-inflammatory lyso-

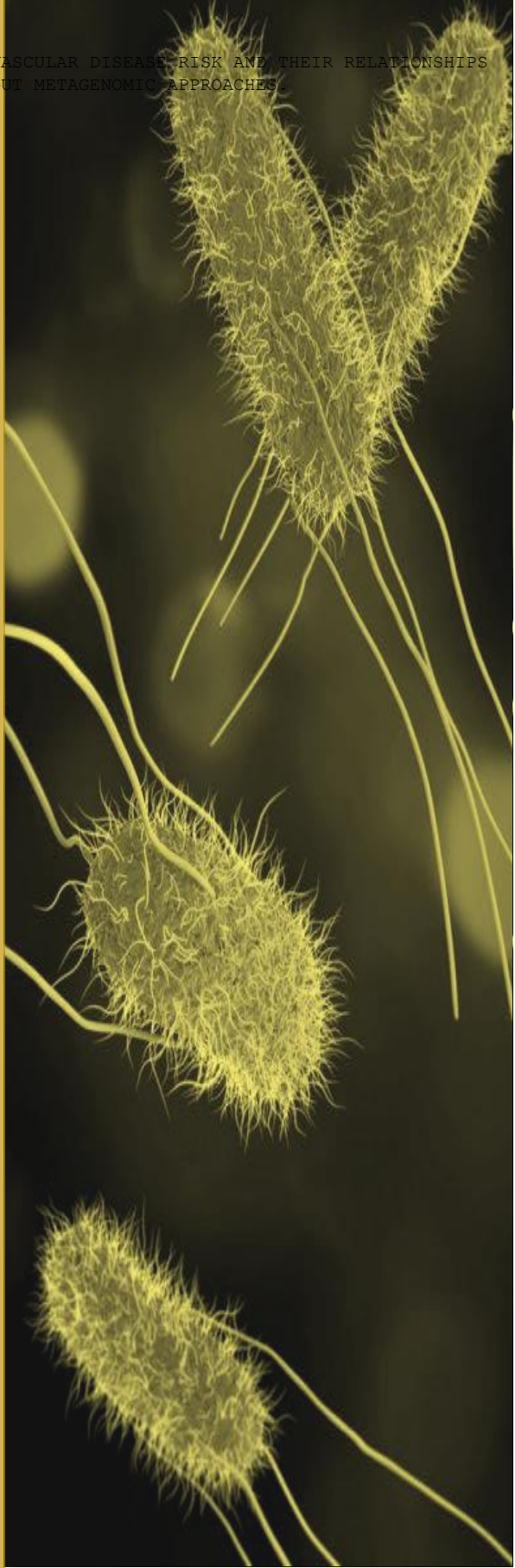
PC(16:0 and 18:0) in obese subjects, while decreased plasma Lp-PLA2 precursor mass in healthy, dyslipidemic and coronary artery disease subjects. Therefore, daily n-3 PUFA supplementation, provided as EPA+DHA, consumed from 1 to 6 months, exhibit positive effects on the plasma Lp-PLA2 mass in healthy subjects, with CVD and with CVD risk factors, suggesting an anti-inflammatory effect. However, an impaired saturated lyso-PC response to n-3 is manifested in obese subjects.

CONCLUSIONS

The identification of particular gut bacterial signatures, such as *B. coprocola*, *B. plebeius*, *Intestimonas* and genera of Lachnospiraceae, and higher faecal levels of acetate, propionate, butyrate and valerate provide novel biomarkers of the gut microbiota ecosystem that could be useful to discriminate HT individuals from NT individuals. In addition, knowledge of these biomarker relationships with BP, coffee and olive fruit PCs highlights the complex dietary pathways involved in the pathogenesis or prevention of hypertension. Additionally, increased serum lyso-PC 15:0 or decreased lyso-PE 18:2, together with intrahepatic accumulation of lyso-PE 18:2, could represent promising susceptibility/risk biomarkers of hypercholesterolemia progression. Dietary FAs, particularly n-3 PUFAs, provided as supplements or oily fish ED, modulate circulating lyso-PC moieties and decrease plasma Lp-PLA2 mass. These findings favour the management of the main causal and modifiable CVD risk factors. Overall, our results offer novel promising health/disease biomarkers that may help more accurately detect the main causal and modifiable CVD risk factors in their preliminary stages. Nevertheless, further prospective studies are required for successful predictions of CVD risk.

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

ABBREVIATIONS



UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

ACE	angiotensin-converting enzyme
ADMA	asymmetric dimethylarginin
ALA	alpha-linoleic acid
ALT	alanine transaminase
ANP	atrial natriuretic peptide
AOS	arabinooligosaccharides
ApoB-100	apolipoprotein B 100
ApoE	apolipoprotein E
AST	aspartate transaminase
ASVs	amplicon sequence variants
AUC	area under the curve
BAs	bile acids
BCAAs	branched-chain amino acids
BMI	body mass index
BNP	brain natriuretic peptide
BP	blood pressure
Ca2+	calcium
CAD	coronary artery disease
CBG	β-glucosidase
Cer	ceramides
CFU	colony forming unit
CI	confidence interval
CRP	C-reactive protein
CV	cardiovascular
CVDs	cardiovascular diseases
CYP7A1	cholesterol 7-α hydroxylase
DAG	diacylglycerol
DALYs	disability-adjusted life years
DBP	diastolic blood pressure
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DPBs	dietary pattern biomarkers
EAS	European Atherosclerosis Society
ECF	extracellular fluid

ABBREVIATIONS

ED	enriched diets
EF-C	enriched food-components
EPA	eicosapentaenoic acid
ESC	European Society of Cardiology
ESH	European Society of Hypertension
ESI	electrospray ionization
FAs	fatty acids
FCIBs	food compound intake biomarkers
FFQ	food-frequency questionnaire
FIBs	food intake biomarkers
FMO3	flavin monooxygenase 3
FMT	faecal microbiota transplantation
FOS	fructooligosaccharides
FXR	farnesoid X receptor
GBD	global burden of disease
GC	gas chromatography
GC/FID	gas chromatography coupled to flame ionization detector
GGT	gamma-glutamyl transferase
GI	gastrointestinal
G3P	glycerol-3-phosphate
GOS	galactooligosaccharides
GPRs	G-protein-coupled receptors
HDL	high-density lipoprotein
HFD	high-fat diet
HMG-CoA	hydroxymethylglutaryl-coenzyme A
HT	grade 1 hypertensive
ICH	International Conference of Harmonization
IHD	ischaemic heart disease
IL	interleukin
IOM	Institute of Medicine
ISAPP	International Scientific Association for Probiotics and Prebiotics
8-iso-PGF2a	8-Iso prostaglandin F2 alpha
LA	linolenic acid
LC	liquid chromatography

LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LC-Q-TOF	liquid chromatography coupled to a time-of-flight detector
LCT	long chain triglyceride
LDA	liner discriminant analysis
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
L-LDL-c	low LDL cholesterol
LEfSe	liner discriminant analysis and effect size algorithm
LFD	low-fat diet
LPH	lactase phlorizin hydrolase
LPLATs	lysophospholipid acyltransferases
Lp-PLA2	lipoprotein-associated phospholipase A2
LPO	lipid peroxidation
LPS	lypopolysaccharides
Lyso-PLs	lysophospholipids
Lyso-PC	lysophosphatidylcholine
Lyso-PE	lysophosphatiylethanolamine
Lyso-PI	lysophosphatidylinositol
MACs	microbiota accessible carbohydrates
MCFAs	medium-chain FAs
MH-LDL-c	moderate to high LDL cholesterol
MPO	myeloperoxidase
MS	mass spectrometry
MUFAs	monounsaturated fatty acids
Na^+	sodium
NIBs	nutrient intake biomarkers
NMR	nuclear magnetic resonance spectroscopy
NO	nitric oxide
NoNIBs	non-nutrient intake biomarkers
NSAID	non-steroidal anti-inflammatory drug
NT	normotensive
n-3	omega-3
n-6	omega-6
Olfr78	olfactory receptor 78

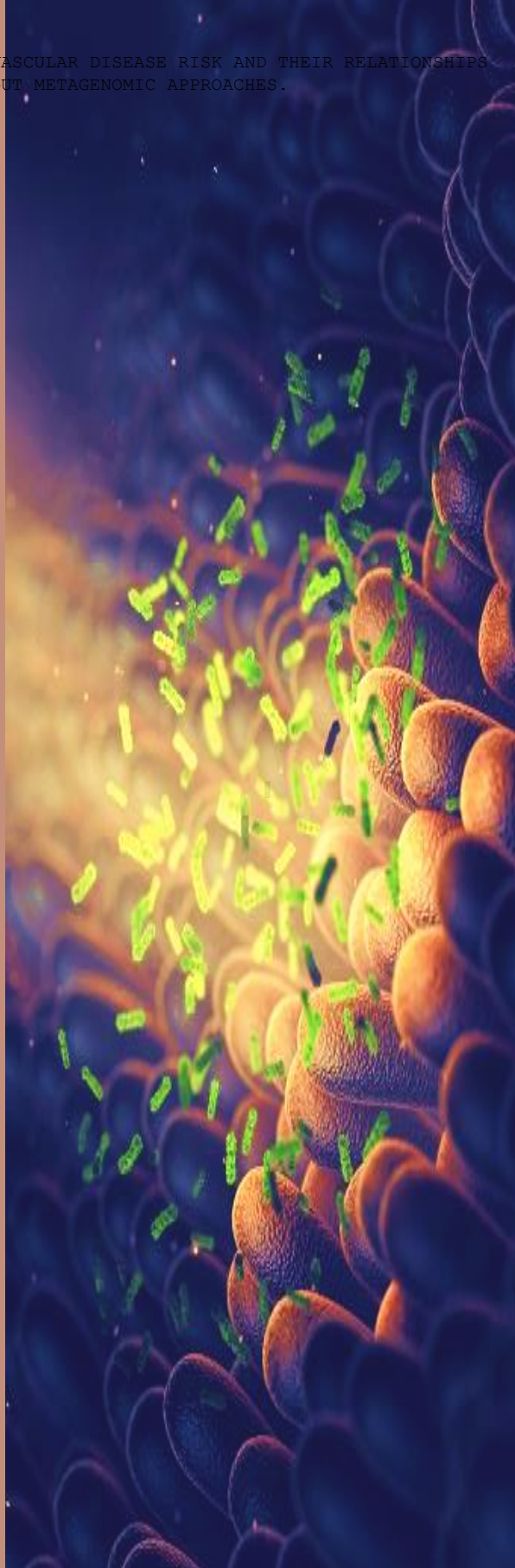
ABBREVIATIONS

OR51E2	olfactory receptor 51E2
OSA	obstructive sleep apnea
OTUs	operational taxonomic units
Ox-LDL	oxidized low-density lipoprotein
PA	phosphatidic acid
PAI-1	plasminogen activator inhibitor-1
PCs	phenolic compounds
PCA	principal component analysis
PCoa	principal coordinates analysis
PCR	polymerase chain reaction
PCSK9	proprotein convertase subtilisin/kexin type 9
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PICOS	Population, Intervention, Comparison, Outcomes and Study design
PLAs	phospholipases
PLs	phospholipids
PLS-DA	partial least squares discriminant analysis
PS	phosphatidylserine
PSQI	Pittsburgh Sleep Quality Index
PUFAs	polyunsaturated fatty acids
QqQ	triple quadrupole mass spectrometer
RAA	renin-angiotensin-aldosterone
RCTs	randomized controlled trials
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROC	receiver operating characteristic
ROS	reactive oxygen species
RR	relative risk
RTs	non-controlled randomized trials
rRNA	ribosomal ribonucleic acid
SBP	systolic blood pressure
SCFAs	short-chain fatty acids
SD	standard deviation

SDMA	symmetric dimethylarginin
SE	standard error
SFAs	saturated fatty acids
SLs	sphingolipids
SM	sphingomyelins
SMD	standard mean difference
SNS	sympathetic nervous system
sPLA2	secretory phospholipase A2
SR-A1	steroid receptor RNA activator 1
TEI	total energy intake
TMA	trimethylamine
TMAO	trimethylamine N-oxide
TNF-α	tumor necrosis factor-α
TPE	total polyphenol excretion
TPI	total polyphenol intake
TFAs	trans-fatty acids
VIP	variable importance in projection
VLC-PUFAs	very long chain polyunsaturated fatty acids
V0	visit 0
V1	visit 1
WHO	World Health Organization
WP	work package
XOS	xylooligosaccharides

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

JUSTIFICATION



UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

There are known knowns and unknowns in the research of cardiovascular diseases (CVDs). **We know** that several risk factors contribute to CVD burden, including primary causal and modifiable metabolic risk factors, such as hypertension and hypercholesterolemia (Roth et al., 2020). These risk factors have led to the development of risk prediction models and to major developments in therapy. However, not all traditional risk factors are enough to identify all at-risk individuals. Indeed, not all individuals with CVDs may manifest traditional risk factors, and once the risk is evident, a residual CVD risk could remain even after initial lifestyle changes and risk factor treatment (Visseren et al., 2021). Moreover, classical CVD risk factors are restricted by their limited predictive value in risk assessment models (van Bussel et al., 2020), and the current biomarkers are actually endpoints of disease progression. Thus, pharmacological treatments are the only way to alleviate these alterations (Ali et al., 2016).

Novel disease biomarkers, referred to as a broad subcategory of quantifiable and reproducible characteristics of biological signs, have emerged to enhance the current risk-stratification metrics for CVDs and improve the selection of therapeutic strategies for individuals (J. Wang et al., 2017). Whereas disease biomarkers play a critical role in the definition, prognosis, and decision-making in the clinical setting, dietary intake biomarkers, such as food compound intake biomarkers (FCIBs), are of particular interest because of their accurate associations with disease risk (Gao et al., 2017). Hence, the identification of both new disease risk and dietary intake biomarkers can conform together to provide a multimarker approach that would ease the implementation of preventive strategies to

avoid the continuous advancement of the main causal and modifiable CVD risk factors through their early identification.

In recent years, technological progress in 'omics' sciences, such as metabolomics, has provided key tools for biomarker discovery, employing techniques such as nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) (Olivier et al., 2019). In addition, lipidomics and metagenomics have emerged as metabolomics and environmental genomics subfields in the identification of lipid-based metabolites or microbial communities with important roles in CVD development (Brial et al., 2018). The integration of metabolomics, including lipidomics, and metagenomics data suggests a promising analytical strategy for the identification of target microbes and bioactive metabolites able to mediate physiological and metabolic functions in the host (Agus et al., 2017). For instance, this integrative data analysis may explain how particular microbes influence on host physiology through production, modification, or degradation of bioactive metabolites, allowing the generation of testable hypothesis regarding the mutual interactions between microbial communities and disease causality.

The gut microbiome constitutes a complex system of mutualistic microorganisms that are susceptible to be modified by a number of environmental and behavioural factors, including diet (Singh et al., 2017). It has emerged as a pivotal regulator of CV pathophysiology and has raised interest in the development of microbiota-targeted therapies aimed at modulating the composition and/or metabolism of the microbial community

(L. Jin et al., 2021). Therefore, understanding the composition and functional capabilities of the gut microbiome represents a major challenge.

Remarkably, the human gut microbiome is a critical component of digestion, breaking down complex carbohydrates, proteins, and to a lesser extent fats that reach the gastrointestinal tract. Thus, it plays a crucial role in macronutrient metabolism and produces major fermentation byproducts, such as trimethylamine-N-oxide (TMAO) and short-chain fatty acids (SCFAs), which have a complex impact on human health (Oliphant et al., 2019). On the one hand, these bioactive metabolites have shown beneficial effects in maintaining cardiovascular function (Z. Wang et al., 2018), but on the other hand, their dysregulation has been potentially causally linked to CVDs (Koeth et al., 2013; Tang et al., 2013; Z. Wang et al., 2011). It is, therefore, of much interest to understand the complex relationships between the gut metabolome and exogenous factors able to disturb its functions in the host.

It is known that the human gut metabolome serves as a functional readout of the gut microbiome, it can be used as an intermediate phenotype mediating host-microbiome interactions (Zierer et al., 2018). Beyond the gut, the circulating metabolome, which reflects systemic metabolic processes, represents a niche of small groups of metabolites, some of which originate from enzymatic activity in the intestinal lumen. This is the case for lysophospholipids (lyso-PLs), which constitute a diverse group of circulating bioactive lipids involved in a broad range of physiological and pathological processes in humans (Tan et al., 2020). In the intestine, hydrolysed lyso-PLs become a substrate for the synthesis of TMAO precursors (Hui, 2016). Once they are absorbed into enterocytes, they could be able to promote lipid disorders, eliciting CVDs risk (Akerele et al., 2015; Suárez-García et al., 2017).

Overall, both the microbiome and the metabolome are susceptible to being modulated by external bioactive dietary compounds. In this sense, phenolic compounds (PCs), a large group of phytochemicals found in plants and certain plant-based foods (Pérez-Jiménez et al., 2010), can exert modulatory activities on gut microbiota composition and function. PCs have 'prebiotic-like effects' that promote the growth of beneficial anaerobic bacteria involved in SCFA production in the gut, similar to dietary fibres (Fernandez-Navarro et al., 2018). However, it is necessary to fully clarify the microbiota-dependent catabolism of PCs in the large intestine and to determine their mechanisms of action on the gut microbiota. Dietary fatty acids (FAs) are known to be important determinants of lyso-PL structure, and their contribution depends on the fatty acyl chain composition (Akerele et al., 2015). Thus, the effects of lyso-PLs on lipid disorders could be protective or harmful depending on the type of FA intake. Indeed, free n-3 PUFAs have been shown to bind to plasma phospholipids (PLs) and lyso-PLs following the intake of n-3-rich oily fish (Raatz et al., 2013; Žáček et al., 2018) or supplements (Del Bas et al., 2016), although the resultant effects of these lipid profile modifications on the healthy state or the main causal and modifiable CVD risk factors are unclear.

Finally, although diet has long been known to contribute to the pathogenesis of CVD, the extent to which habitual diet, and particularly bioactive dietary compounds, interact with the gut microbiome and metabolome, and how consequently the risk of developing CVD is modified, is unknown. Therefore, the understanding of diet-microbe/metabolite-host interactions and their links to CVD risk factors susceptibility represent exciting gaps in knowledge.

The present thesis aims to fill these gaps by identifying early disease biomarkers that are able to detect the main causal and modifiable CVD risk factors using an observational approach. Accordingly, two cross-sectional studies (the *Cardiogut* study and the *Bioclaims* study) involving healthy, HT and moderate to high hypercholesterolaemic individuals, are presented. In the *Cardiogut* study (***Article 1*** and ***Article 2***), to discover novel gut microbiota- and metabolite-based biomarkers related to hypertension development, a comprehensive metagenomic characterization of the gut microbiota composition and function was performed comparing HT with NT individuals. Moreover, faecal microbe-derived metabolites, mainly SCFAs, were determined by the application of targeted metabolomics. Additionally, the relationships between dietary PC intake, targeted gut microbes and SCFAs were tested to study the role of dietary PCs in the gut microbiota composition and its derived metabolites in hypertension pathogenesis or prevention. In the *Bioclaims* study (***Article 3***), to assess whether circulating bioactive lipids and microbe-derived metabolites, such as lyso-PLs and TMAO, may be suitable susceptibility/risk biomarkers of hypercholesterolemia, targeted lipidomics and metabolomics approaches were applied in L-LDL-c and MH-LDL-c individuals. In addition, the relationships between targeted lyso-PLs, liver transaminases and FA intake were investigated. Then, to verify the human lipidomic results and elucidate the possible mechanisms of action of lyso-PLs in relation to hypercholesterolemia, a secondary *in vivo* study was conducted in hamsters. Finally, a systematic review and meta-analysis of randomized clinical trials (***Article 4***) involving healthy subjects, with CVD and CVD risk factors, was performed to further evaluate the effects of dietary FAs on circulating

bioactive lipids as intermediate biomarkers of health, CVD disease or CVD risk factors.

As shown in ***Figure 1***, this research provides a comprehensive overview of the complex interplays between diet, gut microbial composition, metabolites and the host CVD risk factors through the integration of multiomics approaches results. Moreover, the application of multivariate discriminant analyses have allowed to reveal candidate susceptibility/risk biomarkers for early detection of the main causal and modifiable CVD risk factors with promising applicability in future preventive nutrition-based therapies.

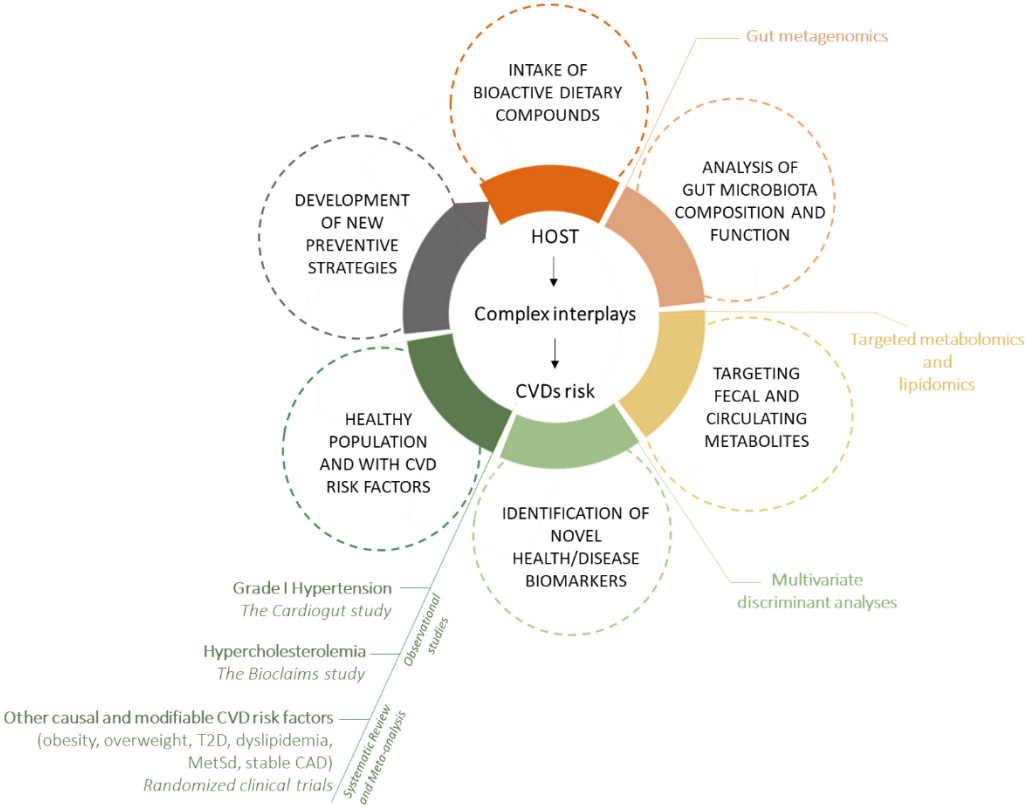
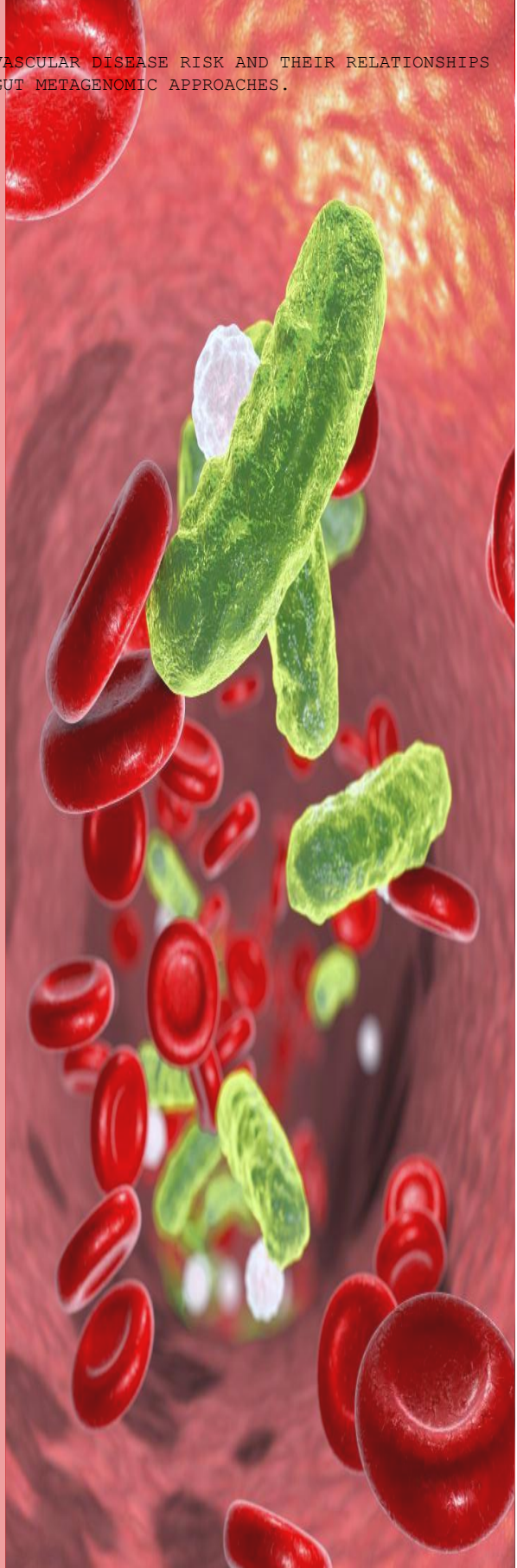


Figure 1 | Graphical representation of the main topics encompassed in the present thesis. Abbreviations: CAD, coronary artery disease; CVDs, cardiovascular diseases; MetSd, metabolic syndrome; T2D, type 2 diabetes.

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

INTRODUCTION



UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

Chapter 1. Cardiovascular diseases: present and future

1.1. Global burden and definition

More people die from CVDs worldwide than from any other cause. An estimated 18.6 million people died from CVDs in 2019, signifying 31% of all global deaths (Roth et al., 2020). In the coming years the CVD mortality burden is expected to increase; it is expected to reach more than 23 million deaths by 2030 (Amini et al., 2021).

CVDs cover a broad group of medical disorders that affect the circulatory system, mainly the heart and blood vessels. Ischaemic heart disease (IHD) (inadequate supply of oxygen-rich blood to a portion of the myocardium) and stroke or cerebral ischaemia (interruption in blood supply to the brain tissue by cerebral blood vessel blockage or intracranial bleeding) are the most common disorders and the leading cause of mortality and disability (Antman et al., 2018; Smith et al., 2018). Atherosclerotic disease of epicardial coronary arteries and cerebral vessels is the most common cause of both IHD and stroke. According to the latest analysis of the Global Burden of Disease (GBD) Study 2019, the global trends of disability-adjusted life years (DALYs) due to IHD and stroke have significantly increased over the last 30 years, reaching 182 and 143 million total DALYs and 9.14 and 6.55 million deaths, respectively, worldwide in 2019 (Murray et al., 2020; Roth et al., 2020).

Causal and modifiable CVD risk factors are those that can be reduced or controlled with altered behaviour, including cardiometabolic, behavioural, environmental, and social risk factors. In contrast, nonmodifiable CVD risk factors are those that cannot be changed, such a person's age, sex, ethnicity

and family history. When different causal and modifiable risk factors, such as hypertension, diabetes, hypercholesterolemia, obesity, tobacco use, unhealthy diet, physical inactivity and harmful use of alcohol, are combined, they substantially increase the risk of suffering acute events, such as heart attacks and strokes (Roth et al., 2020). Thus, the proper monitoring, management and early detection of these risk factors are key primary preventive actions for global initiatives aiming to reduce the incidence, morbidity and mortality of CVDs. This is the case for the *Global HEARTS* initiative promoted by the WHO Programme on CVDs (WHO, 2016).

In addition, prevalent cases of CVD are likely to increase as a result of population growth and aging, especially in low and middle income countries where the share of older people is projected to double between 2019 and 2050. Thus, also increased attention to promoting ideal CV health and healthy aging across the lifespan is necessary (Reynolds et al., 2019).

1.2. Tackling cardiovascular disease: causal and modifiable cardiovascular disease risk factors

CVDs are considered to be caused by multiple causal and modifiable risk factors that can have a direct relationship (causation) or act as indirect predictors or intermediates for CVDs (J. et al., 2004). The concept of CVD risk factors was introduced for the first time by Dr. W. Kannel in the context of the Framingham Heart Study (pioneer in the study of CVDs) (Kannel, 1961). Typically, the concept is defined as a measurable trait that predicts an individual's likelihood of developing CVDs, including particular habits, behaviours, circumstances or conditions.

In the last few decades (1990-2019), among traditional causal and modifiable CVD risk factors, high SBP and high LDL cholesterol have been considered leading metabolic risk factors for CVDs (***Figure 2***) (Roth et al., 2020). According to the last ESC Guidelines on CVD prevention in clinical practice (2021), the levels of apolipoprotein-B-containing lipoproteins, mainly LDL, in the blood and high BP remain the main causal and modifiable CVD risk factors (Visseren et al., 2021). Regardless of the multifactorial origin of both risk factors (e.g., obesity, diabetes, and sedentary lifestyle), they both directly contribute to CVD risk and prediction. A large number of randomized clinical trials have shown that medications that lower blood pressure (BP) and LDL cholesterol reliably reduce the risk of major CVD events, including coronary heart disease, stroke and heart failure (Bibbins-Domingo et al., 2016; Ettehad et al., 2016). These findings suggest a causal link between these risk factors and CVDs. In this way, recent studies indicate that in the 40 to 69 year-old population, each 20 mmHg increase in SBP is associated with a more than 2-fold difference in stroke-related death and death due to IHD (Oliveros et al., 2020).

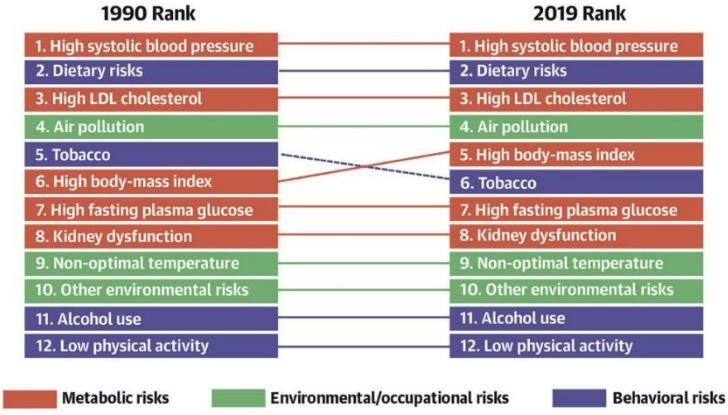


Figure 2 | CVD burden attributable to causal and modifiable risk factors. Source: GBD study, 2019 (Roth et al., 2020).

Other metabolic CVD risk factors include high body mass index (BMI), high fasting plasma glucose levels and kidney dysfunction. Additionally, behavioural risks, such as dietary risks, mainly unhealthy dietary habits, tobacco or alcohol use and low physical activity, are among the primary causal and modifiable risk factors contributing to CVD burden. In addition, environmental risks, such as air pollution, constitute the fourth largest modifiable risk factor for CVDs. Conversely, the male sex and increasing age are both considered traditional nonmodifiable CVD risk factors that contribute to risk in largely unknown and complex ways, yet these are clearly not causal in the same way as BP and LDL cholesterol levels.

Although lifestyle changes and pharmacological therapy represent evidence-based recommended treatments for the main causal and modifiable CVD risk factors, a residual CVD risk could remain even after initial lifestyle changes and risk factor treatment (Visseren et al., 2021). Thus, more efficient risk reduction strategies should be developed that take into account the biomarkers of exposure to causal and modifiable CVD risk factors over the time.

The top three modifiable metabolic and behavioural risk factors substantially contributing to CVD burden, on which this thesis is based, are hypertension, hypercholesterolemia and dietary risks, which are described in greater detail in the following subsections.

1.2.1. Metabolic risk factors

1.2.1.1. Hypertension

Hypertension is defined as increased values in both SBP and diastolic blood pressure (DBP). It is one of the key modifiable risk factors for CVDs and is

present in all populations, especially in industrialized societies. It doubles the risk of CVDs, including IHD, congestive heart failure, stroke, renal failure, and peripheral arterial disease. Current hypertension guidelines (European Society of Cardiology and European Society of Hypertension; ESC/ESH, 2018) (Williams et al., 2018) recommend BP criteria for classifying the office BP as optimal, normal and high-normal and defining the hypertension grades as grade 1 to 3 hypertension or isolated systolic hypertension, which is frequent among elderly individuals (***Table 1***). The prevalence of hypertension increases with age, and it is more common in black populations than in nonblack populations living in Europe (Williams et al., 2018).

Blood pressure category	*Systolic (mmHg)*		*Diastolic (mmHg)*
Optimal	<120	and	<80
Normal	120 - 129	and/or	80 - 84
High-normal	130 - 139	and/or	85 - 89
Grade 1 hypertension	140 - 159	and/or	90 - 99
Grade 2 hypertension	160 - 179	and/or	100 - 109
Grade 3 hypertension	≥180	and/or	≥110
Isolated systolic hypertension	≥140	and	<90

Table 1 | Office blood pressure classification and definitions of hypertension grade. Adapted from: ESC/ECH 2018 Hypertension Guidelines (Williams et al. 2018).

Elevated BP affects more than one billion individuals and causes an estimated 9.4 million deaths per year (Kotchen, 2018). Although antihypertensive therapy reduces the risks of CVD and renal disease, large segments of the population are either untreated or inadequately treated. According to the Annual Report on the National Health System (2018/19), high BP is among the most frequent chronic diseases affecting 19.8% of adults in Spain. Of

those affected, only 39.7% of those with high SBP received treatment, and the remaining 60.3% that were untreated had it under control on their own (Spanish Ministry of Health, 2018).

Adequate BP control reduces the incidence of acute coronary syndrome by 20–25%, stroke by 30–35%, and heart failure by 50%. This scenario suggests that effective health care approaches at individual and population level as well as health policies need to be set to achieve an optimal control and management of hypertension. Diet and lifestyle modifications (e.g., sodium restriction, increased intake of dietary potassium, weight loss if overweight/obese, physical activity prescription, moderation of alcohol intake, and following a healthy DASH-like diet rich in fruits, vegetables, whole grains and low-fat dairy products), are considered essential strategies to reduce CVD risk associated to hypertension (Flack et al., 2020). However, greater efforts should be taken to predict and diagnose the pathology at its preliminary stages prior to clinical manifestation.

1.2.1.2. Hypercholesterolemia

Dyslipidaemia is characterized by an alteration in essential lipid profile components (mainly blood lipoproteins) such as LDL cholesterol, HDL cholesterol and triglycerides, and is an important causal and modifiable risk factor for the development of CVDs and atherosclerosis. Isolated LDL cholesterol traditionally has been considered one of the best lipid-related markers for CVD prediction as fully described in Section 1.3.2.1. Numerous human epidemiologic, genetic and clinical studies have consistently demonstrated that high LDL cholesterol levels, or hypercholesterolemia, are causally associated with atherosclerotic plaque formation and related

subsequent CV events (Ference et al., 2017). In fact, lowering LDL cholesterol levels reduces the risk of atherosclerotic CVD proportional to the absolute reduction in LDL cholesterol levels (Baigent et al., 2010). It is important to note that hypertension and hypercholesterolemia act synergistically in a way that elevated BP has been demonstrated to increase the atherogenicity of cholesterol-rich lipoproteins (Martin et al., 2003). According to the 2019 ESC/EAS Guidelines for the Management of Dyslipidaemias (Mach et al., 2020), new LDL cholesterol goals are proposed to reduce CVD risk through lipid modification (***Table 2***). A more intensive reduction in LDL cholesterol according to CVD risk categories is suggested, with target levels ranging from <70 to <116 mg/dL for patients with high to low CVD risk.

CVD risk category	*LDL cholesterol goal*		
	mmol/L		*mg/dL*
*Very high-risk**	<1.4		<55
*High risk**	<1.8	or	<70
Moderate risk	<2.6		<100
Low risk	<3.0		<116

Table 2 | Lower LDL cholesterol goals across CVD risk categories. *An LDL cholesterol reduction of ≥ 50% from baseline is recommended. Adapted from: 2019 ESC/EAS Guidelines for the Management of Dyslipidemias (Mach et al., 2020).

Hypercholesterolemia remains a major menace to public health, and the overall burden in terms of the number of DALYs and deaths is increasing globally. Since 1990, the number of DALYs and deaths has risen steadily reaching 98.6 and 4.40 million, respectively, in 2019 (Kotchen, 2018). More total DALYs from elevated LDL are experienced by males than females, especially in males younger than 65 years (Roth et al., 2020). Therefore, health systems and countries may need to focus on new approaches to

reverse these trends for CVD prevention. Among the typical determinants of hypercholesterolemia, aproximately 80% are related to diet and lifestyle, including tobacco use and sedentary lifestyle, with the remainder 20% being familial. Familial hypercholesterolemia is the most common of the monogenic lipid disorder and is strongly related to premature CVD (Mach et al., 2020). It usually appears asymptomatic and if left untreated, it develops into early coronary artery disease (CAD) before the ages of 55 and 60 years.

Primary treatment of hypercholesterolemia can reduce LDL cholesterol burden, improve endothelial function, attenuate the development of atherosclerosis, and improve coronary outcomes (Mach et al., 2020). Intervention strategies vary depending on the total estimated CVD risk and the levels of LDL cholesterol (***Table 3***). They include lifestyle advice alone, lifestyle intervention with consideration of adding drug intervention if uncontrolled, or lifestyle intervention with concomitant drug intervention. Drug therapy mainly consists of statin prescription, which has a dose-dependent effect on LDL cholesterol reduction.

On the other hand, the use of nutraceutical supplements and functional foods, such as plant sterols/stanols, red yeast rice, berberine, dietary fibers, β-glucans, soy isoflavones, n-3 PUFAs and several PCs, among others, have emerged as alternative therapies to the conventional drug-based therapy. These therapies have reported beneficial effects and good tolerability in hypercholesterolemic subjects (Santini & Novellino, 2017). Therefore, diet and lifestyle modifications are a priority to improve the overall lipoprotein profile, as described in Section 1.2.2.1.

	Total CV risk (SCORE) %	Untreated LDL-C levels					
		<1.4 mmol/L (55 mg/dL)	1.4 to <1.8 mmol/L (55 to <70 mg/dL)	1.8 to <2.6 mmol/L (70 to <100 mg/dL)	2.6 to <3.0 mmol/L (100 to <116 mg/dL)	3.0 to <4.9 mmol/L (116 to <190 mg/dL)	≥4.9 mmol/L (≥ 190 mg/dL)
Primary Prevention	<1 low-risk	Lifestyle advice	Lifestyle advice	Lifestyle advice	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	I/C	I/C	I/C	I/C	IIa/A	IIa/A
	≥1 to <5, or moderate risk	Lifestyle advice	Lifestyle advice	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	I/C	I/C	IIa/A	IIa/A	IIa/A	IIa/A
	≥5 to <10, or high- risk	Lifestyle advice	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	IIa/A	IIa/A	IIa/A	I/A	I/A	I/A
	≥10, or at very-high risk due to a risk condition	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	IIa/B	IIa/A	I/A	I/A	I/A	I/A
Secondary Prevention	Very-high risk	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	IIa/A	I/A	I/A	I/A	I/A	I/A

Table 3 | Intervention strategies according to total cardiovascular risk and untreated LDL cholesterol levels. Abbreviations: CV, cardiovascular; SCORE = Systematic coronary risk estimation. [a] Class of recommendation; [b] Level of evidence. Source: 2019 ESC/EAS Guidelines for the Management of Dyslipidemias (Mach et al., 2020).

1.2.2. Behavioural risk factors and nutritional strategies

1.2.2.1. Dietary risks

CVDs are the primary consequence of dietary risks, with 7.94 million annual deaths and 188 million annual DALYs attributed to dietary risks (Kotchen, 2018). Dietary risks comprise the sum of adverse effects of both individual foods and dietary patterns. Excessive sodium intake, insufficient intake of nuts/seeds, high intake of processed meats, and low intake of seafood rich in n-3 PUFAs have been associated with higher rates of cardiometabolic mortality due to IHD, stroke, and type 2 diabetes (Micha et al., 2017). Consistent evidence from epidemiological studies indicates that 15 food types are either underconsumed (fruits, vegetables, nuts and seeds, legumes, whole grains, seafood, milk, fibre, calcium and PUFAs) or overconsumed (red and processed meat, sugar-sweetened beverages, trans-fatty acids (TFAs), and sodium) (Emmett et al., 2019), leading to a higher incidence of CV events.

Different dietary patterns, such as the Dietary Approaches to Stop Hypertension (DASH) diet and the Mediterranean diet, have shown direct effects on major metabolic CVD risk factors, such as hypertension and hypercholesterolemia (Zampelas & Magriplis, 2020). Both patterns are hypothesis-driven diets characterized by key components. The DASH diet takes into account macro- and micronutrients that have been demonstrated to be effective in reducing risk of hypertension such as increased potassium, magnesium and calcium from fruits, vegetables, nuts, whole grains, low-fat dairy and lean protein, and reduced sodium (Sacks et al., 2001). The Mediterranean diet relies on the dietary habits adopted by populations traditionally living in the Mediterranean area and is characterized by high

contents of fruit, vegetables, legumes, fish, nuts, and unsaturated FAs from vegetable sources, including a large amount from olive oil (Estruch et al., 2018; Urpi-Sarda et al., 2012). In this sense, the *Prevención con Dieta Mediterránea* (PREDIMED) trial indicated that participants allocated to a Mediterranean-type diet supplemented with extra-virgin olive oil or nuts, had a significantly lower (approximately 30%) incidence of major CV events than those who were on a low-fat diet (Estruch et al., 2018). The underlying mechanisms for the CV health benefits of the DASH and Mediterranean diets are complex but can be explained by the improvement of cardiometabolic profiles, including insulin resistance, lipid profiles, BP, and inflammatory markers (Grosso et al., 2014). Hence, it is plausible that adherence to these dietary patterns might be positively associated with a metabolically healthy phenotype. In contrast, a Western diet, which is characterized by increased dietary intake of saturated fats, refined grains, sugar, processed meats, alcohol and salt and low intake of fibre, represents a growing dietary risk that contributes to the increased occurrence of CVDs, especially in countries adapting a westernized lifestyle (Kopp, 2019).

Table 4 summarizes the currently available evidence of the overall influences of lifestyle modifications on plasma LDL cholesterol and BP levels, indicating the magnitudes of the effects and the levels of evidence.

Lifestyle modification	*Magnitude of the effect* (+++ =>10%, ++ = 5-10%, + =<5%)	*Level*
To reduce LDL cholesterol levels		
Avoid dietary trans fats	++	A
Reduce dietary saturated fats	++	A
Reduce dietary cholesterol	+	B
Increase dietary fibre	++	A
Use functional foods enriched with phytosterols	++	A
Use red yeast rice nutraceuticals	++	A
Reduce excessive body weight	++	A
Increase habitual physical activity	+	B
To reduce blood pressure levels		
Reduce salt intake	+++	A
Eat a DASH style diet	+++	A
Moderate consumption of coffee, green and black tea	++	A
Lower alcohol intake	+	B
Reduce excessive body weight	++	A
Increase regular physical activity	++	A
Stop smoking	++	A
Reduce stress and induce mindfulness	+	B

Table 4 | Impact of lifestyle modifications on LDL cholesterol and BP levels. The magnitude of the effect and level of evidence (A = data derived from multiple randomized controlled trials, B = data derived from a single clinical trial or large non-randomized studies) refer to the impact of each lifestyle modification on plasma LDL cholesterol or BP levels. Adapted from: 2019 ESC/EAS Guidelines for the Management of Dyslipidemias and ACC/AHA 2020 Hypertension Guidelines (Flack & Adekola, 2020; Mach et al., 2020).

Effects of dietary fat. The replacement of animal fats, including dairy fat, with vegetable sources of fats and PUFAs have shown to decrease the risk of major CVDs (Chen et al., 2016). A current meta-analysis of cohort studies has suggested a cardio-protective effect of PUFAs in studies followed up for more than 10 years, however, higher TFAs intake has been associated

with greater risk of CVD in a dose-dependent manner (Y. Zhu et al., 2019). Avoiding any consumption of TFAs, which are derived from the partial hydrogenation of vegetable oils, is a key measure of the dietary prevention of CVD. As for saturated fatty acids (SFAs), its consumption should be reduced to less than 10 % of total energy intake (TEI), and should be further reduced (less than 7 % of energy) in the presence of hypercholesterolemia (Mach et al., 2020). Thus, fat intake should mainly come from sources of monounsaturated fatty acids (MUFAs) and PUFAs, including omega-6 (n-6) and n-3 PUFAs – also known as alpha-linoleic acid (ALA) and linolenic acid (LA) – from vegetable oils, nuts, seeds and oily fish.

Effects of dietary carbohydrates. A high-carbohydrate diet, especially rich in added sugar, has been associated with elevated BP levels due to increased angiotensin II and sympathetic pathway activation which stimulate aldosterone production, causing sodium retention and a subsequent vascular vasoconstriction (Mansoori et al., 2019). In this sense, a recent cross-sectional study in older females has reported that decreasing sugar by 2.3 teaspoons a day could result in 8.4 and 3.7 mmHg drop in SBP and DBP respectively (Mansoori et al., 2019). In contrast, dietary carbohydrate has a 'neutral' effect on LDL cholesterol, although excessive consumption is represented by untoward effects on plasma triglycerides and HDL cholesterol levels (Tay et al., 2015). Total carbohydrate intake should range between 45-55% of TEI, whereas added sugar should not exceed 10% of TEI. Conversely, dietary fibre intake, particularly soluble fibre present in legumes, fruits, vegetables, and wholegrains (e.g. oats and barley), is associated with lower BP (Aleixandre & Miguel, 2016) and with reduced plasma LDL cholesterol levels. Principally, intake of oat β-glucan, a soluble

dietary fibre found in the endosperm cell wall of oats, has demonstrated LDL cholesterol-lowering effects (by 5-10% reduction in normocholesterolemic or hypercholesterolemic subjects) when consumed at daily doses of at least 3 g (Othman et al., 2011).

Effects of alcohol intake. In healthy adults, consuming low-to-moderate amounts of alcohol each day typically has no short-term substantial effects on BP or LDL cholesterol. However, evidence suggest that binge drinking (more than 5 standard drinks a day) is associated with transient increases in both BP and LDL cholesterol levels (Piano, 2017). According to the ESC/ECH 2018 Hypertension Guidelines and the ESC/EAS 2018 Guidelines for the Management of Dyslipidaemias, the recommended limit for alcohol consumption is 2 standard drinks for males and 1.5 for females (10 g alcohol/standard drink).

Effects of sodium intake. There is strong evidence for a relationship between high sodium intake and increased BP (Gay et al., 2016). Low-sodium dietary interventions (<5 g salt/day) have revealed an overall pooled net effect on BP ranging from -3.07 mmHg on SBP to -1.81 mmHg on DBP (Gay et al., 2016). The main source of sodium in the diet is salt, which is commonly used as food ingredient or condiment. However, most of the sodium intake (70-75%) comes from processed foods that, besides salt, contain other added sodium compounds including sodium nitrate, monosodium glutamate and sodium phosphate (Flack & Adekola, 2020). According to the WHO Guideline on Sodium Intake (WHO, 2012), healthy adults should consume less than 5 g (just under a teaspoon) of salt per day, which is equal to 2000 mg of sodium. For individuals with established hypertension or pre-hypertension further reduction to 1500 mg of sodium per day can result in greater BP reduction.

1.2.2.2. Effects of food bioactive compounds and functional foods

Previously described dietary food sources are considered important dietary risks which can be reverted or modified by "interventional medicine" as the first therapeutic step. However, a large body of evidence reveals a number of functional foods and plant bioactive which are potentially efficacious in preventing and reducing main causal and modifiable CVD risk factors, such as hypertension and hypercholesterolemia (***Table 5***).

Plant bioactive component	*Attributed effect*	*Daily dose*
Lipid - lowering effect		
Plant stanols and sterols (phytosterols)	↓ 8 - 12 % LDL cholesterol	1.6 - 3 g
Red yeast rice (Monacolin - HMG-CoA reductase)	↓ > 20 % LDL cholesterol	3 - 10 mg
Soy proteins (B-conglycinin globulin)	↓ 3 % LDL cholesterol	30 g
Berberine	↓ 9 - 22 % total cholesterol ↓ 7 - 22 % LDL cholesterol	500 - 1500 mg
Blood pressure - lowering effect		
Green and black tea catechins, tannins and flavonoids	↓ 2 mmHg SBP ↓ 1.2 mmHg DBP	2 - 6 cups
Beetroot juice (inorganic nitrates)	↓ 3.5 mmHg SBP ↓ 1.3 mmHg DBP	70 - 500 mL
Pomegranate juice tannins, anthocyanins and flavonoids	↓ 5 mmHg SBP ↓ 2 mmHg DBP	> 240 mL
Cocoa flavanols	↓ 1.76 mmHg SBP ↓ 1.76 mmHg DBP	30 - 1218 mg
Resveratrol	↓ 9 mmHg SBP ↓ 6 mmHg DBP	≥ 300 mg
Lycopene	↓ 5 mmHg SBP	10 - 50 mg
Aged garlic extract (S-allylcysteine)	↓ 9 mmHg SBP ↓ 4 mmHg DBP	1200 - 2400 mg

Table 5 | Main effects of plant bioactive components observed in clinical trials and their dose range. Abbreviations: DBP, diastolic blood pressure; HMG-CoA, hydroxymethylglutaryl-coenzyme A; SBP, systolic blood pressure. Own source, adapted from: (Bahadoran et al., 2017; Caliceti et al., 2016; Cicero et al., 2017, 2019; Gerards et al., 2015; X. Li & Xu, 2013; Méndez-del Villar et al., 2014; Ried et al., 2017; Rohner et al., 2015; Sahebkar et al., 2017; Tokede et al., 2015).

Some lipid- and BP-lowering bioactive have been studied for their impact on human vascular health, particularly as regards endothelial function and arterial stiffness (Cicero et al., 2017).

1.3. Pathophysiology of the main causal and modifiable cardiovascular disease risk factors

1.3.1. Pathophysiology of primary hypertension

The pathogenesis of primary or essential hypertension (also called idiopathic hypertension) is multifactorial and highly complex. Primary hypertension is the term applied to 95% of hypertensive patients in which elevated BP results from complex interactions between multiple genetic and environmental factors (Carretero & Oparil, 2000; Sutters, 2021). The onset of this condition is usually between the ages of 25 and 50 years, and it is uncommon before 20 years of age. The kidney is both the contributory and the target organ of the hypertensive process, in fact, primary hypertension can develo into secondary hypertension as renal function decreases.

BP homeostasis is regulated by two components; **cardiac output** and **peripheral resistance**. Cardiac output is determined by heart rate and stroke volume, which is related to myocardial contractibility and to the size of the vascular compartment. Peripheral resistance is determined by functional and anatomic changes in small arteries and arterioles involving vascular structure and function (Kotchen, 2018). Therefore, arterial BP is a product of both cardiac output and peripheral vascular resistance, and these components must be autoregulated to maintain a set BP in an individual. Autoregulatory mechanisms maintain the blood flow of most tissues over a wide range of BP according to their specific needs. Kidney play a key role regulating

intravascular volume contraction and expansion, although BP regulation also occurs by way of transfer of transcapillary fluid by the mechanism of pressure-natriuresis (Granger et al., 2002). Through this mechanism salt and water balance is achieved at heightened systemic pressure, whereby increases in renal perfusion pressure lead to decreases in sodium (Na^+) reabsorption and increases in Na^+ excretion.

Primary hypertension involves the interaction of multiple organ systems and mechanisms of independent or interdependent pathways. Among the best understood pathways that interact to produce hypertension, the following ones have a greater contribution:

- Overactivation of the sympathetic nervous system (SNS).
- Overactivation of the renin-angiotensin-aldosterone (RAA) system.
- Defect in natriuresis.
- Abnormal cardiovascular or kidney development.
- Increased intracellular sodium (Na^+) and calcium (Ca^{2+}) concentrations.

The specific causes of the alterations produced in the main BP regulatory pathways remain largely unknown, although genetic influences are evident. Additionally, behavioural and lifestyle factors can exacerbate the effects of genetic factors. As shown in ***Figure 3***, the overall genetic and environmental factors involved in the pathophysiology of primary hypertension include increased plasma and extracellular fluid (ECF) volume, elevated vascular reactivity, vascular wall thickness and vascular smooth muscle tone. The resultant increases in cardiac output and peripheral resistance notably contribute to primary hypertension.

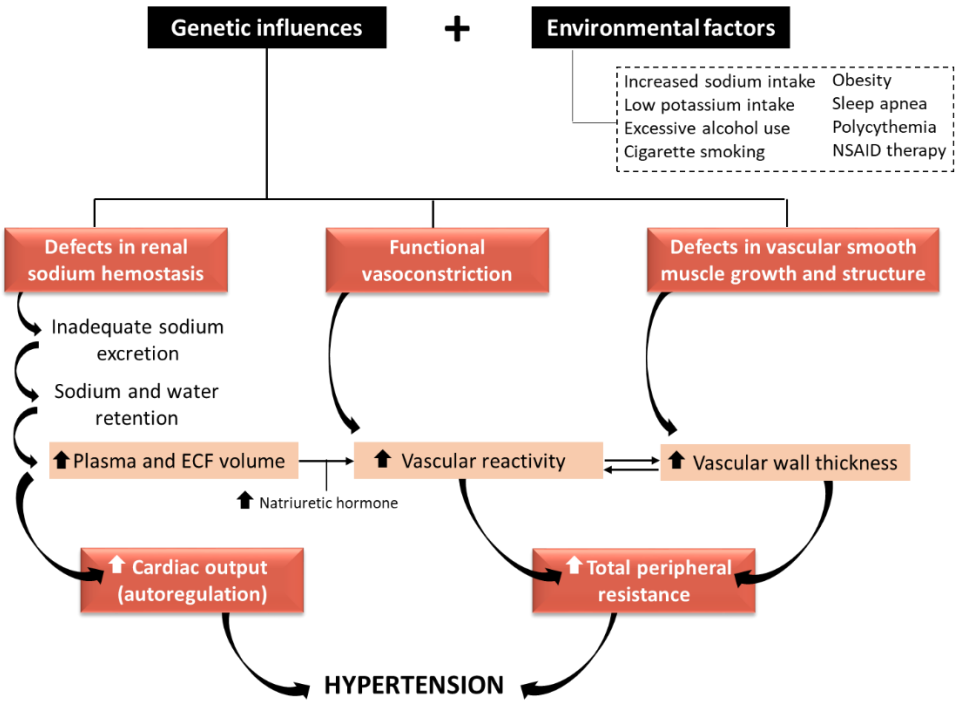


Figure 3 | The pathophysiology of primary hypertension. A hypothetical scheme for the pathogenesis of primary hypertension, implicating genetic defects in vascular and renal systems and environmental factors. Abbreviations: ECF, extracellular fluid; NSAID, Non-Steroidal Anti-inflammatory Drug. Adapted from: Robbins & Cotran, Pathologic basis of disease, 7th edition (Damjanov, 2005).

1.3.1.1. Emergent biomarkers in primary hypertension

Biomarkers are objective, quantifiable characteristics of biological processes, that can be measured accurately and reproducibly, as explained in Chapter 4. Multiple circulating biomarkers for hypertension have been identified over the years and may shed light on the underlying processes involved in the onset, development and progression of primary hypertension. Discovering and utilizing emergent validated biomarkers offer insight into disease pathogenesis and may allow for the prediction, early diagnosis and stratified treatment of disease (Shere et al., 2017).

Among the isolated circulating biomarkers that predict the risk of developing primary hypertension, high levels of C-reactive protein (CRP), interleukins (IL)-1 and IL-6, tumour necrosis factor-α (TNF-α) (all well-established proinflammatory proteins), plasminogen activator inhibitor-1 (PAI-1; a serine protease inhibitor), fibrinogen (a fibrin precursor), B-type natriuretic peptide (BTNP; secreted by cardiac ventricles in response to excessive elongation of heart muscle cells), homocysteine (a sulfur-containing amino acid derived from methionine), endothelin 1 and 2 (vasoconstrictor peptides), oxidized LDL (Ox-LDL; a critical factor in the atherosclerosis process), 8-isoprostaglandin (8-iso-PGF2 alpha; an isoprostane involved in lipid peroxidation), leptin (hormone involved in energy balance regulation), aldosterone, angiotensin and vitamin D3, among others, have been associated with an increased risk of high BP in both normotensive and prehypertensive individuals (***Figure 4***) (Shere et al., 2017; Touyz & Burger, 2012).

However, disease progression and control biomarkers are used when there exists an evident alteration in BP levels and, in some cases, vascular or renal damage. This is the case for CRP, PAI-1, TNF-α, Ox-LDL, asymmetric dimethylarginin (ADMA), symmetric dimethylarginin (SDMA), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), reactive oxygen species (ROS), NO, renin, and CXCR2 protein (***Figure 4***).

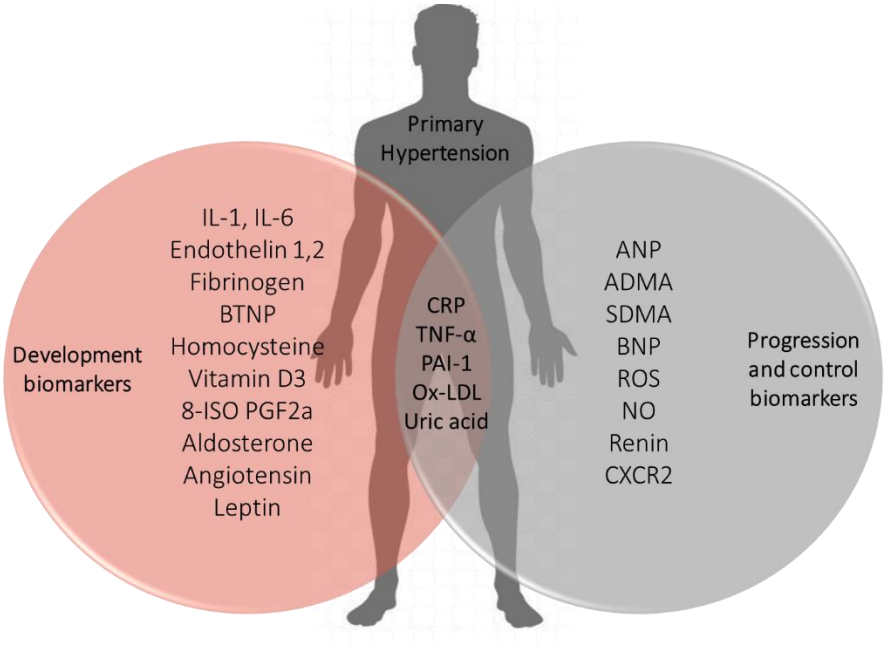


Figure 4 | Circulating biomarkers in the development and progression of primary hypertension. Abbreviations: ADMA, asymmetric dimethylarginin; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; BTNP, B-type natriuretic peptide; CRP, C-reactive protein; IL, interleukine; NO, nitric oxide; Ox-LDL, oxidized LDL; PAI-1, plasminogen activator inhibitor-1. Adapted from Shere et al., and Touyz & Burger (Shere et al., 2017; Touyz & Burger, 2012).

Overall, these circulating biomarkers play important roles in inflammation, endothelial dysfunction and oxidative stress contributing to increased peripheral vascular resistance and vascular dysfunction. Thus, monitoring and targeting the mechanisms by which they are expressed opens new therapeutic opportunities to develop disease-modifying therapies to reduce or delay the incidence of primary hypertension.

Beyond blood biomarkers, novel strategies to account for primary hypertension in the preclinical stages are emerging. In this context, gut microbiota composition in conjunction with their derived bioactive

metabolites could have a potential role as early biomarkers in the pathophysiological development of this disease (G. et al., 2021). In Chapter 2, these emergent biomarkers will be described in greater detail.

1.3.2. Pathophysiology of hypercholesterolemia

Hypercholesterolemia is characterized by high plasma LDL cholesterol levels and normal triglycerides. Primary hypercholesterolemia includes autosomal dominant hypercholesterolemia, which is caused by mutations of the LDL receptor (LDLR), familial defective apolipoprotein B 100 (ApoB-100), or proprotein convertase subtilisin/kexin type 9 (PCSK9). Primary hypercholesterolemia also includes autosomal recessive hypercholesterolemia; polygenic hypercholesterolemia and hyperlipoproteinemia(a) (Martinez-Hervas & Ascaso, 2019).

There are several distinct patterns of LDL metabolism responsible for primary hypercholesterolemia, and both genetics and diet are important contributing factors (Huff et al., 2021). Genetic defects mainly involve mutations in LDLR, which mediates the uptake of LDL into the liver. Under normal conditions, endocytosis of LDL is the primary way that the body decreases cholesterol levels, so it is plausible that a decrease in LDLR function would also increase LDL concentrations in plasma. On the other hand, diet has a variable effect on LDL cholesterol depending on the fat composition. For example, diets high in SFAs and TFAs can increase small, medium and total LDL particles and concentrations (Chiu et al., 2017), and diets rich in MUFAs and n-3 PUFAs have positive effects on LDL composition and oxidizability (Kratz et al., 2002).

Even though a high LDL level is of particular clinical importance, it should be noted that hypercholesterolemia can also include very-low-density

lipoprotein (VLDL) and intermediate-density lipoprotein (IDL), i.e., non-HDL-cholesterol (Martinez-Hervas & Ascaso, 2019). Overall, these classes of particles differ in their sizes, densities, chemical composition, or charges. Indeed, in recent years, LDL particles have been divided into two phenotypes: type A (large and light LDL; particle diameter ≥ 25.5 nm), and type B (small and dense LDL; particle diameter < 25.5 nm). In this regard, subjects with predominantly small dense LDL particles have been recently characterized to have an unfavorable cardiometabolic risk profile, as shown by the results of the *Liposcale* population-based study in Spain (Pichler et al., 2018). In addition, small dense LDL has a stronger ability to cause atherosclerosis and has been accepted for a long time as a risk factor for CVD by the American Cholesterol Education Program and Adult Treatment Program III (NCEPIII, 2002).

1.3.2.1. Hypercholesterolemia and the atherosclerosis process

High levels of LDL cholesterol in the blood are a major contributor to the increased risk of atherosclerotic lesion formation. The atherosclerotic lesion develops progressively with the accumulation of small dense LDL particles in the arterial wall, and their successive oxidation and modification to provoke chronic inflammation and ultimately thrombosis or stenosis (detailed process in ***Figure 5***).

The atherogenic potential of small dense LDL particles has been linked to the specific biochemical and biophysical properties of these particles (Ivanova et al., 2017). Given their small size, they can easily enter the arterial wall serving as a source of cholesterol and lipid storage. Moreover, their longer circulation time increases the probability of atherogenic modifications in the

blood plasma. Native LDL does not occasion lipid accumulation in cultured cells, however, modified particles, such as oxidized, desialylated, glycated, and electronegative LDL, possess proinflammatory properties and are highly atherogenic (Ivanova et al., 2017).

Oxidation is one of the first atherogenic modifications of small dense LDL particles. It occurs when vascular wall cells, including endothelial cells, smooth muscle cells, and macrophages, stimulated by the attack factors, produce and release a large number of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which induce the immune response and inflammation. Then, the initial modified LDL activates the endothelial anti-apoptosis signaling pathways, induces endothelial cells to express tissue factors and chemokines, promotes the aggregation on inflammatory cells, and generates a large number of free radicals which rapidly oxidize PUFAs on the surface of the LDL particles (Mushenkova et al., 2021). Finally, Ox-LDL is formed and after complete oxidation it loses its affinity to LDLR and binds specifically to scavenger membrane receptors, such as CD36 or TLR-4 (Syväranta et al., 2014).

Apart from oxidation, another atherogenic modification of small dense LDL particles is ***desialylation***. It is performed in blood plasma by trans-sialidase that transfers the sialic acid moiety from the LDL particle to various acceptors such as plasma proteins, sphingolipids, or gangliosides (Tertov et al., 2001). Desialylation rises the affinity of the small dense LDL particles to proteoglycans in the arterial wall, favouring the lipid storage and atherosclerotic plaque development.

Also, ***glycosylation*** of LDL is a non-enzymatic reaction of glucose and its metabolites with the free amino groups of lysine in which LDL is rich. Glycosylation reduces the affinity of LDL to LDLR, promotes the uptake of LDL by scavenger receptors on the surface of macrophages, and induces the formation of foam cells. In parallel, this process enhances the susceptibility of LDL particle to further oxidation (Younis et al., 2008).

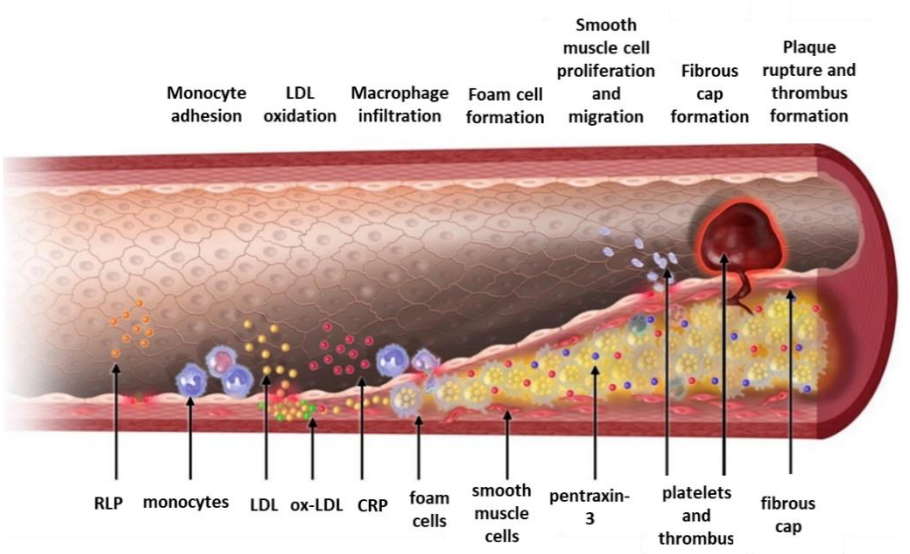


Figure 5 | Atherosclerosis as a multistep pathogenic process. The pathophysiological process through which atherosclerotic plaques develop begins with endothelial damage in the inner arterial surface. Endothelial damage leads to the dysfunction of endothelial cells, which express adhesion molecules that capture leukocytes (monocytes) on their surfaces by means of vascular adhesion molecule-1, E-selectin, P-selectin and intracellular adhesion molecule-1. Then, an increasing in the number of LDL particles that can permeate through the vascular wall is produced. LDL is then oxidized into pro-inflammatory particles (ox-LDL) and taken up via scavenger receptors on macrophages resulting in foam-cell formation. Simultaneously, macrophages produce biomarkers of vascular inflammation like pentraxin-3 favoring the progression of atherosclerotic plaque. As more lipid accumulates within the vessel wall, smooth muscle cells begin to migrate into the lesion. Ultimately, smooth muscle cells encapsulate the newly formed plaque forming the fibrous plaque, the protector of the lesion, preventing the lipid core from being exposed to the lumen of the vessel. Atherosclerotic plaques can lead to occlusion of the vessel (decreasing blood flow distally and causing ischemia) or, more commonly because of abundant lipid and macrophages (vulnerable plaque) rupture, inducing the formation of a thrombus which can completely block the flow of blood. Abbreviations: ox-LDL, oxidized LDL; RLP, Remnant-like particle cholesterol; CRP, C-reactive protein. Sources: (Libby, 2018; Nelson et al., 2017).

1.3.2.2. Emergent biomarkers in atherosclerosis

The pathogenesis of atherosclerosis is very complex. Biomarkers of atherosclerotic lesions are often risk factors for its occurrence. Identification of classical biomarkers, such as LDL cholesterol, may not be helpful in patients with moderate or unusual cardiovascular risk. For more accurate management in these patients group, evidence suggest novel modern biomarkers that can become routine in the laboratory detection and diagnosis of CVD in the future (***Figure 6***) (Surma et al., 2020).

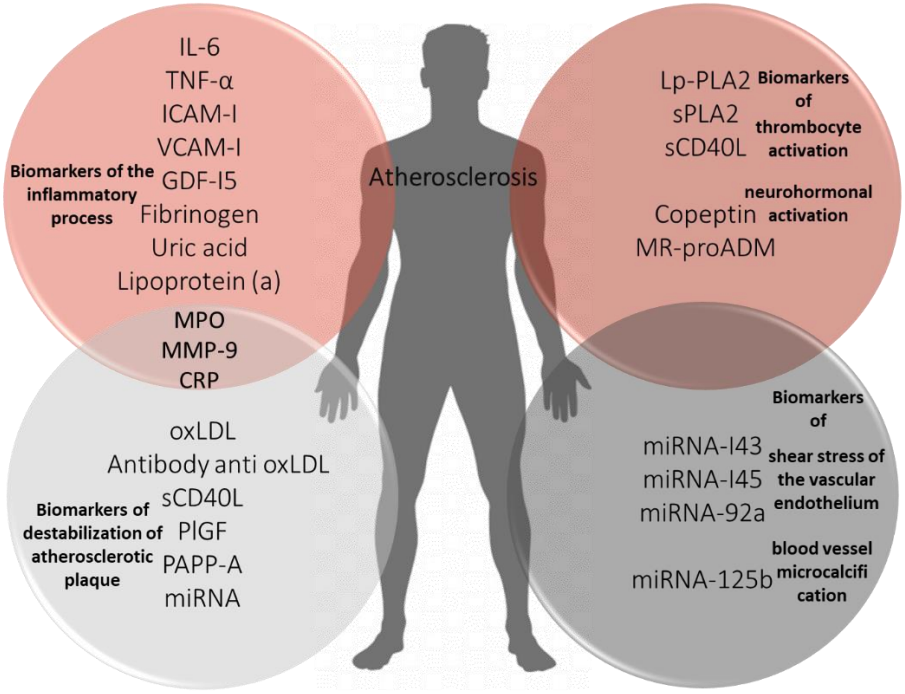


Figure 6 | Novel biomarkers in the development of atherosclerosis. Abbreviations: MPO, myeloperoxidase; MMP-9, Matrix metalloproteinase 9; GDF-I5, Growth/differentiation factor I5; sCD40L, soluble CD40 ligand; PlGF, placental growth factor; PAPP-A, Pregnancy-associated plasma protein A; LpPLA2, Lipoprotein-associated phospholipase A2; sPLA2, secretory phospholipase A2; MR-proADM, Midregional proadrenomedullin. Adapted from Surma et al. (Surma et al., 2020).

These biomarkers include proteins, enzymes and microRNAs involved in different stages of the atherosclerotic process. From IL-6, TNF-α, myeloperoxidase (MPO) or CRP to lipoprotein-associated phospholipase A2 (Lp-PLA2) or secretory phospholipase A2 (sPLA2; mainly produced by monocytes and macrophages, take part in oxidation of LDL and thrombocyte activation). Also, various microRNAs are involved in shear stress of the vascular endothelium and microcalcification of the atherosclerotic plaque.

Future advances are expected in high-technology medicine to combat CVDs, although the fundamental message is that any major reduction in deaths and disability from CVDs will come from prevention, not cure. This must involve novel strategies focused on an early detection and robust reduction of risk factors.

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

Chapter 2. The contributory role of the human gut microbiota in cardiovascular diseases

In recent years, accumulating evidence has indicated the importance of the gut microbiota in maintaining human health. The gut microbiota is a complex system of mutualistic microorganisms and hosts approximately 100 trillion bacteria from 500 to 1000 species. It represents 1-3% of body mass and encodes over 4M genes (Tang et al., 2017). In the initial microbiological studies, Antonie van Leeuwenhoek (1681) was the first to investigate the microorganisms in his own stools, observing *"more than 1000 living animalcules"* (Lane, 2015). Over the last 12 years, the use of modern molecular techniques that permit unbiased sequencing of the gut microbiome has allowed researchers to identify novel microorganisms to be studied to a large extent without the need for culturing. The Human Microbiome Project is a National Institutes of Health initiative to characterize the microbiomes of healthy humans that launched in 2007. It has catalogued more than 750 genomes of pure bacterial strains from multiple body sites, with the gut microbiome being the most representative (Consortium, 2012). This project, involving a large cohort of 300 individuals, allowed for the first time an understanding of the relationships among microbes and between the microbiome and clinical parameters (Consortium, 2012). Moreover, it has elucidated high microbial diversity, especially in gut microbial communities.

In CVDs, the composition of the gut microbiota is altered. In turn, the gut microbiota composition is susceptible to modulation by a number of environmental and behavioural factors, meaning that this is a dynamic organ able to contribute in CVD development. It functions as a bioreactor with

enormous metabolic capacity and cooperates with the host in many biological functions to conform to a 'symbiotic mammalian superorganism' (Fischbach & Segre, 2016). In addition, gut bacteria release bioactive metabolites in the gut that can be used by gut mucosal cells or absorbed into the circulation and transported to the liver where they are transformed. Therefore, the role of the gut microbiota and its bioactive metabolites in the development or prevention of the main causal and modifiable CVD risk factors is an ongoing area of research and one of the topics of the present thesis.

2.1 Introduction to the human gut microbiota

2.1.1 Gut microbiota development

The development of the microbiota ecosystem is a fascinating, complex and dynamic phenomenon (Thursby & Juge, 2017). The origin of the gut microbiota is largely believed to start from birth since all mammals are normally born from a sterile environment. However, in recent years, new theories have supported that the origin lies in womb tissues, such as the placenta (Aagaard et al., 2014). The mode of delivery also appears to affect the pattern of gut microbiota composition during the first months of life (Rutayisire et al., 2016). While the microbiota of vaginally delivered infants contains a higher abundance of *Lactobacilli* and *Bifidobacterium* genera, the microbiota colonization in infants delivered by caesarean section is delayed and is dominated by facultative anaerobes, such as the *Clostridium* genus. Through the first year of life, microbiota diversity progressively increases, and the composition converges towards a distinct adult-like microbial profile that is affected by health and diet behaviours, stress factors and genetic

control (Thursby & Juge, 2017). By approximately 2.5 years of age, the composition, diversity and functional capabilities of the infant microbiota resemble those of the adult microbiota. Notable lines of research have been focused on the study of microbiome acquisition *in utero*. In this context, a recent cross-sectional study involving a large cohort of pregnant women found no evidence to support the existence of a placental microbiome in term pregnancies (Sterpu et al., 2021) (this work was part of the international stay of the PhD student, and further description is provided in ***Annex I***).

Thus, the knowledge of the native composition of the gut microbiota and how external factors disrupt on its establishment in the early life are intriguing and may in the future help to understand and interfere in the pathophysiology of many diseases including CVDs.

2.1.2 Composition and structure of gut microbiota

The healthy human gut microbiota is integrated by a complex community of microorganisms. It is mainly composed of two dominant bacterial phyla, Firmicutes and Bacteroidetes that represent more than 90% of the total community, and by other subdominant phyla including Proteobacteria, Actinobacteria, and Verrucomicrobia (Jandhyala et al., 2015). Even though this general profile remains constant, gut microbiota can exhibit both temporal and spatial differences in distribution at the genus level and beyond along the length of the gastrointestinal (GI) tract. This is mostly attributed to different conditions in the gradient of oxygen, antimicrobial peptides (including BAs), and pH. As shown in ***Figure 7***, the bacterial load is limited in the small intestine community, whereas the colon carries a high bacterial load (from 10^1 bacteria per gram of contents in the oesophagus, stomach and

small intestine to 10^{12} in the colon and distal gut) (Donaldson et al., 2016; Jandhyala et al., 2015).

In the distal oesophagus, duodenum and jejunum, *Streptococcus* appears to be the dominant genus. *Helicobacter* genus, in particular *Helicobacter pylori*, inhabits the stomach as a commensal. The whole large intestine constitutes of over 70% of all microbes in the body and is dominated by beneficial Firmicutes and Bacteroidetes phyla, with genus like *Bacteroides*, *Clostridium* and *Lactobacillus* in the small intestine, or *Prevotella*, *Ruminococcus* and *Eubacterium* in the colon. Human colon also harbors primary pathogens, such as *Campylobacter jejuni*, *Salmonella enterica* and *Escherichia coli*, but with a lower abundance. The reduced abundance of the phylum Preoteobacteria along with high abundance of genera such as *Bacteroides*, *Prevotella* and *Ruminococcus* suggests a healthy gut microbiota (Jandhyala et al., 2015).

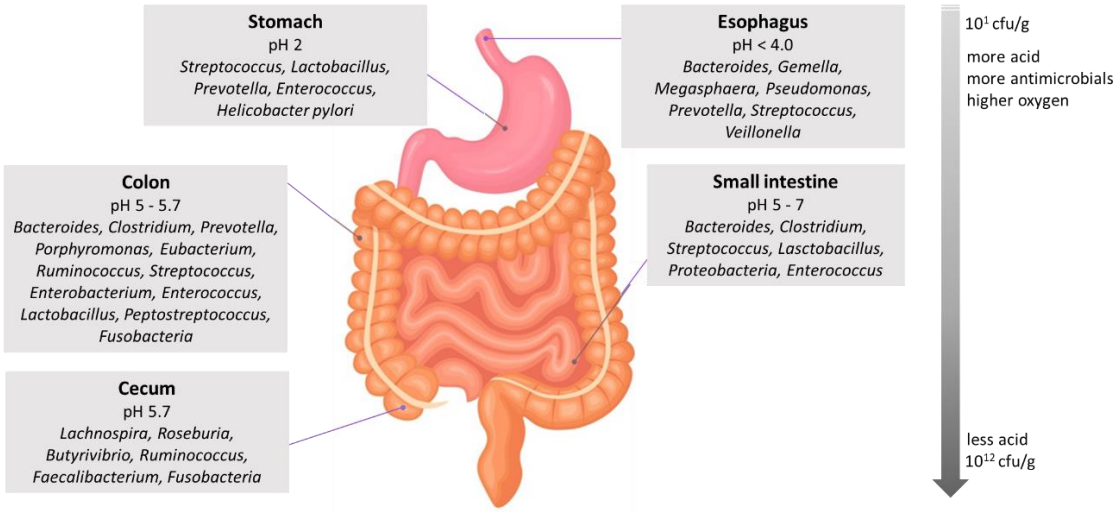


Figure 7 | Composition of the normal gut microbiota. Abbreviations: cfu, colony forming unit. Adapted from Donaldson et al. and Jandhyala et al. (Donaldson, et al. 2016; Jandhyala et al. 2015).

2.1.3 Host-microbiota symbiosis in health and disease

It is clear that it is important to have a holistic vision of our microbial ecosystem that takes into account all the components of the community. The mammalian intestine is a complex and rich ecosystem that provides multiple levels of intercellular signalling among i) the microbiota components; ii) the microbiota and the host; iii) the microbiota and exogenous pathogens; and iv) the host and exogenous pathogens. The knowledge gap in the gut microbiota medicinal approach is not bridged completely. Microbes residing in the intestinal tract influence local and systemic processes of the host. Such processes include supplying vitamins, the maturation of mucosal immunity, nutrient transformation, and the influence on the brain and neurotransmitters (Bajinka et al., 2020).

Under normal and healthy conditions, gut microbes and the host coexist in a state of balance, forming an ecological system or 'superorganism' of microbiota and mammals in symbiosis. The gut microbiota is in a constant state of flux, yet the ecosystem can continue to adjust and function in full health with this balanced imbalance (or unbalanced balance) – a state known as **eubiosis**. Under eubiosis, the GI barrier minimizes contact between ther luminal microbial community and the intestinal epithelial cell surface. This protective effect is possible by the production of several integrated components, including physical (the epithelial and mucus layers), biochemical (enzymes and antimicrobial proteins), and immunological (IgA and epithelial immune cells) factors (Hooper & Macpherson, 2010). Moreover, gut microbes must be adapted to a certain type of lifestyle due to the relatively fewer number of biochemical niches available in the gut, compared with other microbial-rich systems. However, when the balance in

the gut microbial community is altered, often through factors from the external environment, a damaging disturbance can arise, which is known as **dysbiosis**. Dysbiosis could be due to the gain or loss of community members or changes in the relative abundance of the dominant genus. Usually, a reduction in microbial diversity is observed, with a consequent loss of beneficial bacteria, such as Bacteroides strains and butyrate-producing bacteria such as Firmicutes, and a rise in pathobionts (symbiotic bacteria that become pathogenic under certain conditions), such as Proteobacteria (Humphreys, 2020). Therefore, the dysbiotic gut microbiota alters intestinal epithelial barrier function and can trigger inflammation due to the increase in proinflammatory species. Several pathologic conditions associated with dysbiosis have been described, and the list of disorders is continuosly growing. Such pathological conditions include those affecting the GI tract (diarrhoea and irritable bowel disease), the immune system (allergies, multiple sclerosis, type 1 diabetes, inflammatory bowel disease, and rheumatoid arthritis), the central nervous system (Alzheimer and Parkinson diseases and autism), and energy metabolism in the host (obesity, type 2 diabetes, and atherosclerosis) (Wilkins et al., 2019). Despite this, it is not yet clear whether these alterations are the cause or consequence of these disorders.

2.1.4 Factors shaping the gut microbiota

The microbiota composition is subject to alterations by the host and environmental selective pressures. Diet is considered to be one of the main factors that influences the gut microbiota throughout the lifetime of the host. Different dietary patterns, including the intake of fibre, animal-derived saturated fats and dietary cholesterol, are involved in changes to the

microbiota composition in humans (Singh et al., 2017), as further described in Section 2.4. Additionally, drugs, such as antibiotics and other medications, alter the taxonomic, genomic and functional capacity of the gut microbiota, and their effects can be persistent (Modi et al., 2014). Broad-spectrum antibiotics are known to reduce bacterial richness and diversity and promote the growth of specific indigenous taxa. However, recent evidence suggests that some antibiotics can act positively on the gut microbiota, providing a so-called 'eubiotic' effect by increasing the abundance of beneficial bacteria, such as Actinobacteria (Ianiro et al., 2019). Other factors that influence the gut microbiota involve exercise training (Allen et al., 2018); food additives exposure (Cao et al., 2020); smoking status (Lee et al., 2018); stress and depression disorder (Madison & Kiecolt-Glaser, 2019); disruption in circadian rhythms or sleep deprivation (Y. Li et al., 2018); and ethnicity or geographic location (Gaulke & Sharpton, 2018). In addition, the method of delivery at birth (Rutayisire et al., 2016) and the infant feeding, especially the mother's milk-associated microbes (Fitzstevens et al., 2017), shape the gut microbiota composition at the early stages of life. Also, the gut microbiota is influenced in part by the genetic makeup of the host (Goodrich et al., 2014).

2.1.5 Current methods for the study of the gut microbiota

To study the gut microbiota, a stool specimen must be collected from individuals and DNA from stool is isolated. Isolation, identification and enumeration of the vast majority of GI microorganisms using conventional culture based techniques is a laborious task. Currently, with the availability of new culture-independent approaches, such as high throughput gene sequencing technology, the study of the gut microbiota mainly consists of

two major stages: (1) 16S rRNA-based sequencing of bacterial genes and (2) bioinformatics analysis.

Bacterial gene sequencing. Sequencing of bacterial genes involves metagenomic analysis of DNA that codes for the 16S rRNA gene (Claesson et al., 2010). This gene is present in all bacteria and archaea and contains nine highly variable regions (V1–V9), which allows species to be easily distinguished. More recent efforts have attempted to catalogue not only the microbial species but also the microbial genes in the gut by using whole-genome shotgun metagenomics. These next-generation techniques provide higher resolution and sensitivity in the taxonomic characterization and genetic make-up of the gut community and reveal new pathways and functions of gut microbes (Costea et al., 2017; W.-L. Wang et al., 2015), as further described in *Chapter 5*.

Bioinformatics analysis. Bioinformatics analysis enables the cleaning of the data and the identification of bacterial taxa. This can also allow us to obtain information on metabolic functions using a wide array of bioinformatics platforms. Furthermore, statistical analysis of the sequencing data also helps in identifying alpha diversity (diversity of species within the same individual),

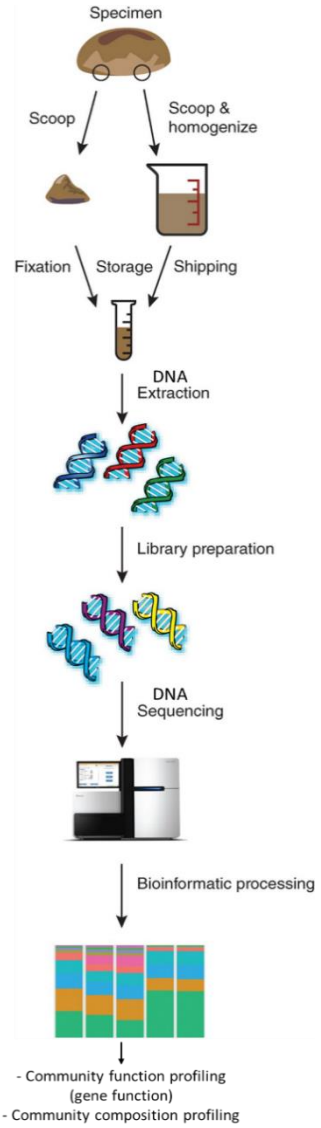


Figure 8| Schematic workflow of the study of the gut microbiota. Abbreviations: ASVs, amplicon sequence variants. Adapted from Costea et al. 2017.

beta diversity (interindividual species diversity), relative abundance, and several other parameters related to the organisms (Jandhyala et al., 2015). ***Figure 8*** shows the schematic workflow of study of the gut microbiota from faecal samples. Additionally, metabolomics is another rapidly expanding field of gut microbiota research that evaluates small molecules associated with the interrelationship of host-bacterial metabolism that has implications in health and disease. Combined data from the gut microbiota and the metabolome provides the most powerful evidence that can demonstrate the closest association with health and disease states. This topic will be further detailed in *Chapter 5*.

2.2 The metabolic potential of the gut microbiota

The gut microbiota is the central regulator of mammalian fuel intake by that processes nutrients into absorbable compounds. It functions as an endocrine organ, producing vitamins and bioactive metabolites that are not synthesized by the host and can impact host physiology. Among the gut microbiota-derived metabolites, SCFAs, secondary metabolites of bile acids (BAs), branched-chain amino acids (BCAAs), and TMAO, among other cometabolites, such as indoles or 4-cresol, are important modulatory factors for CVDs (Brial et al., 2018). All of these factors are derived from dietary substrates that are subject to the saccharolytic and proteolytic actions of the gut microbiota. They are transported to the liver, where they can undergo enzymatic modification, prior to transfer to the circulation and to other organs (***Figure 9***). These bioactive metabolites have promising clinical potential as postbiotic biomarkers in the diagnosis and treatment of human CVDs.

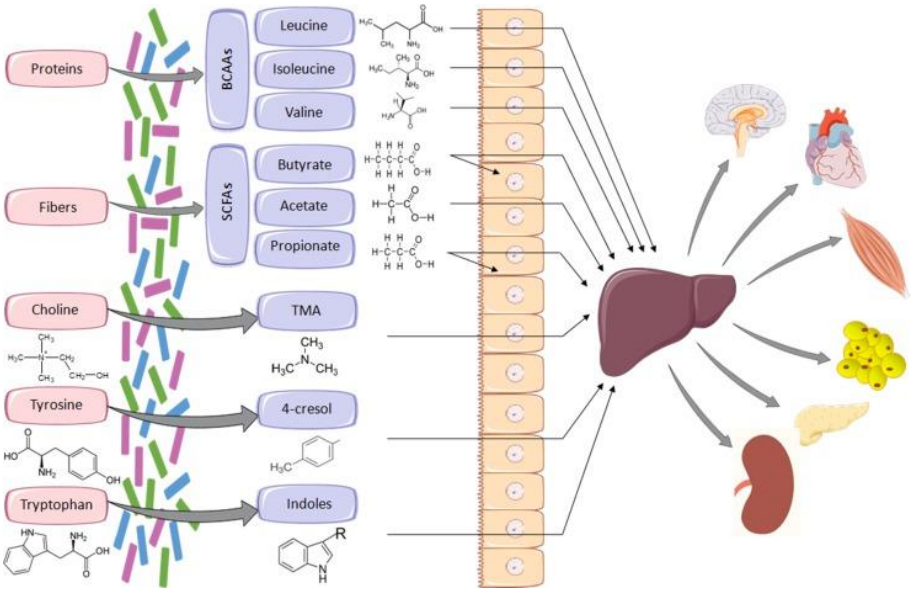


Figure 9 | Bioactive metabolites synthesized by the gut bacteria. Metabolites produced by the gut microbiota from dietary substrates are transported to the liver where they can undergo enzymatic modification (e.g., TMA to TMAO), prior to transfer to the circulation and to other organs. SCFAs (predominantly butyrate and propionate) can be used locally as energy source by gut mucosal cells. Abbreviations: BCAAs, branched-chain amino acids; SCFAs, short-chain fatty acids; TMA, trimethylamine. Source: (Brial et al. 2018).

In the following sections, the main gut microbiota bioactive metabolites investigated in this thesis are described and detailed their associated physiological actions.

2.2.1 Short-chain fatty acids

SCFAs are produced by anaerobic gut bacteria in the caecum and the proximal colon through the saccharolytic fermentation of dietary fibres (e.g., nonstarch polysaccharides and low-digestible saccharides), and to a lesser extent, proteins and peptides (Koh et al., 2016). Three predominant SCFAs, propionate, butyrate and acetate, are typically found in a ratio of 1:1:3 in the GI tract (Morrison & Preston, 2016). In the human gut, acetate is produced

by most anaerobes, propionate is mainly produced by Bacteroidetes, and butyrate production is dominated by Firmicutes. These SCFAs, mainly propionate and butyrate, have local effects as primary energy sources in gut mucosal cells and activate intestinal gluconeogenesis through distinct mechanisms (Koh et al., 2016). Once absorbed by epithelial cells in the GI tract, they mediate several distal effects, including propionate stimulation of liver gluconeogenesis, de novo lipid synthesis, and protein synthesis or cholesterol synthesis stimulated by acetate (Brial et al., 2018). Thus, SCFAs have multiple regulatory roles in energy homeostasis, and glucose and lipid metabolism, and their increased circulating levels have been associated with a reduced risk of CV and metabolic diseases, including obesity, insulin resistance, hypertension and atherosclerosis (Canfora et al., 2015; Ohira et al., 2017; Pluznick, 2017). SCFAs also play a role in regulating the immune system and inflammatory response (Morrison & Preston, 2016). The cellular mediators of the effects of SCFAs have been partly elucidated, and best described pathways involve the expression of G-protein-coupled receptors (GPRs), mainly GPR41 and GPR43 (Brial et al., 2018).

2.2.2 *Trimethylamine N-oxide*

Another means by which the microbiota can interact with the host is through the trimethylamine (TMA)/TMAO pathway (***Figure 10***). TMA is an organic compound that is generated by the gut microbiota. Specifically, this refers to the microbial metabolism of dietary nutrients that possess a TMA moiety, such as choline, phosphatidylcholine, and L-carnitine, all of which are present in high amounts in animal products, such as red meat, fish, eggs and dairy products (Tang & Hazen, 2014; Z. Wang et al., 2014). TMA is then produced as a waste product by a variety of microbial enzymes (TMA lyases) that are

contained in several anaerobic gut bacteria belonging to the Firmicutes, Actinobacteria and Proteobacteria phyla (Rath et al., 2017). Outside the intestinal tract, TMA is rapidly oxidized into TMAO by flavin-containing monooxygenase enzymes, mainly FMO_3, in the liver and then released into the circulation (Z. Wang & Zhao, 2018). TMAO is mainly cleared from circulation by the kidneys, and thus, renal function is also important to consider when examining the levels of TMAO in systemic circulation (Tang et al., 2015).

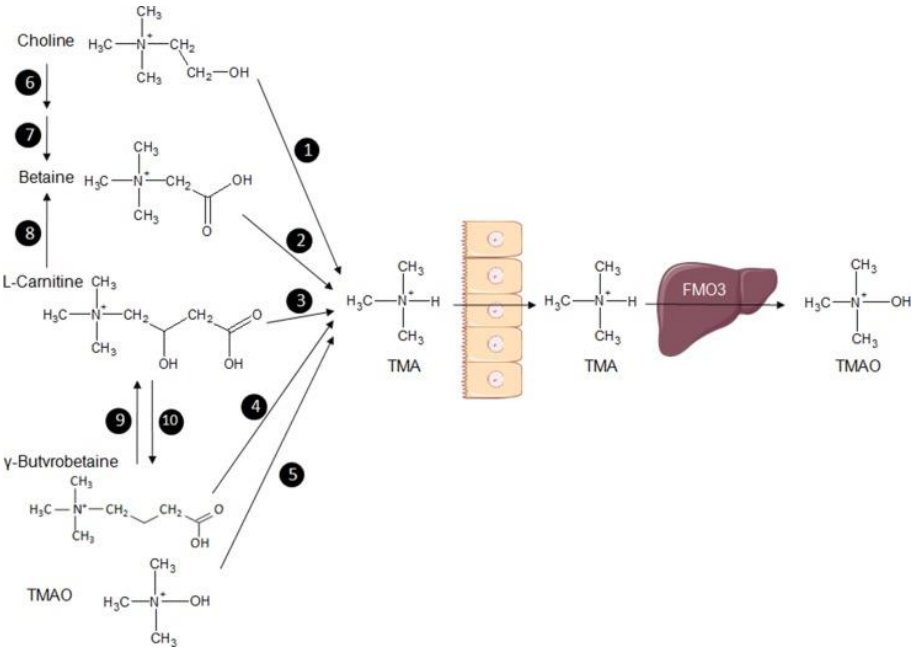


Figure 10 | The trimethylamine/trimethylamine N-oxide pathway illustrating microbiota-host metabolism. Bacterial enzymes use dietary substrates to synthesize trimethylamine (TMA), which is transferred across the intestinal endothelium to the circulation and transported to the liver where it is metabolized into TMAO by the enzyme flavin-containing monooxygenase (FMO3). The TMA substrate betaine can be synthesized from choline and L-carnitine. Different enzymes are involved: 1: Choline TMA lyase; 2: betaine reductase; 3: carnitine reductase; 4: carnitine TMA lyase; 5: TMAO reductase; 6: choline dehydrogenase; 7: betaine aldehyde dehydrogenase; 8: L-carnitine dehydrogenase; 9: γ-butyrobetaine dioxygenase; 10: γ-butyrobetainyl-CoA: carnitine CoA transferase. Abbreviations: TMA, trimethylamine; TMAO, trimethylamine N-oxide. Source: (Brial et al. 2018).

Elevated plasma levels of TMAO have been associated with increased risk of type 2 diabetes mellitus (Zhuang et al., 2019), CVD and cerebrovascular diseases (Tang & Hazen, 2014), incident thrombosis risk (W. Zhu et al., 2016), and atherosclerosis (Randrianarisoa et al., 2016) in population-based and intervention studies. In this sense, TMAO molecule has been recently proposed as a novel biomarker of CVD risk (Schiattarella et al., 2018), although evidence is inconsistent, especially in broad population observations. Despite growing interest in TMAO biology, its cellular ligands remain unknown and their identification is becoming the next breakthrough to demonstrate causality with diseases. On the other hand, the existing body of literature is sparse regarding TMAO and hypertension.

2.3 Contribution of the gut microbiota-derived metabolites to the development of the main causal and modifiable cardiovascular disease risk factors

Notable evidence has revealed that gut microbiota-derived metabolites play essential roles in the development of CVDs (Brown & Hazen, 2018). Gut microbial dysbiosis can exert proatherosclerotic and hypertensive effects by activating multiple metabolite-dependent pathways affecting the generation of TMAO, SCFAs and BAs (Lau et al., 2017; Ma & Li, 2018). Moreover, many findings suggest that the gut microbiota has the capacity to alter blood lipid composition, in particular cholesterol, through their role in BA metabolism (Kriaa et al., 2019).

Table 6 shows a summary of experimental studies demonstrating the effects of microbial-derived metabolites in the development of CVDs, atherosclerosis and hypertension in recent years.

Gut metabolite	Experimental model	Main observed effect	*Reference*
TMAO	C57BL/6 mice	TMAO promotes pathological process of atherosclerosis by impairing endothelial self-repair capacity and enhancing monocyte adhesion	(Warrier et al., 2015)
	ApoE (-/-) female mice	Gut microbial metabolite δ-butyrobetaine is converted into TMAO, and accelerates atherosclerosis	(Koeth et al., 2014)
	ApoE (-/-) mice	Dietary choline or TMAO supplementation enhances atherosclerotic lesion	(Z. Wang et al., 2011)
	ApoE (-/-) mice	Dietary L-carnitine supplementation alters gut microbial composition, enhances production of TMA/TMAO, and increases atherosclerosis	(Koeth et al., 2013)
	817 young adults	TMAO may not significantly contribute to early atherosclerotic disease risk	(Meyer et al., 2016)
	7447 participants (aged 55-80 years)	Plasma metabolites from choline pathway are associated with an increased risk of CVD	(Guasch-Ferré et al., 2017)
	4007 participants (mean age 63 years)	Increased TMAO levels are associated with increased risk of CVD	(Tang et al., 2013)
	18 healthy subjects	Gut microbe-generated TMAO from dietary choline is prothrombotic	(W. Zhu et al., 2017)
SCFAs	205 obese pregnant women	Blood pressure is associated with alterations in gut microbiota and production of butyrate	(Gomez-Arango et al., 2016)
	Hypertensive male C57Bl/6 mice	Acetate supplementation changes the development of hypertension	(Marques et al., 2017)
	Olfr78 (-/-) mice	SCFAs produced by the gut microbiota modulate BP via Olfr78 and Gpr41	(Pluznick, 2013)
	Gpr41 knockout mice	Microbial SCFAs lower BP via endothelial GPR41	(Natarajan et al., 2016)
	Dahl salt-sensitive; Dahl salt-resistant rats	Altered gut microbiota composition affects hypertension by increasing plasma acetate in Dahl salt-sensitive rats	(Mell et al., 2015)
	155 pre-hypertensive	Gut microbiota dysbiosis participates in the pathogenesis of hypertension	(J. Li et al., 2017)

	and hypertensive individuals		
	Spontaneously hypertensive rats; 7 hypertensive patients	High BP is associated with gut microbiota dysbiosis, both in animal and human hypertension	(T. Yang et al., 2015)

Table 6 | Effects of gut microbiota-derived metabolites on the main causal and modifiable CVD risk factors. Abbreviations: BP, blood pressure; SCFAs, short-chain fatty acids´; TMAO, trimethylamine N-oxide; BAs, bile acids; Gpr41, G-protein-coupled receptor 41; Olfr78, olfactory receptor 78; FXR; farnesoid X receptor; LDLR, lipoprotein receptor; APOE (-/-), apolipoprotein E-deficient. Adapted from: Ma et al. 2018 (Ma & Li, 2018).

2.3.1 Link between gut microbiota metabolites and hypertension

In recent years, the involvement of the gut microbiota in the development of hypertension has become of notable interest. The first evidence linking intestinal bacteria to hypertension was provided by Honours et al., who, in a series of experiments in the early 1980s, demonstrated that antibiotic treatment attenuated steroid-induced hypertension in rats (Honour, 2015). In more recent studies, the role of the gut microbiome in BP regulation has been reconsidered. Yang et al. compared the microbiota of normotensive Wistar-Kyoto and spontaneously hypertensive rats, and found that hypertensive rats displayed reduced microbial richness, diversity and evenness, as well as an increased Firmicutes:Bacteroidetes ratio (T. Yang et al., 2015). These changes were associated with reductions in SCFA-producing bacteria that may have contributed to the higher BP. Although the underlying mechanisms that link the gut microbiota and hypertension pathogenesis have not yet been fully elucidated, the existing evidence highlights the critical roles of SCFAs and Ox-LDL (***Figure 11-A***).

SCFAs are able to stimulate host GPR-regulated pathways to affect renin secretion and therefore increase BP (Pluznick, 2017). Natarajan et al.

reported that GPR41 knockout mice exhibited systolic hypertension compared with wild-type mice, and that SCFAs reduced BP by regulating GPR41 in the vascular endothelium (Natarajan et al., 2016). Additionally, olfactory receptor 78 (Olfr78 in mice and OR51E2 in humans), another type of GPR in the kidney, can be modulated by SCFAs, such as acetate and propionate, and affect BP (Pluznick, 2017). In addition, GPR41- and Olfr78-null mice have been shown to play opposite roles in BP regulation. While GPR41-null mice are hypertensive, Olfr78-null mice are hypotensive, suggesting the physiological importance of SCFA signaling pathways in BP control (Natarajan et al., 2016). Both Olfr78 and GPR41 are expressed in the smooth muscle cells of small resistance blood vessels.

On the other hand, gut dysbiosis can contribute to hypertension through vasoconstriction mediated by Ox-LDL (Ma & Li, 2018). Dysbiosis can induce the expression of pro-inflammatory cytokines and NO promoting oxidative stress, which stimulate Ox-LDL production.

2.3.2 Link between gut microbiota metabolites and atherosclerosis

TMAO and secondary BAs are involved in the atherosclerosis pathogenic process (***Figure 11-B***). The underlying mechanisms of TMAO have been extensively investigated. As suggested by Koeth et al., TMAO can lead to atherosclerosis by suppressing reverse cholesterol transport and modulating the activity of cholesterol transporters in macrophages (Koeth et al., 2013). Moreover, experimental studies in apolipoprotein E (ApoE) (-/-) mice supplemented with dietary choline have shown overexpression of CD36 and steroid receptor RNA activator 1 (SR-A1), which are two macrophage scavenger receptors that are highly involved in atherosclerosis (Z. Wang et

al., 2011). These receptors could increase the formation of foam cells and promote TMA production. In addition, TMAO can accelerate the development of atherosclerosis by blocking the BA pathway in the liver (Koeth et al., 2013). Furthermore, secondary BAs can promote atherosclerosis when BA receptors are altered (Ma & Li, 2018).

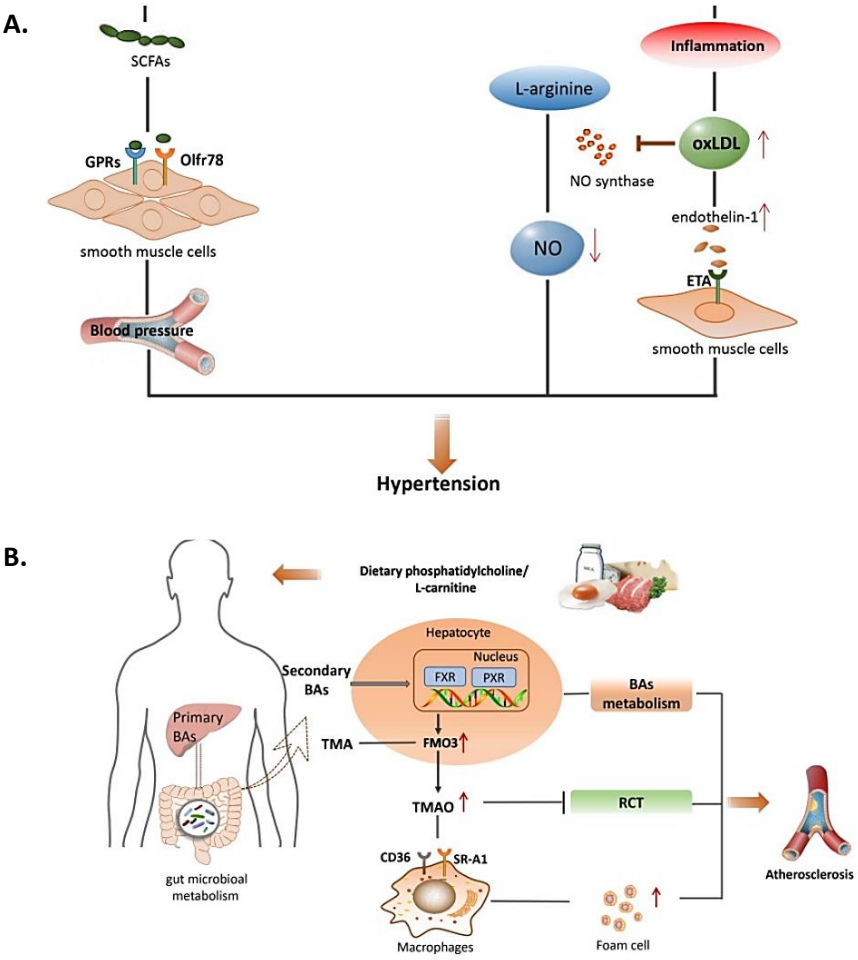


Figure 11 | Mechanisms between gut microbiota-derived metabolites, atherosclerosis and hypertension. Abbreviations: BAs, bile acids; CD36, the monocyte differentiation antigen; ETA, endothelin receptor A; FMO3, flavin monooxygenase 3; FXR, farnesoid X receptor; GPRs, G-protein-coupled receptors; NO, nitric oxide; Olfr78, olfactory receptor 78; oxLDL, oxidized low density lipoprotein; PXR, pregnant X receptor; RCT, reverse cholesterol transport; SCFAs, short-chain fatty acids; SR-A1, steroid receptor RNA activator 1; TMA, trimethylamine; TMAO, trimethylamine N-oxide. Source: (Ma & Li, 2018).

Despite the evidence on these interplays, the exact role of gut microbiota in mediating atherosclerosis and hypertension still deserves further extensive investigation.

2.4 Novel therapeutic strategies for targeting the gut microbiome and improving cardiovascular outcomes

The emergence of the gut microbiota as a pivotal regulator of CV pathophysiology has spawned interest in the development of microbiota-targeted therapies aimed at modulating the composition and/or metabolism of the microbial community (L. Jin et al., 2021). Some of the potential strategies involve dietary intervention, probiotic, prebiotic, and antibiotic treatments, as well as faecal transplantation or bioengineering (***Figure 12***). These strategies have been shown to improve BP and normalize the lipid profiles in CVD patients.

Dietary intervention. Dietary habits influence the composition and function of the gut microbiota. A low intake of dietary fibre is associated with reduced microbial diversity and SCFA production and higher production of metabolites that are detrimental to the mucins of the colonic barrier (Singh et al., 2017). High adherence to the Mediterranean diet, has been associated with increased levels of faecal SCFAs, *Prevotella* bacteria, and other fibre-degrading *Firmicutes*. Conversely, low adherence to this dietary pattern is associated with elevated urinary TMAO, which is related to an increased risk of CVDs (De Filippis et al., 2016). Additionally, a diet high in whole grains results in improvements in the lipid profiles and BP accompanied by concomitant reductions in gut opportunistic pathogens of the *Enterobacteriaceae* family (Xiao et al., 2014). However, a Western diet is

known to be associated with increased CVD risk. This diet reduces microbial diversity, decreases the levels of beneficial *Bifidobacterium* and *Eubacterium* species, and increases the abundance of the mucin-degrading bacterium *Akkermansia muciniphila* (Singh et al., 2017).

Dietary interventions are relatively inexpensive and easily administered for CVD prevention; however, the mechanisms for most of their reported effects remain unclear (L. Jin et al., 2021). The modulatory effects of dietary changes on the microbiota occur quickly, usually within the first 24 h of initiation, and reverse to baseline within 48 h of cessation of dietary manipulation (Battson, Lee, Weir, et al., 2018).

Prebiotics. Prebiotics are dietary constituents that can cause specific changes in the composition and activity of intestinal microbiota to elicit beneficial effects on the host (Holscher, 2017). Prebiotics can incur atheroprotective effects and reduce the risk of CVDs by promoting the growth of beneficial gut microbiota, mainly *Bifidobacteria* and *Lactobacilli* (Singh et al., 2017). Most prebiotics can be classified as dietary fibres and cover multiple sources such as soybeans, inulins, unrefined wheat and barley, raw oats, and nondigestible oligosaccharides, including fructans, fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), and arabinooligosaccharides (AOS) (Pandey et al., 2015). In addition, plant PCs have been shown to have prebiotic properties. They can influence gut microbiota composition and function, and gut metabolism (Kumar Singh et al., 2019), as further described in Chapter 3. Diets that are scarce in prebiotic components have been shown to reduce total bacterial abundance (Halmos et al., 2015).

Consumption of dietary prebiotics can lead to indirect stimulation of a cross-feeding process whereby the products produced from fermentation of a polysaccharide by one bacterial specie provide substrates for growth of other bacteria present in the community (Holscher, 2017). For example, lactate and acetate by-products from the metabolization of fructans by lactic acid bacteria, like *Bifidobacteria*, can be then used by many other bacteria, like *Roseburia* or *Faecalibacterium* species, that produce butyrate (Holscher, 2017). These prebiotic-gut microbiota interactions point out a potent therapeutic strategy to modulate gut microbiota composition and protect from CVDs.

Probiotics. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Sanders, 2008). The most common probiotics are *Lactobacilli* and *Bifidobacteria* strains, which are natural residents of the gut microbiota. Probiotics are a novel potential therapy for treating hypercholesterolemia through their ability to modulate the host gut microbiota and metabolism (Z. Guo et al., 2011). In addition, probiotic strains can prevent the formation, progression, and eventual rupture of atherosclerotic plaques (Z. Guo et al., 2011). The probiotic-related health effects are strain specific. *Lactobacillus reuteri* and *Lactobacillus plantarum* strains have elicited significant lipid-lowering effects through their ability to hydrolyse bile salts in a number of double-blinded randomized controlled trials (RCTs) (Lau et al., 2017), demonstrating their viability as a treatment option for CVD risk (Tuohy et al., 2014). In addition, probiotic mixtures have conferred antihypertensive effects in spontaneously hypertensive rat models (Gómez-Guzmán et al., 2015). Moreover, as recently shown by our research group, heat-killed forms

of *Bifidobacterium animalis* have proven to be more efficient than living strains in reducing DBP in abdominally obese individuals (Pedret et al., 2019).

Whereas the term probiotic implies that microorganisms should be alive at the time of ingestion, current works support the use of nonviable cells or substances released by or produced through the metabolic activity of microorganisms – also called postbiotics – that have shown potential therapeutic applications (Żółkiewicz et al., 2020). In addition, the synergic application of probiotics and prebiotics as a selective substrate, also known as synbiotics, improves the viability of probiotic microorganisms (Markowiak & Śliżewska, 2017).

Antibiotics. Antibiotics have been suggested as a secondary therapeutic strategy for remodelling certain microbial communities and prevent CVD in animal models. As recently shown by Battson et al., in C57BL/6J male mice the use of broad-spectrum antibiotic with a Western diet intervention reverse the diet-induced endothelial dysfunction and arterial stiffness, which are preclinical steps in CVD progression, by suppression of gut dysbiosis (Battson, Lee, Jarrell, et al., 2018). However, the antibiotic therapeutic use for CVD treatment in humans is not supported since they can promote bacterial resistance and increase the susceptibility to opportunistic pathogens (Battson, Lee, Weir, et al., 2018).

Faecal microbiota transplantation (FMT). FMT has emerged as a core therapy for remodelfing the gut microbiota and improving its functional diversity (F. Zhang et al., 2019). It consists of transferring donor faecal samples into the GI tract of a patient with a depleted microbiota. It has been shown tested for a broad range of disease indications, including intestinal

diseases, such as *Clostridium difficile* infection, and CVDs. Atherosclerosis susceptibility have shown to be transmitted via transplantation of the gut microbiota in ApoE null mice in which resident intestinal microbes are suppressed with antibiotics (Gregory et al., 2015). Durgan et al. found that transplantation of dysbiotic faecal contents from obstructive sleep apnoea (OSA)-induced hypertensive rats notably increased BP and induced gut dysbiosis in normotensive rats after only 7 days. These findings, suggest that manipulation of gut microbiota may be a viable treatment for OSA-induced and probably other forms of hypertension (Durgan et al., 2016). Conversely, transfer of faecal contents from normotensive Wistar Kyoto rats to spontaneously hypertensive rats significantly reduced BP in recipients (Toral et al., 2019). Recently, washed microbiota transplantation, in which faecal particles, parasite eggs, and fungi are removed, has shown a BP-lowering effect in nontreated hypertensive patients, offering a novel approach for hypertension treatment (Zhong et al., 2021). However, despite the successful application of FMT, it could also induce several adverse effects in the recipient. Thus, careful additional evaluations must be carried out before FMT can be widely implemented in humans (L. Jin et al., 2021).

Bioengineering. In recent year's, advances in precision medicine have resulted in novel genetic tools that have been designed to modify bacterial properties and cope with GI disorders. It is possible to engineer bacteria to possess more beneficial effects by enhancing particular metabolic activity and end products (Lam et al., 2019). The strategies for precision engineering of host-associated gut microbiota can be grouped into four broad categories: (1) inhibiting metabolic activities carried out by gut bacterial enzymes; (2) removing specific bacterial species or strains; (3)

introducing or engrafting engineered strains into the gut; and (4) directly genetically modifying bacterial cells that are present within the GI tract (Lam et al., 2019). For instance, engineered *Escherichia coli* have been applied to express genes that could complement absent host functions in human metabolic diseases caused by genetic mutations. One such as is phenylketonuria, which is caused by a defect in the human gene encoding phenylalanine hydrolase (Isabella et al., 2018). It has been shown that a modified strain of *Escherichia coli* is able to express proteins involved in phenylalanine degradation induced both aerobically and under anaerobic conditions in the gut of a mouse model of phenylketonuria (Isabella et al., 2018).

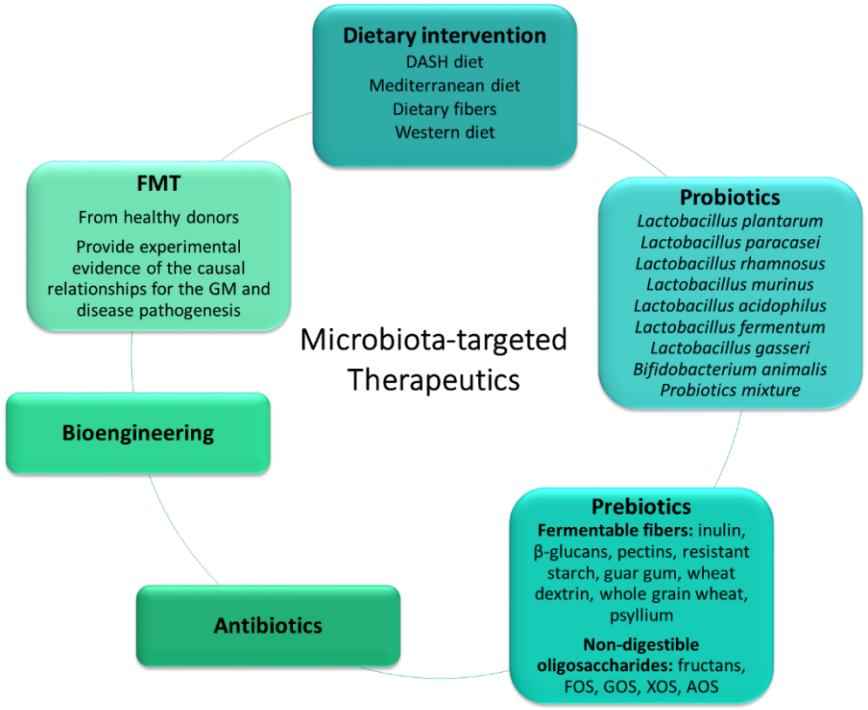
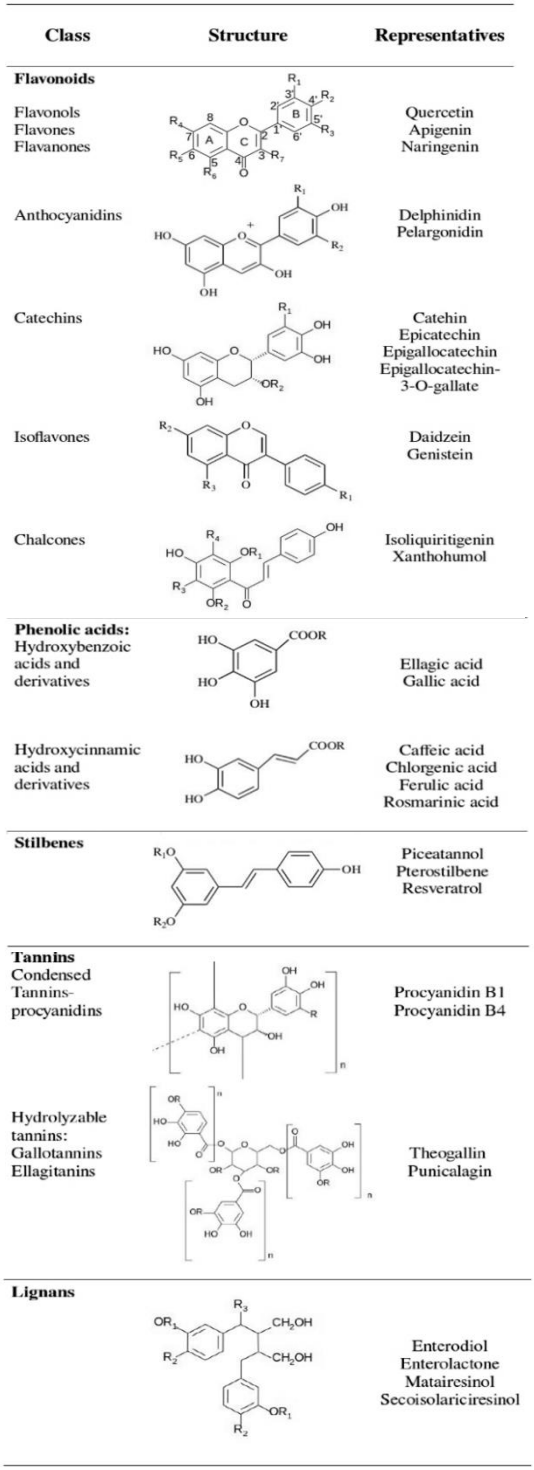


Figure 12 | Potential strategies for modulating the gut microbiota and improving CVDs. Abbreviations: AOS, arabinooligosaccharides; FMT; faecal microbiota transplantation; FOS, fructooligosaccharides; GOS, galactooligosaccharides; XOS, xylooligosaccharides. Adapted from Jin et al. (Jin et al. 2021).

A number of gut microbiota-derived bioactive metabolites have been recognized as microbial biomarkers involved in CVD. The study of the imminent link between microbial composition, their metabolites and CVD risk factors, represent the true exciting and attractive pieces of the puzzle needed for the next stage of this growing field.

Chapter 3. Impact of dietary phenolic compounds on gut microbiota composition

PCs, also named polyphenols, are a large and heterogeneous family of bioactive molecules that are found in a variety of plants and plant-based products, including fruits, seeds, vegetables, olive oil, cocoa products, tea and wine (Pérez-Jiménez et al., 2010). Several preclinical and clinical studies have shown their antioxidant, anti-inflammatory, antidiabetic, anticancer and neuroprotective effects. These effects, suggest a link between PC-rich food consumption and a reduction in the incidence of numerous chronic disorders, indicating that PCs are good candidates for therapeutic/nutraceutical agents (Grosso, 2018; Vauzour et al., 2010). More than 8000 phenolic structures are currently known. Due to their diversity and wide distribution in plants, different classifications have been suggested, according to their source of origin, biological function, and chemical structure. Hydroxylated phenolic rings bounded with sugars such as glucose, galactose, rhamnose, ribulose, arabinopyrinose and arabinofuranose compose their typical chemical structure. The main groups of PCs include flavonoids, phenolic acids, tannins, stilbenes, and lignans (Brglez Mojzer et al., 2016). Phenolic acids (benzoic and cinnamic acid derivatives) and flavonoids are the most abundant PCs in the diet, accounting for 30% and 60%, respectively, of total dietary PCs (***Figure 13***).



Class	Structure	Representatives
Flavonoids		
Flavonols Flavones Flavanones		Quercetin Apigenin Naringenin
Anthocyanidins		Delphinidin Pelargonidin
Catechins		Catehin Epicatechin Epigallocatechin Epigallocatechin-3-O-gallate
Isoflavones		Daidzein Genistein
Chalcones		Isoliquiritigenin Xanthohumol
Phenolic acids: Hydroxybenzoic acids and derivatives		Ellagic acid Gallic acid
Hydroxycinnamic acids and derivatives		Caffeic acid Chlorgenic acid Ferulic acid Rosmarinic acid
Stilbenes		Piceatannol Pterostilbene Resveratrol
Tannins Condensed Tannins-procyanidins		Procyanidin B1 Procyanidin B4
Hydrolyzable tannins: Gallotannins Ellagitanins		Theogallin Punicalagin
Lignans		Enterodiol Enterolactone Matairesinol Secoisolariciresinol

Figure 13 | Phenolic compound classes with their chemical structure and typical representatives.
Source: (Brglez Mojzer et al. 2016).

Inside the human body, the chemical structure of the majority of PCs is received as a xenobiotic, and thus, the bioavailability of these compounds is highly reduced. The unabsorbed PCs accumulate in the large intestine and are exposed to gut microbial enzymatic activities. Therefore, the possible beneficial effects of PCs are partly dependent on their microbiota-derived metabolites, as these are the molecules reported to cross the gut barrier. On the one hand, PCs could modulate gut microbiota composition through a 'prebiotic-like effect', promoting the growth of beneficial bacteria. On the other hand, the microbiota is able to modify PCs, leading to the production of new molecules that can pass through the membrane of the enterocyte and impact human health (Marhuenda-Muñoz et al., 2019). These issues are pivotal for the present thesis, and are described in more detail in the following sections.

3.1 Biotransformation of phenolic compounds by the gut microbiota

To understand PC bioactivity, increased knowledge of the factors affecting their bioavailability and biotrasformation is paramount. Dietary PCs must undergo diverse intestinal transformations, due to the action of both digestive enzymes and the gut microbiota, and the metabolic capacity of microorganisms in the gut has proven greater than that of the host. The level of bioavailability and the level of biotransformation caused by a specific dietary PC along the GI tract is determined by (i) the specific structural subfamily of the PC; (ii) the food matrix (how the PCs are linked to macromolecular food constituents); and (iii) the individual richness at the level of the gut microbiota (Bohn, 2014; Jandhyala et al., 2015). These factors may explain the differences observed for the positive effects of PC consumption reported in epidemiological studies.

Once sugar moieties are cleaved from the phenolic ring in the small intestine, enzymes, such as lactase phlorizin hydrolase (LPH) or β-glucosidase (CBG), hydrolyse glycosylated forms and pass through biotransformation in enterocytes and hepatocytes via phase I (oxidation, reduction and hydrolysis) and especially phase II (conjugation) reactions. These transformations produce a chain of water-soluble conjugated metabolites (glucuronide, sulfate and methyl derivatives) that are readily released in the systemic circulation (Kumar Singh et al., 2019) (***Figure 14***). Treated as xenobiotics, PC metabolites are rapidly removed from the bloodstream into the urine

The absorption of the ingested PCs in the small intestine is very low (approximately 5-10%). Therefore, 90-95% of unabsorbed PCs accumulate in the large intestine for long periods of times and are exposed to gut microbial enzymatic activities. Thus, they are extensively biotransformed by the resident microbiota and consecutively generate low-molecular weight metabolites that are absorbed. This step can enhance PC absorption and bioavailability. The microbiota can hydrolyze glycosides and transform aglycones into new molecules. Their transformation into simpler components involves different reactions, such as ring fission, hydrolysis, demethylation, reduction, decarboxylation, dihydroxylation and isomerization (Mosele et al., 2015).

Some specific biotransformations of PCs can be carried out by diverse gut microbial species and genera (such as deglycosylations), but other more specific chemical reactions require particular species that contain specific genes coding for precise enzymes (such as those responsible for intestinal generation of urolithins or *(S)*-equol from inactive soy isoflavones) (Marín et

al., 2015). Thus, the gut transformation process of PCs highly depends on their recognition by specific enzymes produced by intestinal microbial taxa under eubiosis conditions. This explains in great part the different PC metabolism observed among individuals, since everyone has their own microbiota compositions. Therefore, a similar daily intake of PCs can have different effects on the health of people due to their particular microbial scenario.

Some PCs, such as flavonoids linked to a rhamnose moiety, proanthocyanidins or hydroxycinnamic acids, are resistant to the action of the small intestine enzymes and must reach the colon and be hydrolyzed by enzymes secreted by the colon microbiota, such as *α*-rhamnosidases, in order to proceed to its absorption. The unabsorbed metabolites are eliminated via faeces (***Figure 14***).

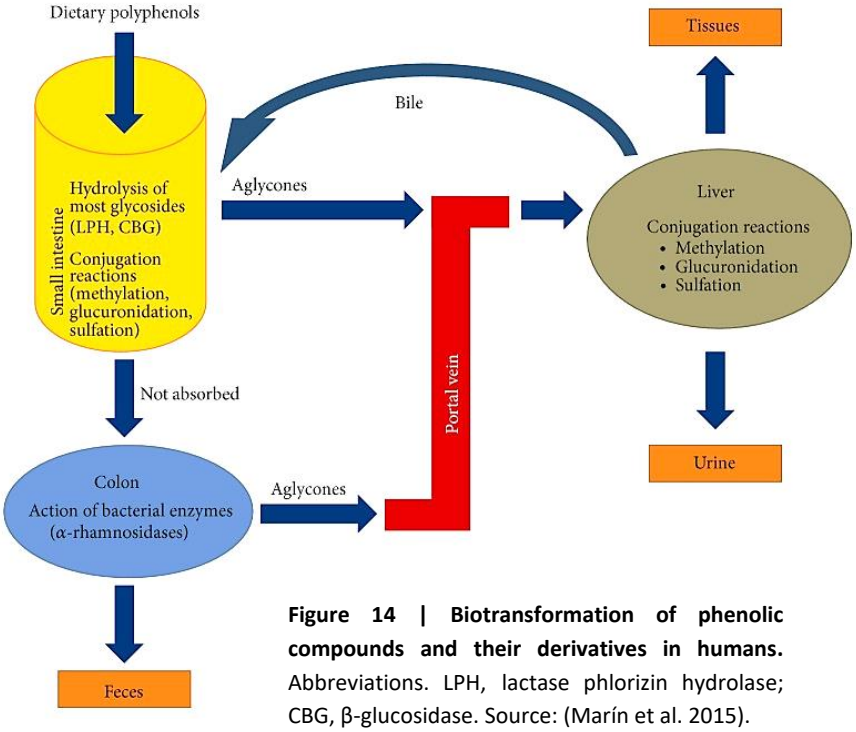


Figure 14 | Biotransformation of phenolic compounds and their derivatives in humans. Abbreviations. LPH, lactase phlorizin hydrolase; CBG, β-glucosidase. Source: (Marín et al. 2015).

Different gut microorganisms are involved in the degradation of particular PCs. At the same time, simpler PC metabolites are produced in the gut with important modulatory effects on gut microbiota composition and function. ***Table 7*** shows a list of dietary PCs present in various food sources, the gut bacteria involved in their transformation, and main metabolites identified.

Phenolic compound	Class	Food source	Gut bacteria	Metabolites identified
Flavonols	Kaempferol Quercetin Myricetin	Onions, capers, apples, broccoli, grapes, plums	*Bacteroides distasonis, Bacteroides uniformis, Clostridium orbiscidens, Enterococcus casseliflavus, Eubacterium ramulus*	2-(3,4-Dihydroxyphenyl) acetic acid 2-(3,5-Dihydroxyphenyl) acetic acid 2-(3-Hydroxyphenyl) acetic acid 2-(4-Hydroxyphenyl) propionic acid 3-(3,4-Dihydroxyphenyl) propionic acid 3-(3-Hydroxyphenyl) propionic acid
Flavanones	Hesperetin Naringenin	Citrus fruits, tomatoes	*Clostridium* spp, *E. ramulus*	(Sayin et al., 2013) 3-(4-Hydroxyphenyl) propionic acid
Flavan-3-ols	Catechin Epicatechin Epigallocatechin	Green tea, cocoa, kola, banana, pomegranate	*Bifidobacterium* spp, *Clostridium coccides*	3-(3-Hydroxyphenyl) propionic acid 3-(3,4-Dihydroxyphenyl)propionic acid 5-(3,4-Dihydroxyphenyl) valeric acid 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone 5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone
Anthocyanins	Cyanidin Peonidin Pelagonidin Malvidin	Bilberries and all red, blue and purple fruits (especially berries)	*Lactobacillus plantarum, Lactobacillius casei, Lactobacillus acidophilus, Bifidobacterium longum Bifidobacterium lactis* BB-12	3,4-Dihydroxybenzoic acid 3-Methoxy4-hydroxybenzoic acid 4-Hydroxybenzoic acid 3,4-Dimethoxybenzoic acid
Isoflavones	Daidzein Genistein Formononetin	Soy, beans, lentils, chickpea	*Lactobacillus* spp, *Clostridium* spp,	(*S*)-Equol O-Demethylangolensin

		(Fabaceae family)	*Bifidobacterium* spp	6'-Hydroxy-O-desmethylangolensin Daidzein
Flavones	Luteolin Apigenin	Cereals, parsley, thyme, celery, citrus fruits	*Clostridium orbiscindens, Enterococcus avium*	3-(3,4-Dihydroxyphenyl)-propionic acid 3-(4-hydroxyphenyl)-propionic acid 3-(3-hydroxyphenyl)-propionic acid 4-hydroxycinnamic acid
Tannins	Ellagitannins Gallotannins	Raspberries, cranberries, strawberries, walnuts, grapes, pomegranate	*Butyrivibrio spp*	Urolithins
Lignans	Secoisolariciresinol Metaresinol Pinoresinol Larciresinol Isolarciresinol Syringiresinol	Flax seeds, cereals, strawberries, apricots	*Bacteroides spp, Clostridium spp, Peptostreptococcus spp, Eubacterium spp*	Enterodiol Enterolactone
Chlorogenic acids	Caffeic acid Ferulic acid	Peach, plums, coffee	*Escherichia coli, Bifidobacterium lactis, Lactobacillus gasseri*	3-Hydroxyphenyl propionic acid 3-(4-Hydroxyphenyl) propionic acid Benzoic acid Vanillin

Table 7 | Main phenolic compounds from different foods, and the gut bacteria involved in their transformation and derived metabolites. Adapted from: Marín et al. and Jandhyala et al. 2015 (Jandhyala et al., 2015; Marín et al., 2015).

3.2 Beneficial effects of phenolic compounds on the gut microbiota community

Recent studies support that dietary PCs reaching the gut microbiota, as well as their derived aromatic metabolites, may modify and produce variations in the gut microbiota community by exhibiting prebiotic-like effects, also known as growth-promoting effects. In fact, the expert consensus document of the International Scientific Association for Probiotics and Prebiotics (ISAPP) stated that, apart from established carbohydrate-based prebiotics,

substances such as PCs might fit the updated definition of prebiotics, as indicated by the convincing weight of evidence in the target host (Gibson et al., 2017). Although the molecular mechanisms of these prebiotic effects have not been fully elucidated, they may be associated with their selective antimicrobial action against pathogenic gut bacteria (Kawabata et al., 2019). Specifically, dietary PCs through their prebiotic action, have shown the ability to modulate gut microbiota composition and function, interfering with bacterial quorum sensing (involving bacterial communication through small signalling molecules), membrane permeability, and sensitization of bacteria to xenobiotics. In addition, they can influence gut metabolism and immunity and exert anti-inflammatory properties (Kumar Singh et al., 2019).

PCs have the ability to bind bacterial cell membranes in a concentration-dependent manner. Therefore, they can alter the functional aspects of the membrane and thus prevent or promote their growth (Kumar Singh et al., 2019). This action is variable in gram-positive and gram-negative bacteria. *In vitro* studies involving faecal samples of healthy volunteers have reported that flavan-3-ol monomers, mainly (+)catechin and (-)epicatechin, may influence the bacterial population in the large intestine (Tzounis et al., 2018). In particular, (+)catechins inhibit the growth of pathogenic *Clostridium* spp. and *Escherichia coli*, while the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. remains comparatively unaltered. In the same way, a human intervention reported that cocoa-derived flavonols stimulate the growth and proliferation of *Lactobacillus* spp. and *Bifidobacterium* spp. with a concomitant decline in the plasma concentration of inflammatory markers such as CRP (Tzounis et al., 2011). Regular consumption of red wine PCs, such as anthocyanins, proanthocyanins and flavonols, has been associated with

reductions in BP, as well as, in plasma triglycerides and LDL cholesterol levels. The reduction in such CVD risk factors might be attributable to PC-mediated induction in the proliferation of *Bacteroides* spp. (Snopek et al., 2018). Also, an intestinal interaction between green tea, fruits and vinegar wine PCs and gut *Bacteroides* spp. has been suggested in obese individuals. In this situation, the weight-reduction activity of PCs could be mediated by the increased glycan-degrading ability of *Bacteroides* spp., that is higher than *Firmicutes* spp. (Rastmanesh, 2011). In human microbiota-associated (HMA) animal studies involving germ-free rodents inoculated with human faecal microbiota, green tea PCs reduced blood glucose levels and lipid metabolism biomarkers. These effects were associated with a decrease in the Firmicutes/Bacteroidetes ratio, which is considered a hallmark of gut dysbiosis (L. Wang et al., 2018). In addition, Zanzer et al., in a crossover RCT, reported that PCs from spices, such as turmeric (curcumin), star anise (quercetin, kaempferol and derivatives), ginger (gingerols and shogaols) and cinnamon (procyanidins, cinnamic acid and derivatives), lowered cardiometabolic risk through gut microbiota modulation (Zanzer et al., 2017).

SCFAs are important metabolites that result from the action of colonic bacteria. Various human and animal studies have shown that dietary PCs improve the health effects of the gut microbiota by activating SCFA production (Kawabata et al., 2019). This is the case for flavonoids and phenolic acids, which promote the growth of SCFA-producing bacteria, including *Bifidobacterium* spp. and *Akkermansia muciniphila*. The intake of grapefruit PCs, containing hesperidin and naringin, apple PCs, containing epicathechin, procyanidin, and chlorogenic acids, and pomegranate ellagitannins, has been shown to increase the total SCFA content in the

caecum of rats and in human faecal batch cultures (Aprikian et al., 2003; Bialonska et al., 2010; Fernandez-Navarro et al., 2018; Fotschki et al., 2016). In addition, pomegranate PCs, mainly punicalagin and ellagic acid, are poorly absorbed in the GI tract. They are further catabolized by the gut microbiota into bioavailable derivatives, such as urolithins, that are subsequently absorbed and distributed into systemic tissues where they can confer vasculoprotective effects, including inhibition of platelet aggregation, oxidative stress, macrophage lipid uptake and LDL oxidation (Fahmy et al., 2020; D. Wang et al., 2018).

Overall, several preclinical and clinical studies have shown the positive prebiotic-like effects of PCs on the gut microbiota community and their association with human health. However, further experimental evidence is still warranted to elucidate the precise molecular mechanisms involved. In addition, the low bioavailability and the inability to efficiently achieve the targets (particular gut bacteria) represents a major drawback for PCs, which compromises their gut and health benefits.

Although recent studies suggest PCs are candidates for prebiotics, the relationship between dietary PCs and the gut microbiota is still obscure and deserves further exploration. Therefore, it is necessary to fully clarify the dual process of microbiota-dependent catabolism of PCs as well as the prebiotic actions of PCs in the large intestine. Efficient use of PCs will help target the gut microbiota early to cope with dysbiotic phenotypes linked to CVDs.

Chapter 4. Novel biomarkers for the early detection of cardiovascular disease risk

4.1 Defining biomarkers

Throughout history, humans have looked for signals that allow for the identification of states of disease to be able to act against them in an effective manner. Initially, these signals were limited to externally feasible measurements of the physiological state, such as temperature control. In the last century, the term “biomarker”, also known as “biological marker”, was introduced to refer to molecules determined by biochemical analysis, which are objectively evaluated and could be related to a current alteration of a biological system (Strimbu & Tavel, 2010).

Many definitions exist for the term biomarker, depending on specific branches of science, e.g., medical therapeutics or nutrition (Gao et al., 2017). Despite this, the most common definition is the one proposed by the US National Institutes of Health Committee that defines biomarkers as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (Biomarkers Definitions Working Group, 2001). This definition includes not only circulating molecular biomarkers (in biological specimens) but also genetic, tissue, and cellular markers, imaging results, and physical and electronic measurements. However, in the field of nutrition, a biomarker has been traditionally defined as ‘a biochemical indicator of dietary intake/nutritional status (recent or long term), or an index of nutrient metabolism, or a marker of the biological

consequences of dietary intake' (Potischman & Freudenheim, 2003). Biomarkers of dietary intake can more accurately assess nutritional intake or status versus self-reported methods (Picó et al., 2019).

Recently, to understand the complex relationships between nutrition and health, different types of biomarkers have been used in nutritional studies. In particular, biomarkers of health/disease state can reflect the different intermediate disease phenotypes or the severity of disease (Picó et al., 2019). Thus, they could have promising applicability for CVD risk detection.

As mentioned in *Chapter 1*, the identification of classical biomarkers for CVDs, such as high LDL cholesterol levels, is not enough to detect all patients at risk for CVD. Certainly, these biomarkers may not be helpful in patients with moderate or unusual CVD. For this reason, there is a need for the identification of novel biomarkers that could assess CVD progression at the early stages of their pathophysiological process. The identification of such novel biomarkers of early disease would ease the implementation of strategies to avoid the continuous advancement of CVDs. Thus, they would have a high impact on clinical diagnosis and allow for the optimization of the resources needed once the disease has developed (Ali et al., 2016).

There are large numbers of emerging novel biomarkers related to various pathophysiological processes associated with CVDs. Such biomarkers can represent the inflammatory process, such as CRP, interleukins, fibrinogen and MPO, the destabilization of atherosclerotic plaques, such as Ox-LDL, or thrombocyte activation, such as Lp-PLA2. In addition, biomarkers of inflammation, endothelial dysfunction and oxidative stress are involved in hypertension, as previously described in *Chapter 1*. Overall, the evaluation of

these emerging biomarkers can provide additional information beyond the traditional risk factors for CVD when used in conjunction with other clinical parameters, such as medical history, physical examination, and laboratory and radiographic measures.

Technological advances in omics sciences have made it possible the to identify putative CVD risk biomarkers with potential clinical value that may be informative concerning the early stages of CVDs. This topic will be further introduced in *Chapter 5*.

4.2 Classification of health/disease and dietary biomarkers

Biomarkers comprise a broad subcategory of quantifiable and reproducible characteristics of biological signs. Useful disease biomarkers must meet a series of standards to be potentially used in routine clinical diagnosis. These standards include: (1) sensitivity: that is, the ability to detect a disease or alteration in patients whom the disease is present; (2) specificity: that is, the ability to rule out the disease in patients in whom the disease is truly absent; (3) accuracy: that is, the ability to identify individuals at risk as a relation between clinical sensitivity and specificity; (4) reliability: that is, the stability of results when repeated; and (5) therapeutic impact with early intervention (Califf, 2018). Furthermore, it is important that the biomarkers be easily interpreted by clinicians and could be measured in noninvasive (urine, saliva, faeces) or minimally invasive samples (plasma or serum). Other factors, such as age, sex, and ethnicity, may be of interest depending on the purpose of use for the biomarkers.

There are several relevant hierarchical steps in demonstrating the clinical interest of a novel biomarker, especially of a CVD biomarker (Lyngbakken et al., 2019):

- Demonstrate that the biomarker is significantly modified in diseased patients as compared to control.
- Assess their diagnostic properties.
- Compare their diagnostic properties to existing tests.
- Demonstrate that the diagnostic properties of the biomarker increase the ability of the clinician to make a decision.
- Assess their usefulness, which should be clearly distinguished to the quality of diagnostic information provided. It involves both characteristics of the test itself, such as cost, and characteristics of the clinical context, such as prevalence of the disease or consequences of outcome.
- Demonstrate that the measurement of the biomarker modifies outcome (intervention studies).

For all stages of this process, it is important to concomitantly understand the pathophysiologic mechanisms involved in biomarker's synthesis, production, its kinetic properties, and its physiologic effects.

Several frameworks for classifying health/disease and dietary biomarkers exist (Gao et al., 2017; Picó et al., 2019). One of these frameworks is the overall division into exposure, effect and susceptibility biomarkers, which also include different subclasses of biomarkers (***Figure 15***).

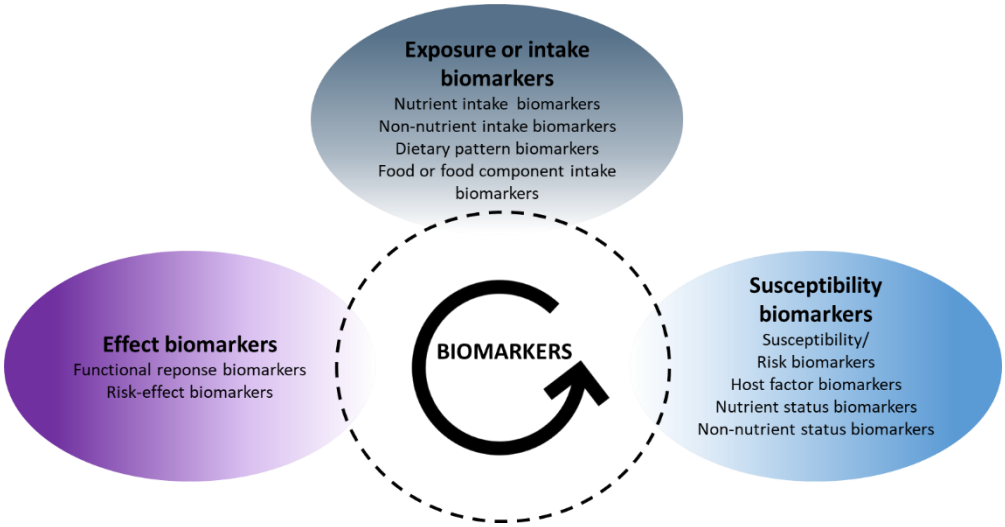


Figure 15 | Classification of health/disease and dietary biomarkers within the space shaped by three hypercategories: exposure, effect and susceptibility. Adapted from Gao et al., 2017 (Gao et al. 2017).

I. **Exposure or intake biomarkers** reflect the level of extrinsic variables that humans are exposed to, such as diets and food compounds, including nutrients, non-nutrients and dietary patterns. Depending on the complexity of what the marker represents in the diet they can be divided into three subclasses: food compound intake biomarkers (FCIBs), where a clear example could be urinary PCs excretion as a biomarker of exposure to dietary intake of PCs; food intake biomarkers (FIBs), that measure the exposure to specific food groups or foods; nutrient intake biomarkers (NIBs), which represent specific nutrient intakes; non-nutrient intake biomarkers (NoNIBs), such as biomarkers of zeaxanthin or organophosphorus pesticide intake as indicators of specific toxics in the diet; and dietary pattern biomarkers (DPBs), which include a set of FIBs and FCIBs that reflect the average diet of an individual (***Table 8***) (Gao et al., 2017). These types of biomarkers are of great interest, as their use

can help to improve the categorization of subjects according to the exposure to a particular nutrient.

Biomarker subclass	Typical uses
FCIBs **NIBs** **NoNIBs**	- Specific intake biomarkers for chemically well-defined food compounds - Intake biomarkers and exposure biomarkers reflecting acute or long-term intakes
FIBs	- Compliance biomarkers - Markers of exposure to food compounds - Biomarkers of dietary exposure - Food intake biomarkers
DPBs	- Representation of 'signal' foods and nutrients in diet (Nutritype biomarkers) - Compliance biomarkers - Biomarkers of dietary exposure

Table 8 | Exposure or intake biomarker classification. Abbreviations: DPBs, dietary pattern biomarkers; FCIBs, food compound intake biomarkers; FIBs, food intake biomarkers; NoNIBs, non-nutrient intake biomarkers; NIBs, nutrient intake biomarkers. Adapted from: Gao et al., 2017 (Gao et al., 2017).

II. **Effect biomarkers** refer to the functional response of the human body to an exposure. They monitor changes in biochemical, physiological and psychological state, i.e. changes in plasma glucose or BP in response to specific dietary exposure. These biomarkers can be divided into (i) those indirectly associated with risk, i.e. functional response biomarkers, and (ii) those directly related to risk, i.e. risk-effect biomarkers, which usually have a recognized cause and effect relationship to disease. A dynamic change in these biomarkers represents an effect as a response to a challenge, a dietary change, a medical treatment, etc. Thus, they are broadly seen in intervention trials, where are identified as surrogate markers of the potential to alter a certain disease risk (***Table 9***) (Gao et al., 2017).

Biomarker subclass	Typical uses
Functional response	- Outcome biomarkers
Risk-effect	- Efficacy biomarkers
	- Impact biomarkers
	- Markers of target function/biological response

Table 9 | Effect biomarker classification. Adapted from: Gao et al., 2017 (Gao et al., 2017).

III. **Susceptibility biomarkers** represent the individual susceptibility or resilience to an exposure predicting the intensity of its effect on the individual. Susceptibility may be seen as the 'background health status', i.e. the sum of intrinsic or 'host' factors explaining current individual or collective health-related risks (***Table 10***) (Gao et al., 2017). In this category, host factor biomarkers and susceptibility/risk biomarkers both represent major subdivisions, followed by food compound status biomarkers, such as nutrient and non-nutrient status biomarkers, which reflect the status for retained nutrients from foods or cumulative intake of other food compounds. While host factors are static and cannot be used to predict risk, i.e. genotypes, susceptibility/risk biomarkers can be used to predict specific aspects of an individual's disease risk or development in preliminary stages when there is still no clinically apparent disease or altered condition (Califf, 2018). Susceptibility/risk biomarkers can be graded on a scale from altered susceptibility to disease diagnostic and prognostic. For instance, SBP, LDL cholesterol or fasting glucose levels in circulation have well-described relationships with risk of disease, and are supported by international guidelines as risk/diagnostic or predictive biomarkers (Califf, 2018; Flack & Adekola, 2020). In addition, susceptibility/risk biomarkers are most often measured in a cross-sectional setting, in order to characterize risk at

baseline in a population (Gao et al., 2017). Thus, these kinds of biomarkers are of great interest for the aim of the present thesis.

Biomarker subclass	Typical uses
Host factor	- Disease risk biomarkers
Susceptibility/Risk	- Health state biomarkers
	- Individual variability biomarkers
	- Biomarkers of phenotypic trait
	- Biomarkers of health/disease
NSBs	- Chronic exposure markers
NoNSBs	- Biomarkers of nutritional status
	- Biomarkers of non-nutrient status or susceptibility
	- Biomarkers of body burden of toxicants

Table 10 | Susceptibility biomarker classification. Abbreviations: NoNSBs, non-nutrient status biomarkers; NSBs, nutrients status biomarkers. Adapted from: Gao et al., 2017 (Gao et al., 2017).

Given the complexity of interacting processes affecting a biological system, which is a dynamic system, biomarkers can capture the state of all the on-going processes and changing balances, thereby giving a full characterization of the current state of the system, including health dynamics and disease risks. Therefore, considering interactions between individual and its environmental exposures, susceptibility and effect has led to a more flexible and less ambiguous classification scheme for biomarkers (Gao et al., 2017). In addition, as the main objective of diet and nutrition is to promote and maintain optimal health, it is highly relevant to have biomarkers of very early stages of alterations that may ultimately progress to disease. Such biomarkers can be considered health and/or prevention markers rather than disease markers (Picó et al., 2019). Biomarkers of health/prevention can guide policies related to food, nutrition and health. In this sense, at present, several projects such as the *BIOCLAIMS* project (FP7-244995); a collaborative research project at the European level, have developed new biomarkers by

exploiting this new concept of biomarkers through quantification of the robustness of the homeostatic mechanisms involved in maintaining optimal health and disease prevention (Bioclaims Group, 2015). Part of the cross-sectional results presented in this thesis have emerged from the *BIOCLAIMS* project, therefore, further description is provided in the Methods and Results section (Part II).

4.3 Cross-sectional studies in novel biomarker research

The process of searching for novel biomarkers is not simple. Once putative molecules are identified, it is necessary to extensively explore the metabolic pathways and biological functions in which these molecules are involved prior to confirming their adequacy as biomarkers. Furthermore, it is necessary to validate its suitability in different models and experiments. In this context, the Institute of Medicine (US) Committee on Qualification of Biomarkers and Surrogate Endpoints in Chronic Disease (IOM, 2010) indicates a need for a unified and transparent process for the evaluation and adoption of biomarkers.

The process of evaluation requires a set of specific and interdependent steps (***Figure 16***): (1) **analytical validation** of relevant biomarkers tests that includes the biomarker's measurability and the test's sensitivity for the biomarker, biomarker specificity, reliability, and lab-to-lab reproducibility; (2) **qualification**, a description of the evidence relating to the biomarker in question and its link with disease pathway, and the clinical endpoint; and (3) **utilization**, the applicability of results from the analytical validation and the description of the evidence to the proposed use of the biomarker given the evidence assessment and proposed purpose and context of use, i.e. risk

stratification, prevention, prediction, screening, diagnosis, prognosis, patient selection or therapeutic monitoring (IOM, 2010).

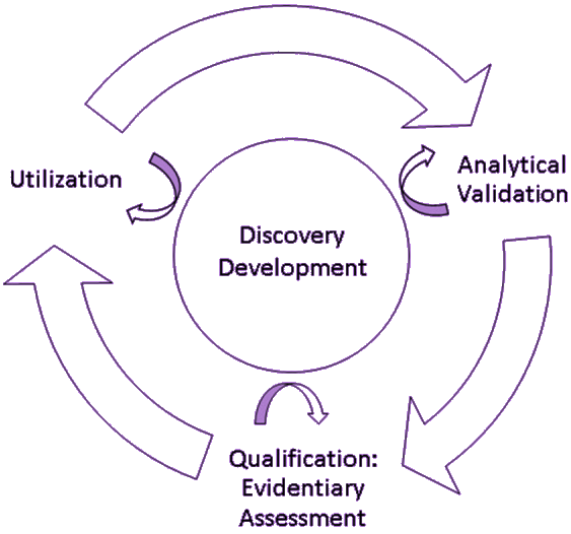


Figure 16 | Steps in the evaluation framework for biomarkers. Adapted from *Institute of Medicine. Evaluation of biomarkers and surrogate endpoints in chronic disease,* (IOM 2010).

While many of the new molecule discoveries are generated in experimental and laboratory research, observational epidemiological studies greatly contribute to exploring the relevance of identified biomarkers in humans (Aleksandrova et al., 2014; Lüdicke et al., 2015; Veugen et al., 2018). Among different study designs in epidemiology, cross-sectional studies have gained special interest in employing biomarker data due to their high feasibility (García-Closas et al., 2011). Such studies are relatively easy, fast and cheap to conduct and provide helpful information for first hypothesis generation. In cross-sectional studies both exposures and health outcome are measured simultaneously at the same point in time (***Figure 17***).

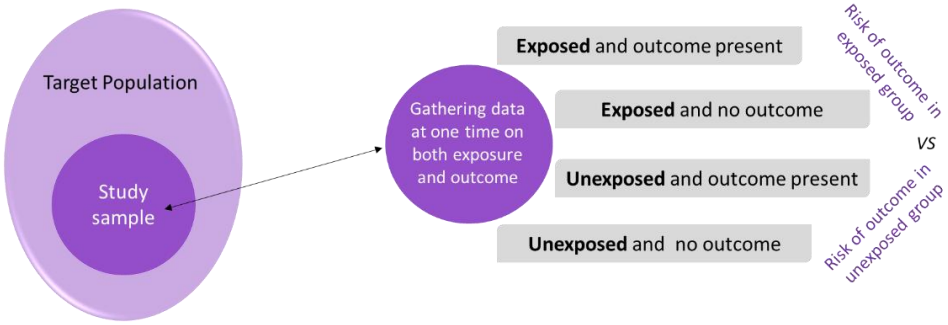


Figure 17 | A schematic representation of a typical cross-sectional study design. Data are collected on both outcomes and exposures of the individuals at a given point in time. Own source.

As such, they provide a 'snapshot' of the population status with respect to exposure variables and intermediate endpoints and, in some instances, disease at a specific moment (García-Closas et al., 2011).

Usually, cross-sectional investigations are performed on healthy subjects exposed to particular exogenous/endogenous factors where the biomarker is treated as the outcome variable. Thus, this observational design has been successfully applied to:

- answer questions whether or not a given population has been exposed to a particular compound;
- evaluate intermediate biologic effects from a wide range of exposures in the environment and diet, for example effects of saturated fats intake on LDL cholesterol levels, where LDL cholesterol is predictive of CVDs occurrence;
- evaluate whether or not there are early biologic perturbations caused by new exposures or recent short-term changes in lifestyle factors to have been evaluated for their associations with disease;

- formulate new hypotheses about observed changes or relationships in exposure and/or intermediate endpoints to be subsequently tested in interventional studies.

However, as previously mentioned, the interpretation and therefore the usefulness of cross-sectional studies heavily depend on the validity of the markers measured. Even though no inferences on causality can be made, cross-sectional studies have proven useful in gaining insights into potential associations between biomarkers and other factors (Wirsching et al., 2018). This is the starting point of the research presented in this thesis.

In recent years, the development of health/disease biomarkers has been driven by medical needs and has largely been focused on identifying and quantifying disease states or progression rather than assessing health status. However, the main objective of diet and nutrition is to promote and maintain optimal health. Therefore, it is of special interest to have biomarkers of the very early stages of alterations that may ultimately progress to disease, particularly in the onset of CVDs, and it is important know how diet interacts with these biomarkers. Such biomarkers could be considered health and/or prevention markers rather than disease markers.

Chapter 5. Application of omic sciences in the discovery of novel biomarkers

A major goal of biomedical research is to identify accurate early indicators of disease. Over the past decades, the application of novel powerful technology platforms, known as omic sciences or "omics", has opened new avenues in biomarker research. Rather than focusing research efforts on individual molecules, pathways, or specific cells, new technologies have helped decipher the genetic code of numerous organisms, including humans (Olivier et al., 2019). A large range of omics technologies have been developed, with 'omics' suffixes referring to the comprehensive study of the roles, relationships, and actions of various types of molecules in cells of an organism (Olivier et al., 2019). This includes platforms such as genomics (focused on the structure, function, evolution, mapping and editing of genes); transcriptomics (the study of the expression of all genes in a cell or organism); proteomics (the analysis of all proteins); metabolomics (the comprehensive analysis of all small molecules); and epigenomics (the study of the epigenetic regulation of the entire genome) (Olivier et al., 2019). In addition, it has also yielded a plethora of other particular 'omics' subfields, including lipidomics (K. Yang & Han, 2016) and metagenomics (W.-L. Wang et al., 2015), which have been usual methods of analysis for the present thesis, and are detailed in the following sections.

The integrative use of multiple omics platforms allows the application of 'multiomics' approaches for precision medicine (Misra et al., 2018). These system approaches allow obtainingcan provide a comprehensive and in-depth view of the physiology and pathology of an individual. They can also

expand the possibility to explore the complex interactions between nutrition and health, particularly to investigate the role of dietary components in health maintenance or in disease development (Fitó et al., 2016). In this way, the application of multiomics along with new bioinformatics tools provides new clues in studies analysing nutrition and CVDs to understand the mechanisms and pathways driving CVDs and related gene-diet interactions (Corella & Ordovás, 2014). Moreover, a deeper integration of omics will allow us to identify a better list of biomarkers that are useful for the prediction of CVDs (Misra et al., 2018) (***Figure 18***).

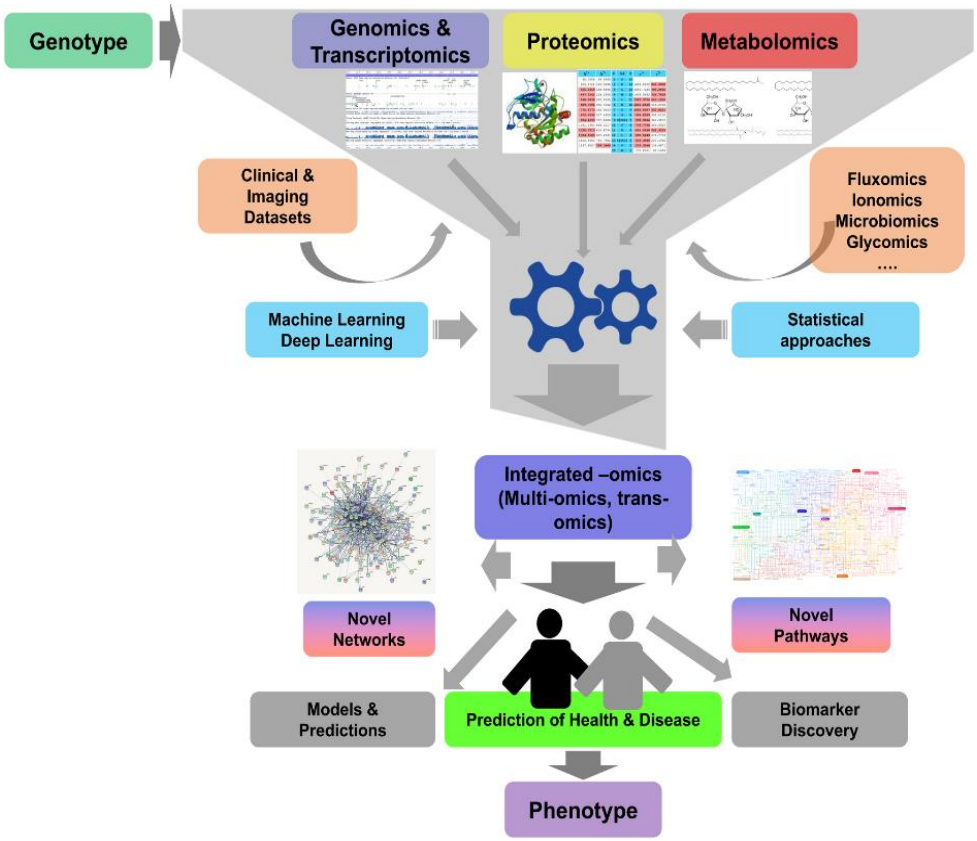


Figure 18 | A typical integrated omics workflow showing input datasets, output datasets and results of novel biomarker discovery. Source: (Misra et al. 2018).

5.1 Metabolomics approach

Metabolomics, the analysis of small molecules or metabolites (with a molecular weight lower than 2,000 Dalton) present in a cell, tissue, or fluid, has been the focus of biomarker discovery studies for a long time. This is because metabolomics is at the last level of "omics cascade" and in consequence is very dynamic and very influenced by the changes that suffers an organism in a determined moment (Newgard, 2017; Patti et al., 2012). The broad metabolite profiles (metabolome) can provide an overview of the metabolism with a level of description that exceeds genetic information and more closely reflects the ultimate phenotype, thus helping to connect genotype to phenotype at the molecular level (Patti et al., 2012). If mechanistically proven, changes in the metabolome may be used to improve disease risk estimates in epidemiological studies. Several authors have claimed that, combining different molecular biomarkers together will help to obtain improved accuracy in the risk prediction, and metabolomics will play a crucial role (Xia et al., 2013). In addition, metabolomics analysis have allowed the identification of food components and their metabolites in biological fluids (Brennan & Hu, 2019).

The main analytical techniques that are used in the omics field are NMR and MS coupled to either liquid or gas chromatography (LC or GC, respectively). The success of these techniques relies in their sensitivity and selectivity, especially in MS which has shown great adequacy to be used in complex samples (X. Zhang et al., 2020).

In metabolomics, targeted and untargeted approaches can be carried out depending on the intended purpose:

- **Targeted metabolomics** is used when only a defined set of known metabolites with similar structures is analyzed. This means that targets of the study are known and, therefore, the analysis will be directed to their detection. It is generally a quantitative tool able to answer specific biochemical questions or hypothesis that motive the investigation of one or more related pathways (Patti et al., 2012). This modality of metabolomics allows high sensitivity and specificity of the measurements, and can typically detect up to 100-200 metabolites at a time. Usually is conducted by using NMR or LC coupled to tandem mass spectrometer detector (LC-MS/MS) such as the triple quadrupole mass detector (QqQ), which perform multiple ionizations of metabolites generating fragments of known weight. Hence, it is possible to quantify metabolites just by monitoring their generated fragments (***Figure 19***).
- **Untargeted metabolomics**, also referred to as "shotgun metabolomics", is focused in the unbiased screening of metabolites in biological specimens and is generally used for global metabolite profiling with the intention of comparing patterns of metabolites among different groups. Therefore, it is capable to identify thousands of spectral features in a biological sample (typically measuring >1000 metabolites at a time) (X. Zhang et al., 2020). It is not a hypothesis-driven but a hypothesis generating approach. In this case, analyses are usually performed by LC coupled to a time-of-flight detector (LC-Q-TOF) and then, the metabolites significantly differing among groups are selected as putative biomarkers thus allowing to generate a hypothesis of the metabolic pathways and processes mediating different effects among samples (Naz et al., 2014) (***Figure 19***).

Accordingly, while untargeted metabolomics is usually used in the first step of novel biomarker discovery, targeted approaches are frequently used in the clinical diagnosis step, when the biomarkers are already established (***Figure 19***).

Several studies have applied these metabolomics approaches in order to identify novel early biomarkers for CVD risk prediction in human. Shan et al. used targeted LC-MS/MS to identify and quantify 69 metabolites in individuals with angiographic CAD, revealing higher levels of BCAAs in association with CAD (Shah et al., 2010). In addition, Wang et al. by using untargeted LC-MS were able to identify thousands of metabolite features in a cross-sectional study of CVD subjects, where the metabolite TMAO was observed to be highly associated with CAD risk after adjusting for several clinical risk factors (Z. Wang et al., 2011). Also, metabolomics has allowed the identification of lipid metabolites. For instance, Würtz et al., in a multi-cohort epidemiological study, by performing high-throughput NMR, identified 68 abundant plasma metabolites of which phenylalanine and MUFA levels were associated with increased CVD risk, whereas higher concentrations of n-6 PUFAs and docosahexaenoic acid (DHA) were associated with reduced CVD risk (Würtz et al., 2015). Thus, these studies highlight the potential for generalizability and validity of metabolomics observations in the search of novel early biomarkers.

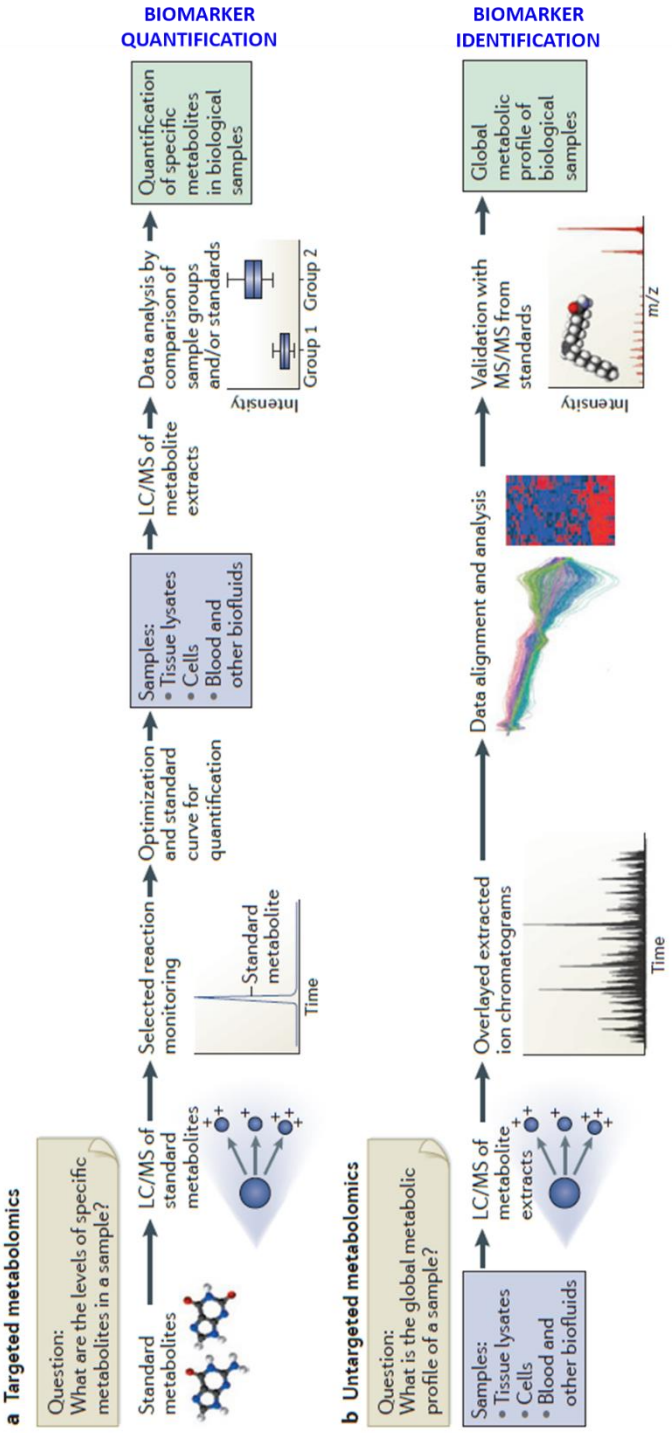


Figure 19 | Targeted and untargeted workflow for LC/MS-based metabolomics. Source: Adapted from Patti et al. (Patti et al. 2012).

5.1.1 Lipidomics in the identification of lipid-related biomarkers

Lipidomics is a subfield of metabolomics that focuses on the global study of the lipidome (Hinterwirth et al., 2014). The lipidome comprise a subgroup of the metabolome, but analytical approaches typically used differs significantly from methods established in metabolomics. The huge diversity of native lipid molecules and their modifications make lipidomic analyses challenging. In last years, lipidomics has been used for lipid-effect analysis (Rombaldova et al., 2017) but also to monitor dietary exposure (O'Gorman et al., 2017) and the relationships between food intake and health parameters (Kuang et al., 2018). Moreover, it has provided insights into metabolic pathways by which food exposure may exert its health effects (Kuang et al., 2018).

The majority of identified lipid molecules contains polar/ionic head groups and nonpolar fatty acyl chain(s), e.g., phospholipids (PLs) and sphingolipids (SLs), which results in the formation of amphiphilic molecules with particular physicochemical properties (Holčapek et al., 2018). According to the Lipid MAPS database, the lipid classification system is comprised of eight categories of representative lipid structures (Fahy et al., 2009) (***Figure 20***).

The main analytical approaches in the lipidomic research are direct infusion MS analysis (also known as shotgun lipidomics), liquid-phase separations coupled to MS (typically LC-MS), and desorption ionization techniques MS approaches (often used for mass spectrometry imaging, MSI) (Holčapek et al., 2018). In last years, the electrospray ionization (ESI)-MS has been widely used as an established ionization technique for medium polar to ionic lipids and also applicable for nonpolar lipid (sub)classes (Holčapek et al., 2018). It allows the quantification of low abundant and isomeric lipid forms.

Moreover, this technique has significant advantages over other techniques, such as excellent sensitivity and easy coupling with liquid-phase separation techniques.

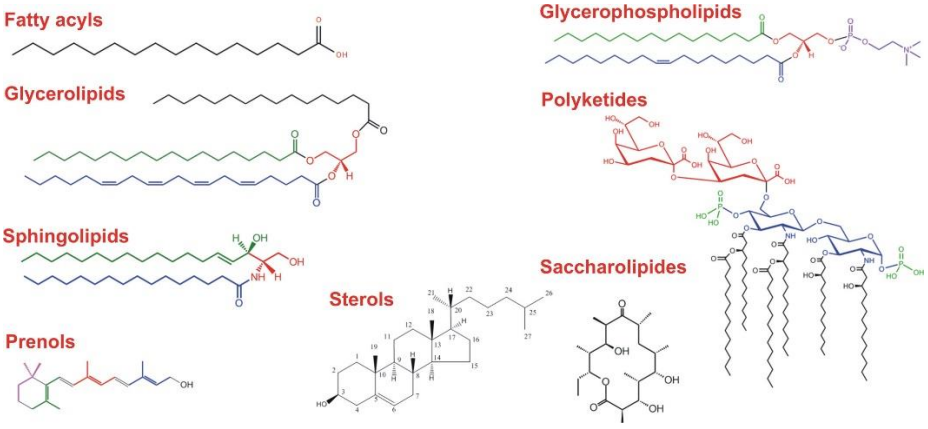


Figure 20 | Lipid categories according to Lipid MAPS classification with representative structures for individual categories. Source: (Holčapek, et al., 2018).

Glycerophospholipids, are one of the major components of cellular membranes which are synthetized from glycerol-3-phosphate (G3P) in a de novo pathway that initially produces phosphatidic acid (PA) and diacylglycerol (DAG) or cytidine diphosphate-DAG (CDP-DAG) (Hishikawa et al., 2014). Through this de novo pathway, a set of different types of glycerophospholipids with different polar heads at the sn-3 position of the glycerol backbone, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) or phosphatidylserine (PS), are generated (Hishikawa et al., 2014). Then, the glycerophospholipid acyl chains are remodeled by subsequent enzymatic acylation reactions, mainly mediated by phospholipases (PLAs), acyl-CoA synthases, transacylases, and lysophospholipid acyltransferases (LPLATs) to conform lysoglycerophospholipids or lyso-PLs (Hishikawa et al., 2014) (***Figure***

21). This class of hydrolyzed lipids, including lysophosphatidylcholine (lyso-PC), lysophosphatiylethanolamine (Lyso-PE) and lysophosphatidylinositol (Lyso-PI), among others, are commonly found in blood and acts as bioactive molecules involved in a broad range of physiological and pathological processes in humans (Tan et al., 2020). Moreover, lyso-PLs, mainly lyso-PC, have recently shown potential as mediators in CVD development in animal models (Kleger et al., 2011; Suárez-García et al., 2017), and as potent pro-inflammatory and deleterious mediators of atherosclerosis process in humans (Knuplez & Marsche, 2020). Contrary, an increasing number of recent studies suggest beneficial properties towards inflammation in the vascular system under various pathological conditions (Knuplez & Marsche, 2020). On the other hand, scarce research suggest that lyso-PLs chain length and the degree of saturation of the fatty acyl moiety, at either sn-1 or sn-2 position, could modulate the direction of the inflammatory response (Akerele & Cheema, 2015). In fact, (n)-PUFAs at the sn-1 position of lyso-PC could modulate the inflammatory response thereby making this bioactive lipid less atherogenic (Akerele & Cheema, 2015). Despite this, the extent to which dietary FAs influence lyso-PLs metabolic profile is unknown.

Therefore, future lipidomic research should deep-in the pathways by means these bioactive lipids are modulated with special attention on the complex interactions with dietary FAs. The knowledge on how diet affect lyso-PLs fatty acyl moieties, and how this structural reconfiguration conditions their intermediate role in CVDs, may provide novel early lipid-related biomarkers for the risk of CVDs. Also, it will allow designing dietary based therapeutic strategies for the prevention and management of CVDs.

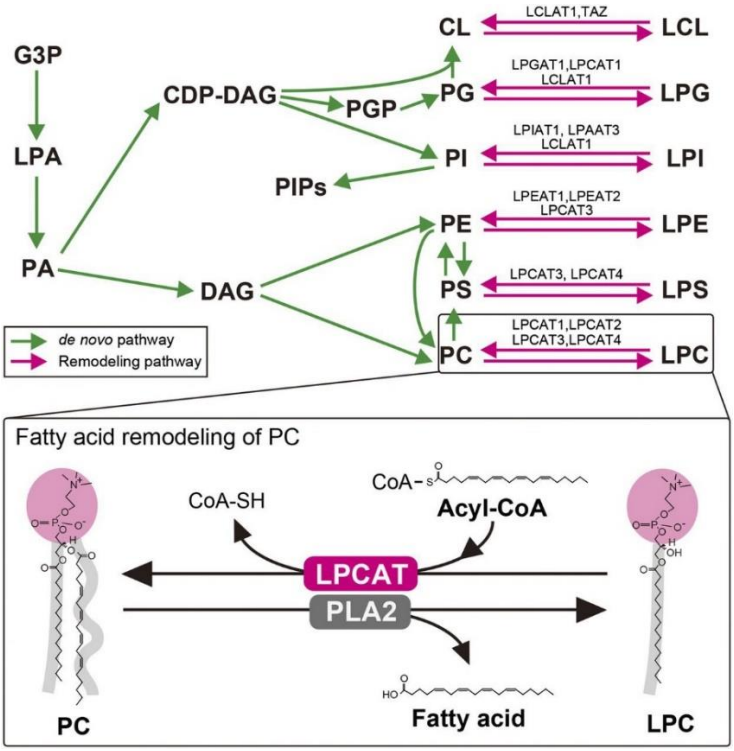


Figure 21 | Biosynthetic pathways of glycerophospholipids. Upper panel shows the de novo synthesis (green lines) and the fatty acid remodeling (magenta lines) of glycerophospholipids. Lower panel shows an example of the fatty acid remodeling of PC. In this reaction, PLA2s release fatty acid (arachidonic acid) from the *sn*-2 position of PC, while LPCATs catalyze the reacylation at the *sn*-2 position of LPC using acyl-CoA (arachidonoyl-CoA). CDP-DAG, cytidine diphosphate-diacylglycerol; CL, cardiolipin; DAG, diacylglycerol; G3P, glycerol-3-phosphate; LCL, lysocardiolipin; LCLAT, lysocardiolipin acyltransferase; LPA; lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; LPC, LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase, LPE, lysophosphatidylethanolamine; LPEAT, lysophosphatidylethanolamine acyltransferase; LPG, lysophosphatidylglycerol; LPGAT, lysophosphatidykgkycerol acyltransferase; LPI, lysophosphatidylinositol; LPIAT, lysophosphatidylinositol acyltransferase; LPS; lysophosphatidylserine; LPSAT, lysophosphatidylserine acyltransferase: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PI, phosphatidylinositol; PS, phosphatidylserine. Source: (Hishikawa et al., 2014).

5.2 Gut metagenomics approach

Gut metagenomics is a subfield of environmental genomics that has emerged as a next-generation sequencing technology from the application of molecular biologic technology in the study of the gut microbiome. It was first described in 1998 by Handelsman and Rondon, and became a novel DNA sequencing approach to study the complex gut microbial community (Handelsman et al., 1998). Metagenomics can be used to study intestinal microbiome diversity and dysbiosis, as well as its relationships to health and disease. Moreover, it enables a more comprehensive understanding of the structure, function, genes and pathways of microbial communities than traditional culture methods (W.-L. Wang et al., 2015). The typical metagenomics sequencing process start from the extraction of total DNA of all microorganisms in faecal samples. Once DNA is extracted, two different metagenomics approaches can be used for decipher microbial community composition and function (***Figure 22***):

I. ***16S-based approach.*** Is based on the amplification and sequenciation of the 16S ribosomal RNA (16S rRNA) gene, which is a marker gene with well characterized and high conserved, ubiquitous sequences with better taxonomic resolution (Johnson et al., 2019). 16S rRNA gene sequencing allows the identification of similar sequences, which are assembled into Operational Taxonomic Units (OTUs), or Amplicon Sequence Variants (ASVs). Then, these grouped sequences are compared to reference 16S databases to identify them as precisely as possible. The community composition can be described in terms of which OTUs are present, their relative abundance, and/or their phylogenetic relationships.

II. ***Shotgun metagenomics approach.*** Is based on the direct metagenomic sequencing of the community total DNA. Before being sequenced, total DNA samples are randomly sheared by a "shotgun" approach. Then, the shotgun sequence reads are filtered to obtain high-quality sequences for the whole genomic profile by metagenomics. The filtered sequences are then assembled to form longer genomic sequence contigs. Computational methods are needed to code sequences into contigs. Finally, sequence contigs are compared to reference genomes or gene catalogs which allow better observation of single nucleotide polymorphisms (SNPs) and other variant sequences. The functional capabilities of the community are determined by comparing the sequences to functional databases (e.g. KEGG or SEED) (Kanehisa et al., 2008). Through this approach the community can be described as relative abundances of its genes and pathways.

These gut metagenomics approaches provide a wide range of applications in different research areas: the detection of microbial composition and diversity, novel genes, microbial pathways, functional dysbiosis of the intestinal microbiome, antibiotic resistance genes, and the determination of interactions and co-evolution between microbiota and host (W.-L. Wang et al., 2015). Moreover, they could assist in the discovery of novel gut microbiota-based biomarkers.

However, despite the power of metagenomics in describe the genetic potential of the microorganisms present in a sample, it has a very limited function in revealing their activity or gene expression (W.-L. Wang et al., 2015). In this context, novel complementary 'omics' have appeared to solve the gut metagenomics weakness. This is the case of metatranscriptomics

(Gosalbes et al., 2011), metaproteomics (Kolmeder et al., 2012) and metabolomics (Shaffer et al., 2017), which allow the determination of the activity of genes in a defined environment (***Figure 22***).

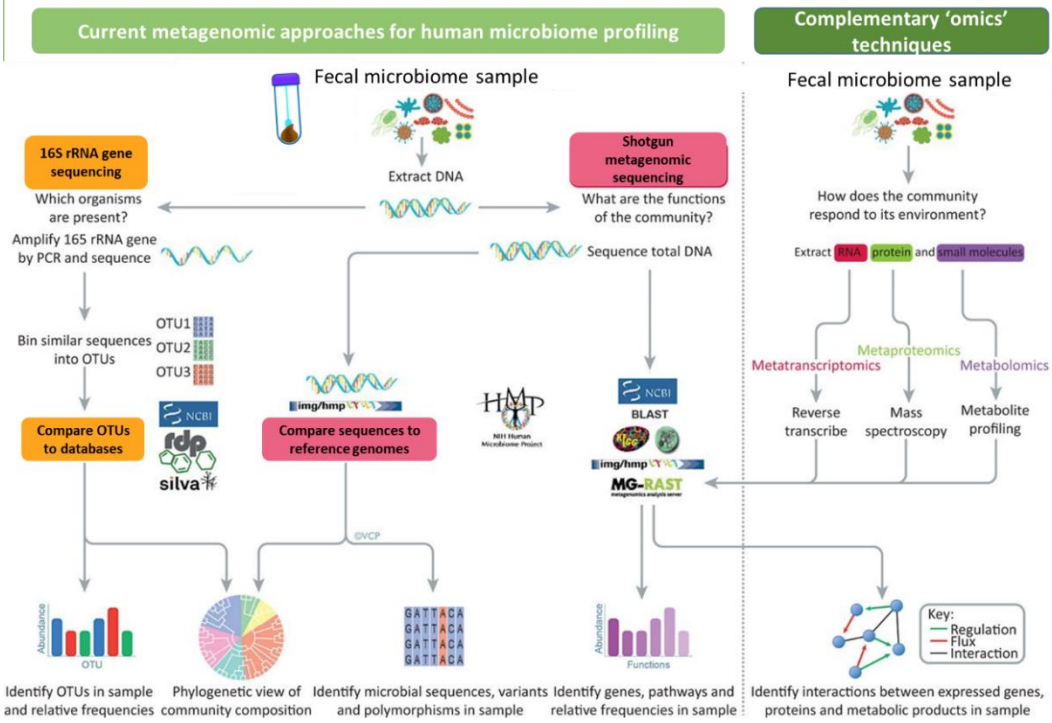


Figure 22 | Overview of next-generation gut metagenomics approaches and bioinformatics methods for human microbiome profiling. Abbreviations: DNA, deoxyribonucleic acid; OTU, operational taxonomic unit; PCR, polymerase chain reaction; rRNA, ribosomal ribonucleic acid. Adapted from Woodhouse et al. (Woodhouse et al., 2020).

5.3 Metabolome and microbiome data integration

The integrate analysis of the metabolome and the microbiome has potential in the discovery of health and disease biomarkers (Shaffer et al., 2017). This integrative data analysis has promise for identifying microbial influence on host physiology thorough production, modification, or degradation of

bioactive metabolites. Moreover, it can help generating testable hypothesis regarding mechanistic links between microbial communities and disease causality. Therefore, the joint application of metabolomics and metagenomics analysis could help to address the gap existing in non-causal association observational studies by understanding the mechanisms, which are essential for translational research.

Some gaps that could be unhindered with the integration of these omics fields are: the understanding of (1) biological properties of microbes and metabolites; (2) which microbial enzymes/pathways produce which metabolites; and (3) the effects of bacterial metabolites on host (Ursell et al., 2014). These gaps have arisen some questions such as; *How the presence of a microbiome effects the host metabolome?* or *How the metabolome and microbiome are linked with the exposome?* The first question has been addressed by several studies which have compared germ free (GF) mice to mice with colonizing microbes including conventional mouse microbiota, human microbiota, or specific individual bacterial species (Marcobal et al., 2013; Shaffer et al., 2017). These studies have found that microbial colonization/composition has a profound impact on both the presence and relative abundances of many metabolites in various specimens including blood, urine, faeces, liver, and ileum (Shaffer et al., 2017). For example, the decreased diversity of gut microbiota community can lead to reduced fermentative activity and less production of SCFAs by gut bacteria which suppose less absorption and higher faecal excretion, with detrimental metabolic effects in the host (Koh et al., 2016). The second question, address the link between microbiome/metabolome with the exposome (e.g. exogenous factors like diet). It is known that diet composition substantially

affects the human microbiome, and its change can be accompanied by alteration in bacterial gene expression (David et al., 2014). In this way, consumption of different diets, such as the Mediterranean diet, have been associated with changes in microbially-produced metabolites, suggesting that the diet influences both gut microbiome and metabolome (Q. Jin et al., 2019). These links have also allowed the identification of microbially-produced compounds found to mediate disease process such as TMAO, a dietary origin microbial-derived metabolite related to CVD risk and atherosclerosis (Z. Wang et al., 2011).

Technological advances have led to the 'omics era', which enables the collection and integration of data at different molecular levels. There is a clear path to the clinical application of multiomics approaches in the management of CVDs. Future omics investigations should focus on the implementation of biomarkers at the early stages of CVD risk factors and associate them with diet and nutritional status.

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

HYPOTHESIS & OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

Hypothesis

The primary hypothesis of the present thesis is that particular gut microbes and faecal or circulating metabolites derived from the gut microbiota and lipid metabolism, broadly identified by the application of metabolomics, including lipidomics and gut metagenomics, could act as novel biomarkers. These biomarkers could assist in the early diagnosis of the main causal and modifiable CVD risk factors, primarily hypertension and hypercholesterolemia, in the preliminary stages of their pathophysiological process.

Second, it is hypothesized that PCs from a habitual diet could be precursors for the occurrence of particular bacterial signatures in the first grade of hypertension, and impair the concentrations of SCFAs by changes in gut microbiota composition. Similarly, dietary FAs could modulate the composition of circulating bioactive lipids, conditioning their intermediate role in hypercholesterolemia progression. Moreover, the multiple-way interactions between dietary compounds, the gut microbiome and the metabolome would help to understand the complex pathways involved in the pathogenesis or prevention of hypertension and hypercholesterolemia.

Objectives

To address the aforementioned hypotheses, a primary objective has been proposed:

- To identify novel early gut microbiota- and metabolite-based CVD risk biomarkers suitable for the preliminary stages of human hypertension, hypercholesterolemia and other main causal and modifiable CVD risk

factors through the integration of multiomics approaches and to assess their relationships with bioactive dietary compounds to speculate about the involved pathways and their possible effects in CVD development.

For this primary purpose, the following specific objectives have been set:

1. To determine the composition of the gut microbiota in grade 1 hypertensive subjects and compare it with the profile of normotensive subjects to establish a possible relationship of certain taxonomic groups with hypertension development. Also, to target novel gut microbiota-based biomarkers by applying gut metagenomics.
2. To determine faecal metabolites of bacterial origin as new metabolite-based biomarkers with potential clinical significance in grade 1 hypertension by applying targeted metabolomics.

Article 1: Calderón-Pérez et al., 2020

3. To investigate potential associations between dietary PC intake, faecal microbiota composition and SCFAs, as target faecal metabolites, in nontreated grade 1 hypertensive subjects compared to normotensive subjects.

Article 2: Calderón-Pérez et al., 2021

4. To assess whether serum lyso-PLs and plasma TMAO may be suitable susceptibility/risk biomarkers of human hypercholesterolemia based on their observed increase prior to LDL cholesterol alteration.
5. To evaluate the relationships between targeted circulating lyso-PLs and TMAO and diet composition; a special focus was placed on FA intake.

6. To examine the relationships between targeted lyso-PLs and serum liver transaminases to speculate possible hepatic mechanisms involved in lyso-PL metabolism in humans.
7. To verify the human results in a secondary *in vivo* study in hamsters to elucidate the possible mechanisms of action of lyso-PLs and their role in hypercholesterolemia risk.

Article 3: Calderón-Pérez et al., Manuscript accepted in Clinical Nutrition on Nov 29th 2021

8. To systematically review evidence from human randomized clinical trials, to evaluate the effect of SFAs, MUFAs, PUFAs and TFAs, provided as supplements, enriched food-components or diets, on the circulating bioactive lipid profile of healthy subjects, with CVD and CVD risk factors.

Article 4: Calderón-Pérez et al., Manuscript submitted to Critical Reviews in Food Science and Nutrition

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

METHODS & RESULTS

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

PART I. Discovering gut microbiota- and metabolite-based biomarkers and their relationships with bioactive dietary compounds in hypertension: *The Cardiogut study*

Article 1. Gut metagenomic and short-chain fatty acids signature in hypertension: A cross-sectional study

Lorena Calderón-Pérez⁺, Maria José Gosalbes⁺, Silvia Yuste, Rosa M Valls, Anna Pedret, Elisabet Llauradó, Nuria Jimenez-Hernandez, Alejandro Artacho, Laura Pla-Pagà, Judit Companys, Iziar Ludwig, Maria-Paz Romero, Laura Rubió, and Rosa Solà.

Scientific Reports. 2020 Apr 15;10(1):6436

Article 2. Interplay between dietary phenolic compound intake and the human gut microbiome in hypertension: A cross-sectional study

Lorena Calderón-Pérez, Elisabet Llauradó, Judit Companys, Laura Pla-Pagà, Anna Pedret, Laura Rubió, Maria José Gosalbes, Silvia Yuste, Rosa Solà, and Rosa M Valls.

Food Chemistry. 2021 May 15; 344:128567

In this first part, the methods and results of the *Cardiogut* study are shown. The *Cardiogut* was a cross-sectional study conducted between June 2016 and November 2017. It focused on the characterization of gut microbiota profile and function in HT compared to NT individuals. In addition, the potential relationships between gut microbiota community, microbe-derived metabolites and diet, particularly dietary PCs, were assessed (***Figure 23***). As a result of this work, two scientific articles have been published (***Article 1 and Article 2***).

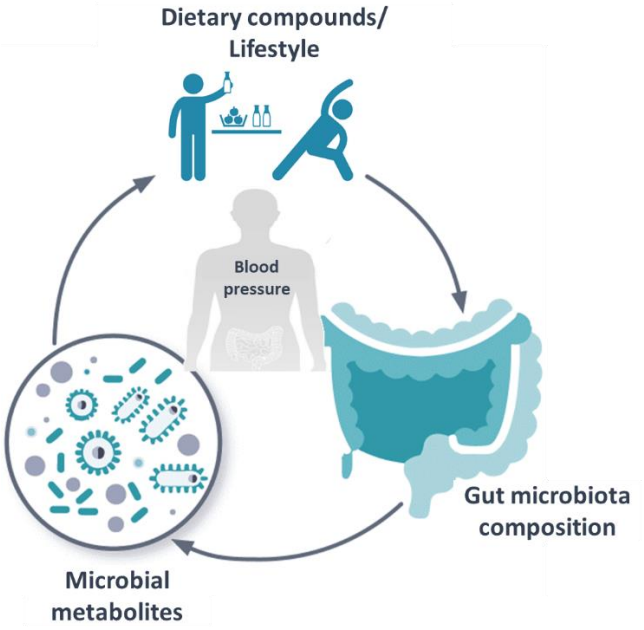


Figure 23 | Diagram of the multiple interactions in hypertension assessed in the *Cardiogut* study. Own source.

METHODS

Study aims

The main purpose was to provide new evidence that could help establish new therapeutic targets focused on the gut microbiota, as well as propose individualized dietary strategies to normalize the gut microbiome and reduce the risk of CVDs. The following specific aims were set:

- To determine the composition of the gut microbiota in HT subjects and compare it with the profile of NT subjects to establish a possible relationship of certain taxonomic groups with hypertension development. Also, to target novel microbiota-based biomarkers, by applying gut metagenomics.
- To identify new metabolite-based biomarkers of bacterial origin with potential clinical significance in grade 1 hypertension throughout the analysis of the metabolite profile derived from the microbiota in faeces (SCFAs) and plasma (SCFAs and TMAO).
- To study the role of dietary PCs in the composition of the gut microbiota and its metabolites in faeces in HT compared to NT subjects.

Study design and population

A cross-sectional study was performed in 29 nontreated grade 1 HT and 32 NT subjects recruited in Reus (Spain). They met the following criteria:

Inclusion criteria:

- SBP ≥ 140 - 159 mmHg (nontreated HT)
- SBP < 120 mmHg (NT)

- Age from 18 to 65 years old
- No family history of CVDs or chronic diseases
- Willingness to provide informed consent before starting the study

Exclusion criteria:

- BMI ≥ 30 kg/m^2
- Fasting glucose > 126 mg/dL
- LDL cholesterol > 190 mg/dL
- Triglycerides > 350 mg/dL
- Using antihypertensive or lipid-lowering medications or supplements
- Using antibiotics or probiotics
- Smoking
- Suffering anemia (hemoglobin ≤13 or ≤12 mg/dL in men and women, respectively)
- Suffering intestinal disorders
- Menopausal, pregnant or breastfeeding women
- Chronic alcohol consumption
- Following a vegetarian or vegan diet

Two face-to-face visits were performed to obtain the study data (V0 and V1). In a first screening visit (V0), a clinical interview to verify that participants met all the eligibility criteria was performed. In addition, a fasting blood test was performed, BP was measured, and anthropometric and physical activity data were collected. In the second visit (V1), in addition to the previous measures, urine and stool samples were collected. Moreover, a 3-day dietary record and a food-frequency questionnaire (FFQ) were provided by participants to assess dietary habits. There were no subject withdrawals between first and second visit so data were available for all subjects.

Study outcomes and measurement

A summary of main study clinical, lifestyle and biological outcomes with the corresponding methodology applied for their analysis is provided in ***Table 11***.

Outcome	Methodology for determination and analysis
Clinical	
SBP DBP	Automatic sphygmomanometer *(OMRON 501 HEM-907; Peroxfarma, Barcelona, Spain)* Two readings and calculation of the average value
Weight BMI Fat mass	Body composition analyzer *(Tanita SC 330-S; Tanita Corp., Barcelona, Spain)*
Waist circumference	150-cm anthropometric steel measuring tape At the umbilicus level
Lifestyle	
Diet composition	3-day dietary record Spanish Food Composition Tables *(Cesnid)*
Dietary PCs intake	3-day dietary record Phenol-Explorer database *(Available online: http://phenol-explorer.eu/)*
Dietary habits	Validated, semi-quantitative, food frequency questionnaire (Fernández-Ballart et al., 2010) Self-reported
Physical activity	Validated questionnaire *(Physical Activity Questionnaire Class AF)* (Vallbona Calbó et al., 2007)
Sleeping habits	Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989)
Biological	
Lipid profile Blood glucose	Serum sample Automated enzymatic methods in an autoanalyzer *(Beckman Coulter-Synchron, Galway, Ireland)*
Gut microbiota composition	Faecal sample DNA purification Sequencing of the 16S rRNA gene Taxonomic classification *(SILVA database, v.132)* ASVs assignation
Gut microbiota function	Faecal sample Metagenomic analysis Functional assignment *(TIGRFAM database of prokaryotic protein family models, v.9.0)*

Microbe-derived metabolites	***Faecal SCFAs:*** Faecal sample Gas chromatography coupled to a flame ionization detector (GC-FID) Internal standard reference peak side ***Plasma SCFAs:*** Plasma sample Gas chromatography coupled to a mass detector (GC-MS) Internal standard method ***TMAO:*** Plasma sample Ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)
Total polyphenol excretion	Urine sample Folin-Ciocalteu method Mass spectrometry (MS)

Table 11 | Outcomes and their analytical measurement in the *Cardiogut* study. Abbreviations: ASVs, amplicon sequence variants; BMI, body mass index; DBP, diastolic blood pressure; PCs, phenolic compounds; SBP, systolic blood pressure; SCFAs, short-chain fatty acids; TMAO, trimethylamine N-oxide. Own source.

In addition to all targeted metabolomics and gut metagenomics analyses, a complete statistical and bioinformatics analysis was performed including the following: alpha diversity and beta diversity calculation; Principal Coordinates analysis (PCoa); sample clustering Principal Component Analysis (PCA); Liner Discriminant Analysis (LDA) and Effect Size algorithm (LEfSe); Random forest analysis; heatmap; correlation; and linear regression models setting ASVs biomarkers as predictors and clinical or dietary variables as response variable.

Article 1

Gut metagenomic and short-chain fatty acids signature in hypertension: A cross-sectional study

Lorena Calderón-Pérez [+], Maria José Gosalbes [+], Silvia Yuste, Rosa M Valls, Anna Pedret, Elisabet Llauradó, Nuria Jimenez-Hernandez, Alejandro Artacho, Laura Pla-Pagà, Judit Companys, Iziar Ludwig, Maria-Paz Romero, Laura Rubió, and Rosa Solà.

[+] These authors contributed equally to the study.

Sci Rep. 2020 Apr 15;10(1):6436

SCIENTIFIC REPORTS
nature research

OPEN

Gut metagenomic and short chain fatty acids signature in hypertension: a cross-sectional study

Lorena Calderón-Pérez[1,2,7], Maria José Gosalbes[3,4,7], Silvia Yuste[5], Rosa M. Valls[1,2*], Anna Pedret[1,2], Elisabet Llauradó[2], Nuria Jimenez-Hernandez[3,4], Alejandro Artacho[3,4], Laura Pla-Pagà[1,2], Judit Companys[1,2], Iziar Ludwig[1], Maria-Paz Romero[5], Laura Rubió[2,5*] & Rosa Solà[1,2,6]

Hypertension is an independent and preventable risk factor for the development of cardiovascular diseases, however, little is known about the impact of gut microbiota composition in its development. We carried out comprehensive gut microbiota analysis and targeted metabolomics in a cross-sectional study of 29 non-treated hypertensive (HT) and 32 normotensive (NT) subjects. We determined fecal microbiota composition by 16S rRNA gene sequencing and bacterial functions by metagenomic analysis. The microbial metabolites analysed were short chain fatty acids (SCFA) both in plasma and feces, and trimethylamine N-oxide (TMAO) in plasma. The overall bacterial composition and diversity of bacterial community in the two groups were not significantly different. However, Ruminococcaceae NK4A214, Ruminococcaceae_UCG-010, Christensenellaceae_R-7, *Faecalibacterium prausnitzii* and *Roseburia hominis* were found to be significantly enriched in NT group, whereas, *Bacteroides coprocola*, *Bacteroides plebeius* and genera of *Lachnospiraceae* were increased in HT patients. We found a positive correlation between the HT-associated species and systolic and diastolic blood pressure after adjusted for measured confounders. SCFA showed antagonistic results in plasma and feces, detecting in HT subjects significant higher levels in feces and lower levels in plasma, which could indicate a less efficient SCFA absorption. Overall, our results present a disease classifier based on microbiota and bacterial metabolites to discriminate HT individuals from NT controls in a first disease grade prior to drug treatment.

Hypertension is one of the leading risk factors for the development of cardiovascular diseases (CVD) globally, and lifestyle measures are effective as a preventive strategies[1]. In 2015, hypertension based on office blood pressure (BP) reported the highest rates with a global prevalence of 1.13 billion cases, and 150 million in central and eastern Europe regardless of the income countries status[2–4].

The identification of the determinants of hypertension is still challenging although it is well recognized its multifactorial etiology[5]. It has been elucidated the interplay of genetic and environmental factors related with risk-conferring behaviors, such as, smoking, lack of physical activity, alcohol consumption and unhealthy diet[6,7].

Recently, it has been evidenced the role of gut microbiota dysbiosis on the modulation of high BP, both in animal and human hypertension[8]. Mell *et al.* (2015) were the first to demonstrate the differential fecal microbial composition of Dahl salt-sensitive and Dahl salt-resistant rats[9]. In the same period, Yang *et al.* (2015) also found a clear gut dysbiosis in spontaneously hypertensive rats (SHR) mediated by a decrease in microbial richness and

[1]Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain. [2]Universitat Rovira i Virgili, Facultat de Medicina i Ciències de la Salut, Functional Nutrition, Oxidation, and Cardiovascular Diseases Group (NFOC-Salut), C/Sant Llorenç 21, 43201, Reus, Spain. [3]Fundación para el Fomento de la Investigación Sanitaria y Biomédica, Valencia, Spain. [4]CIBER en Epidemiología y Salud Pública (CIBEResp), Madrid, Spain. [5]Food Technology Department, XaRTA-TPV, Agrotecnio Center, Escola Tècnica Superior d'Enginyeria Agrària, University of Lleida, Avda/Alcalde Rovira Roure 191, 25198, Lleida, Catalonia, Spain. [6]Hospital Universitari Sant Joan de Reus, Reus, Spain. [7]These authors contributed equally: Lorena Calderón-Pérez and Maria José Gosalbes. *email: rosamaria.valls@urv.cat; laura.rubio@udl.cat

Variable	Hypertensive (n = 29)	Normotensive (n = 32)	p-value
Age, *y*	53.7 ± 9.6	41.1 ± 9.1	<0.001
Gender, *(F/M)*	(10/19)	(16/16)	0.301
SBP, *mm Hg*	153.1 ± 14.6	109.7 ± 7.1	<0.001
DBP, *mm Hg*	91.0 ± 8.8	65.7 ± 6.7	<0.001
Weight, *kg*	75.3 ± 9.3	68.9 ± 10.8	0.017
BMI, kg/m^2	26.2 ± 2.5	23.8 ± 2.7	<0.001
Waist circumference, *cm*	94.4 ± 8.3	84.0 ± 9.0	<0.001
Fat mass, *%*	26.6 ± 7.9	22.1 ± 7.8	0.037
FBG, *mg/dL*	91.2 ± 11.3	81.1 ± 7.5	0.001
Cholesterol, *mg/dL*			
Total	199.6 ± 43.9	181.7 ± 34.7	0.017
LDL	123.7 ± 21.3	100.7 ± 33.2	0.002
HDL	62.6 ± 14.0	64.9 ± 18.0	0.580
Triglycerids, *mg/dL*	97.3 ± 38.8	80.7 ± 42.6	0.067
Physical activity, *%*			0.729
Inactive	6.9	0.0	
Very low activity	10.3	10.0	
Low activity	10.3	6.7	
Moderate activity	20.7	20.0	
High activity	51.7	63.3	
Sleep Quality, *%*			0.299
Good quality	48.3	63.3	
Poor quality	51.7	36.7	

Table 1. Baseline characteristics of participants. Data expressed as mean ± standard deviation or percentage. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; FBG, fasting blood glucose; LDL. low density lipoproteins; HDL, high density lipoproteins. P-value for gender, physical activity and sleep quality was calculated by Fisher's exact test. P-value for age, SBP, DBP, weight, BMI, waist circumference, fat mass, FBG, cholesterol and triglycerides was calculated by Student's t-test and Mann-Whitney U test.

diversity compared to the normotensive Wistar-Kyoto rats[8]. In line with these results, changes in gut microbiota composition also occur in humans at different hypertension grades, and high BP appears to be transferrable from hypertensive donor to germ-free mice through fecal microbiota transplantation[10]. It was also recently reported that, these changes in the composition of SHR rats gut microbiota seems to be at least partially associated with altered integrity of the gut epithelial barrier and altered inflammatory status[11].

Changes in gut bacteria composition precede alterations in the level of metabolic microbiota-derived end products in the bloodstream. A clear example of bacterial metabolites are short chain fatty acids (SCFA) derived from dietary fibers fermentation, being the most abundant butyrate, acetate and propionate[12]. The low abundance of SCFA-producing bacteria in SHR rats disrupt the gut bacterial balance leading to immunological, physiological and metabolic alterations in the host status that can influence BP homeostasis[8]. Although the mechanism by means SCFA regulates BP has not been fully studied, Pluznick *et al.* (2013) showed how BP can be modulated in response to circulating SCFA via G-protein-coupled receptors (GPCRs) expression in the vascular or renal tissues[13,14].

Studies are conflicting as to whether SCFA are beneficial or detrimental to cardiometabolic health. In a recent study de la Cuesta-Zuluaga *et al.* (2018)[15] reported in a human cohort that higher fecal SCFA concentrations were associated with a measure of gut permeability and also hypertension. The same authors also highlighted that more studies are needed analyzing both fecal and circulating SCFA in humans to test the hypothesis that the association of higher fecal SCFA with cardiometabolic dysregulation is due to less efficient SCFA absorption to circulation.

In addition to SCFA, other bacterial metabolites such as trimethylamine N-oxide (TMAO) might also be involved in the pathogenesis of CVD. TMAO is derived from dietary sources of phosphatidylcholine (lecithin), such as red meat, dairy products, eggs and fish[16,17]. Elevated TMAO plasma levels are associated with high atherosclerosis burden, and specific bacterial genera have been identified as a putative choline degraders and TMAO producers[18–20]. However, the existing body of literature is sparse regarding TMAO and hypertension.

To address the questions raised above, we carried out a comprehensive taxonomic and functional analysis and targeted metabolomics in a cross-sectional study of 29 non-treated grade I hypertensive (HT) and 32 normotensive (NT) subjects, with special emphasis on targeting fecal metabolites that appear to be a novel target for hypertension treatment.

Results and Discussion

Clinical and lifestyle characteristics of the study participants. From 80 subjects who were assessed for eligibility, 19 were excluded for not meeting the inclusion criteria for systolic blood pressure (SBP). The remaining 61 participants were assigned to HT group (n = 29) and NT control group (n = 32) (**Additional file 1:** Fig. S1). Anthropometric and clinical characteristics of the total study population segregated by HT and NT, are

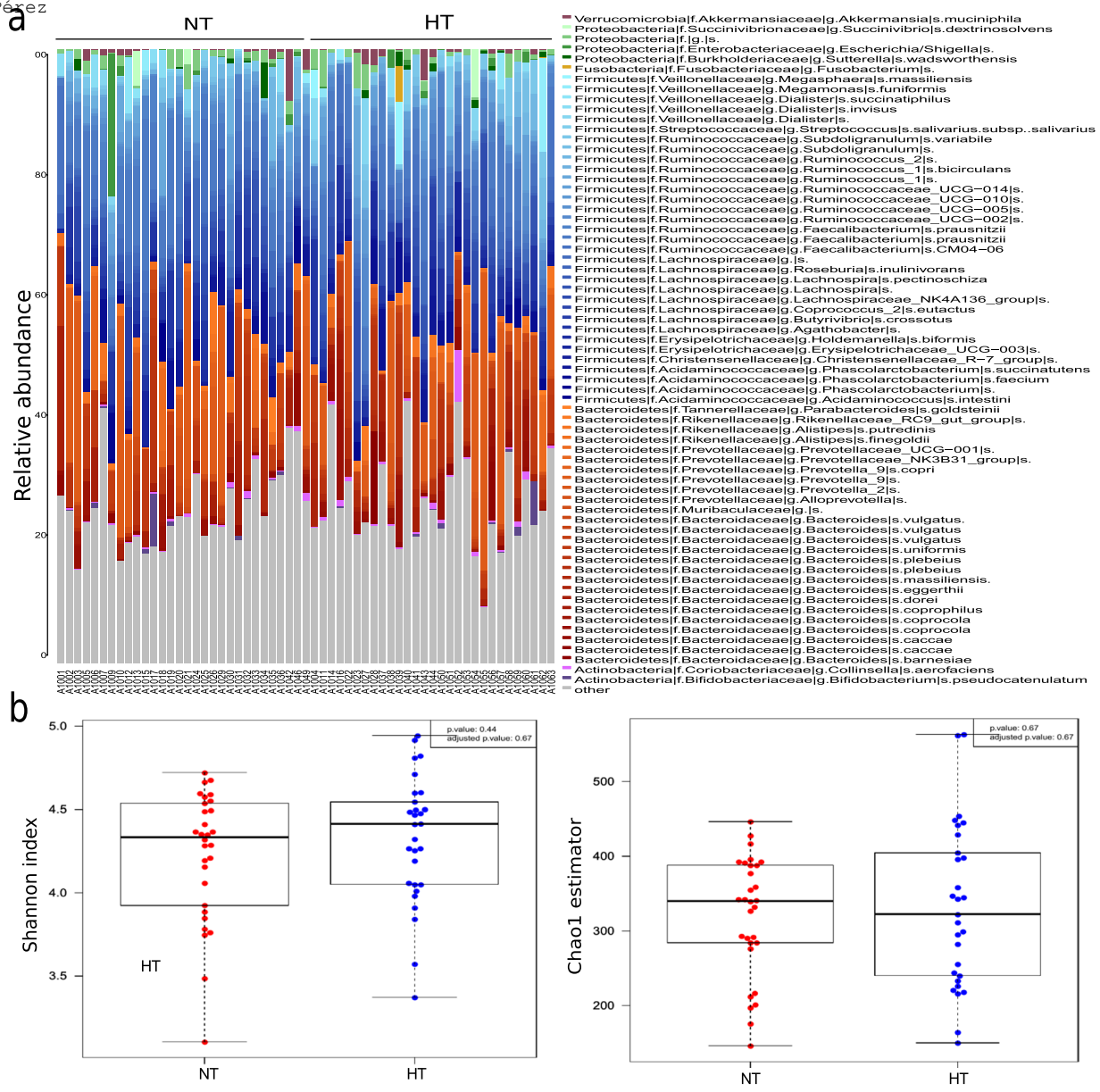


Figure 1. Difference of gut microbial community between hypertensive (HT) and normotensive (NT) groups. **(a)** Bacterial composition between groups at ASV level. **(b)** Differences between groups in diversity (Shanon index) and richness (Chao1 index) of bacterial community.

shown in Table 1. A total of 10 females and 19 males with a mean age of 53.7 years were assigned to HT group, and 16 females and 16 males with a mean age of 41.1 years were designated into the NT group. Although age was significantly different between HT and NT ($p < 0.001$), both groups were in the same range of age (adulthood).

Significant differences between groups were observed in anthropometric parameters as weight (HT, 75.3 ± 9.3 kg; NT, 68.9 ± 10.8 kg; $p = 0.017$), body mass index (BMI) (HT, 26.2 ± 2.5 kg/m^2; NT, 23.8 ± 2.7 kg/m^2; $p < 0.001$), waist circumference (HT, 94.4 ± 8.3 cm; NT, 84.0 ± 9.0 cm; $p < 0.001$) and fat mass (HT, 26.6 ± 7.9%; NT: 22.1 ± 7.8%; $p = 0.037$). Results from analytical parameters showed that HT group had higher levels of fasting blood glucose (HT, 91.2 ± 11.3 mg/dL; NT, 81.1 ± 7.5 mg/dL; $p = 0.001$), total cholesterol (HT, 199.6 ± 43.9 mg/dL; NT, 181.7 ± 34.7 mg/dL; $p = 0.017$) and low density lipoprotein (LDL)-cholesterol (HT, 123.7 ± 21.3 mg/dL; NT, 100.7 ± 33.2 mg/dL; $p = 0.002$) than NT group. Despite these differences between groups, it is important to highlight that all baseline clinical parameters remained within normal values except for SBP (HT, 151.8 ± 16.2 mmHg; NT, 109.7 ± 7.1 mmHg; $p < 0.001$) and DBP (HT, 90.2 ± 9.9 mmHg; NT, 65.7 ± 6.7 mmHg; $p < 0.001$). Previous evidence suggest that gut microbial composition appears to be altered with lifestyle, obesity and cardiometabolic disease[21]. From our results, the differences found in clinical parameters between HT and NT remained among healthy ranges. So, we hypothesize that microbial changes might be mainly related to hypertension, and the other parameters would not contribute to the changes.

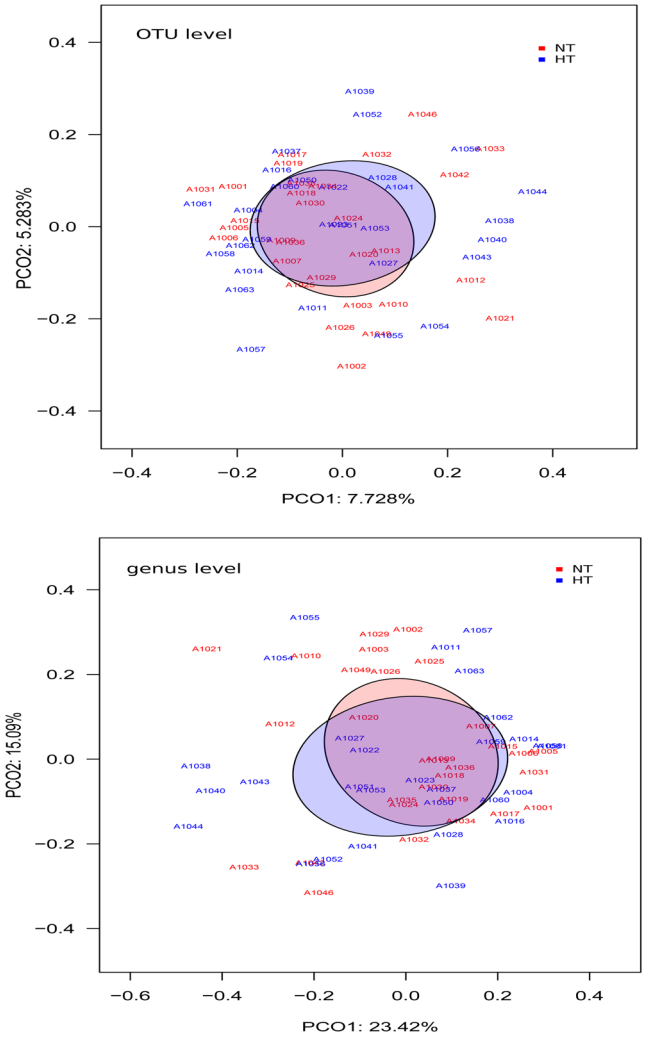


Figure 2. Beta diversity between NT and HT groups. PCoAs based on Bray-Curtis dissimilarity index at ASV and genus level.

No differences were found in physical activity and sleep quality. Diet composition was similar between groups with exception of total dietary fiber intake, which was greater in the NT group (HT, 20.95 ± 9.90 g; NT, 25.90 ± 11.63 g; $p = 0.029$) (**Additional file 2:** Table S1). Reported data from food frequency questionnaire (FFQ) showed differences between HT and NT groups in daily mean intake of processed meat products ($p = 0.016$), potatoes ($p = 0.05$), natural juices ($p = 0.008$) and coffee ($p = 0.033$) showing a higher consumption in HT patients. The whole-grain cereals intake was higher in NT group ($p = 0.034$) (**Additional file 3:** Table S2).

Subjects taking antihypertensive drugs were excluded in order to assess patients in a first disease grade prior to drug treatment and to avoid any drug interference on gut microbiome. It should be noted that 4 NT and 6 HT subjects were taking occasionally other drugs, specifically, analgesic and nonsteroidal anti-inflammatory (NSAIDs), with the last intake at least 30 days before the inclusion visit. Although changes in the composition and biodiversity of the intestinal microbiome have been described after usual NSAIDs administration[22], none of the study participants were chronic NSAIDs users.

Microbiota composition analysis. We obtained 9,609,446 amplicon raw sequences for 59 fecal samples that we filtered by length, quality and chimera giving a total of 7,171,741 filtered sequences with an average of 129,125 sequences per sample in HT and 114,237 sequences per sample in NT. We used the DADA2 pipeline generating 4491 amplicon sequence variants (ASV) available for further analysis. Taxonomic assignment was performed at ASV and genus level. Both groups, NT and HT, presented Firmicutes (45.94% and 45.98%, respectively) and Bacteroidetes (25.62% and 26.35%, respectively) as the major two phyla. Also, Proteobacteria (1.42% and 0.83%) and Actinobacteria (0.98% and 1.2%) were detected in NT and HT groups (Fig. 1a). In Firmicutes phylum, we found that *Faecalibacterium* (12.1% and 9.7%), *Ruminococcus* (3.1% and 1.9%), *Lachnospira* (2.68% and 2.46%), *Phascolarctobacterium* (2.5% and 2.81%), *Roseburia* (5.14% and 4.17%) and *Dialister* (1.05% and 1.01%) were the main genera in NT and HT. For Bacteroidetes phylum, *Bacteroides* (12% and 13.45%), *Prevotella* (9.1% and 8.2%) and *Alistipes* (1.8% and 1.2%) were the most abundant taxa.

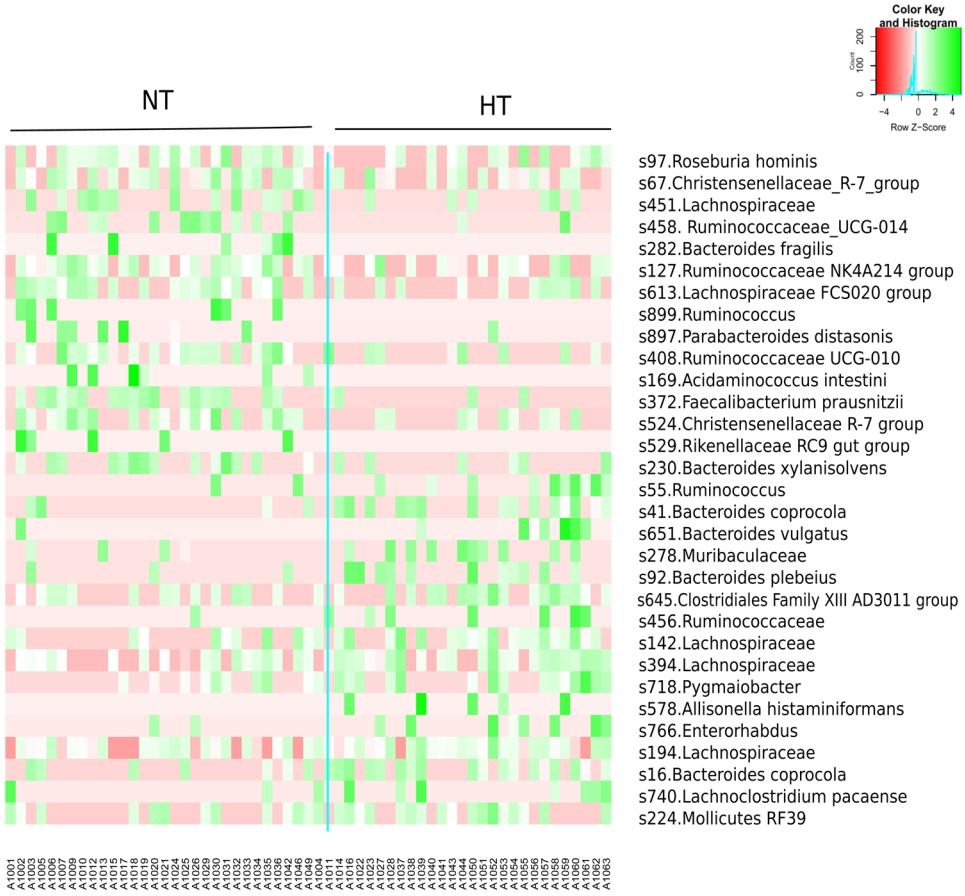


Figure 3. Heatmap based on ASV biomarkers. Only the ASVs having a LDA score >2.5 (log10) are represented. The relative abundance is expressed as Z score.

To characterize the richness and diversity of bacterial community, we calculated, at ASV level, Chao1 estimator and Shannon index, respectively. As shown in Fig. 1b, the richness estimator and diversity index in the two groups were not significantly different. To assess the overall bacterial composition for both groups we performed Principal Coordinates Analysis (PCoA) using Bray-Curtis dissimilarity index at ASV and genus level (Fig. 2). No relevant differences in the gut microbiota at ASV (Adonis test, $p = 0.31$) or genus level (Adonis test, $p = 0,33$) were detected.

In contrast to our results, a previous study[23] reported a significant dysbiotic gut microbiome in HT patients observing a decrease in the microbial richness and diversity compared to a healthy control group. This could be explained because in this previous study, a great part of HT patients had taken antihypertensive drugs and they were in a more advance hypertension grade. It has been recently reported that the hypertensive drug treatment can induce compositional changes in the gut microbiota and also inflammation[24], so drug-induced gut microbiome shifts could have strongly influenced in their results. Moreover, in contrast to our study, in which we assessed diet composition and other lifestyle outcomes such as physical activity and sleep habits showing no significant differences among both groups, they did not control any environmental factor that could also affect the gut microbial community. In the present study, instead of observing a clear dysbiosis in HT, we report that subjects in a grade I of hypertension prior to drug treatment could have specific alterations in particular bacterial taxa.

In order to identify specific taxa as biomarkers, we performed linear discriminant analysis (LDA) effect size (LEfSe) at ASV level. We detected, with LDA score > 2, a total of 67 ASVs biomarkers that had significant different abundance between HT and NT subjects (**Additional file 4:** Fig. S2). Figure 3 shows the normalized relative abundance (Z score) of the biomarkers that presented higher discriminant power (LDA score > 2.5) (Table 2). Particularly, we observed that in HT group three ASVs (s41, s16 and s92) with the highest LDA scores (3.08, 3.05 and 2.9, respectively) belong to *Bacteroides* genus. Two of them (s41 and s16) were classified as *Bacteroides coprocola* while the other ASV (s92) was assigned as *Bacteroides plebeius*.

On the other hand, different genera of Ruminococcaceae, Lachnospiraceae, Acidaminococcaceae and Christensenellaceae families from Firmicutes phylum and Tannerellaceae, Rikenellaceae and Bacteroidaceae from Bacteroidetes, were the most frequent taxa in NT group. *Faecalibacterium prausnitzii* (LDA score = 2.91, $p = 0.0005$) presented the highest discriminant power in the NT group. This specie and *Roseburia hominis* (LDA score = 2.76, $p = 0.02$), both biomarkers for NT, have been described as the main SCFA-producers in healthy status[15,25]. Several species of the Ruminococcaceae genera were also enriched in the NT group (s127, s458, s408, s899). Ruminococcaceae genera can degrade several types of polysaccharides in the lower GI tract, including

ASV	LDA score (log10)	p.value	Taxonomy
Normotensive (NT)			
s897	2.5004	0.0267	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Tannerellaceae;g__Parabacteroides;s__distasonis
s529	2.5557	0.0229	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Rikenellaceae_RC9_gut_group
s613	2.5759	0.0067	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Lachnospiraceae_FCS020_group
s899	2.5799	0.0119	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
s408	2.6117	0.0146	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcaceae_UCG-010
s524	2.6530	0.0083	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae;g__Christensenellaceae_R-7_group
s169	2.6650	0.0119	p__Firmicutes;c__Negativicutes;o__Selenomonadales;f__Acidaminococcaceae;g__Acidaminococcus;s__intestini
s451	2.7061	0.0435	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
s282	2.7181	0.0119	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__fragilis
s458	2.7226	0.0042	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcaceae_UCG-014
s97	2.7644	0.0199	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia;s__hominis
s230	2.7885	0.0339	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__xylanisolvens
s67	2.8589	0.0174	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae;g__Christensenellaceae_R-7_group
s127	2.8661	0.0060	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcaceae_NK4A214_group
s372	2.91	0.0005	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium;s__prausnitzii
Hypertensive (HT)			
s651	2.5177	0.0219	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__vulgatus
s740	2.5255	0.0368	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Lachnoclostridium;s__pacaense
s766	2.5713	0.0260	p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Eggerthellaceae;g__Enterorhabdus
s645	2.5868	0.0494	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Family_XIII;g__Family_XIII_AD3011_group
s456	2.6274	0.0191	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
s718	2.6301	0.0244	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Pygmaiobacter
s578	2.6434	0.0186	p__Firmicutes;c__Negativicutes;o__Selenomonadales;f__Veillonellaceae;g__Allisonella;s__histaminiformans
s394	2.6554	0.0238	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
s55	2.6743	0.0132	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
s194	2.7215	0.0434	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
s224	2.7357	0.0488	p__Tenericutes;c__Mollicutes;o__Mollicutes_RF39
s278	2.8638	0.0345	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Muribaculaceae
s142	2.8795	0.0147	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
s92	2.8831	0.0104	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__plebeius
s16	3.0552	0.0050	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__coprocola
s41	3.0803	0.0017	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__coprocola.

Table 2. Significant differences between the two groups HT and NT in the relative abundance of ASVs that present a LDA score > 2.5 in LEfSe analysis.

starch, cellulose, and xylan[26]. This genera also consume hydrogen and produce acetate that can be utilized by *Roseburia* to produce butyrate[27]. In accordance with previous human cohort studies in which gut microbiome in hypertension status was compared to healthy subjects[10,23], a reduction in all of these bacterial groups in HT patients was also observed. The depletion of these specific bacteria may have functional consequences on SCFA production and therefore the ability of the host to repair the epithelium and to regulate inflammation.

Correlation of biomarkers with clinical variables. We explored the correlations between microbiota composition, by means of ASV biomarkers, and clinical variables taking into account the confounders of age, waist circumference, LDL-cholesterol, BMI, fat mass and dietary fiber intake (Table 3). After adjusting by false discovery rate a few correlations remained significant.

Two ASV biomarkers of HT group, s394 and s718, correlated positively with SBP (r 0.396, p = 0.0018 and p-adjust = 0.007, and r 0.46, p = 0.00026 and p-adjust = 0.039, respectively). These results indicated that higher relative abundance of these ASVs, implied higher values of SBP, suggesting a negative effect of these bacteria on health status. ASV s718 was assigned as *Pygmaiobacter*, a new acidogenic genus of Ruminococcaceae family[28], was positively correlated with both SBP and DBP, and also appeared to be a biomarker of HT group (Table 2). ASV s394 was classified as a genus of Lachnospiraceae family and we found that its 16S rRNA sequence share by BLAST 97.27% of identity with *Faecalicatena orotica*, a new specie described by Sakamoto, M. *et al.*[29]. This fecal bacteria also displayed a positive correlation with DBP (r 0,324; p = 0,012 and p-adjust = 0.1542), fat mass (r 0.356, p = 0.007 and p-adjust = 0.1283) and fasting blood glucose (r 0.42, p = 0.0009 and p-adjust = 0.0492), so further studies would be needed to confirm the contribution of these bacteria in hypertension.

We reported that *Faecalibacterium prausnitzii* (ASV s372), biomarker of NT group with the highest discriminant power, was negatively correlated with SBP (r −0.403, p = 0.0015 and p-adjust = 0.075) and DBP (r −0.43, p = 0.0006 and p-adjust = 0.042) (Table 3). *F. prausnitzii* is a well-known butyrate-producer in the gut and this SCFA has a potent anti-inflammatory effect that could affect to BP[30]. Another NT biomarker (ASV s524) that was

ASV increased in HT group	Clinical variable	Spearman correlation index	p.value Spearman correlation	p.adjust Spearman correlation
s718. Ruminococcaceae, *Pygmaiobacter*	SBP	0.4586	0.0003	0.0399
s718. Ruminococcaceae, *Pygmaiobacter*	DBP	0.3565	0.0056[a]	0.1238
s394. Lachnospiraceae	FBG	0.4208	0.0009	0.0492
s394. Lachnospiraceae	SBP	0.3966	0.0019	0.0755
s394. Lachnospiraceae	DBP	0.3242	0.0122	0.1542
s394. Lachnospiraceae	Fat mass	0.3561	0.0076	0.1283
s1498. Ruminococcaceae	FBG	0.2894	0.0262[b]	0.2304
ASV increased in NT group	**Clinical variable**	**Spearman correlation index**	**p.value Spearman correlation**	**p.adjust Spearman correlation**
s230. *Bacteroides xylanisolvens*	cLDL	−0.3318	0.0103	0.1416
s451. Lachnospiraceae	TG	−0.3346	0.0096	0.1359
s372. *Faecalibacterium prausnitzii*	DBP	−0.4334	0.0006	0.0417
s372. *Faecalibacterium prausnitzii*	SBP	−0.4031	0.0015	0.0750
s524. Christensenellaceae_R-7_group	DBP	−0.3054	0.0186[c]	0.1871
s524. Christensenellaceae_R-7_group	SBP	−0.3075	0.0178[d]	0.1830
S458. Ruminococcaceae_UCG-014	DBP	−0.3402	0.0083[e]	0.1283

Table 3. Correlations between ASV biomarkers and clinical variables. p is probability at $\alpha = 0.05$. SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, tryglicerides; cLDL low density liproprotein cholesterol; FBG, fasting blood glucose. Variables were adjusted for age, waist circumference, cLDL, BMI, fat mass and total fiber intake. [a]Confounding factor: Fat mass (p value = 0.058). [b]Confounding factor: Fat mass (p value = 0.0614). [c]Confounding factors: Age (p value = 0.0514), waist circumference (p value = 0.1676); total fiber intake (p value = 0.0578). [d]Confounding factors: Waist circumference (p value = 0.092); total fiber intake (p value = 0.054). [e]Confounding factor: Fat mass (p value = 0.067).

assigned as Christensenellaceae R-7 group also correlated negatively with SBP (r -0.308, p = 0.018 and p-adjust = 0.18) and DBP (r −0.305, p = 0.0186 and p-adjust = 0.187). Christensenellaceae family has been described also as SCFA producer and associated with low BMI and with reduced weight gain in mice[31].

Identification of hypertension-associated markers from gut microbiome. To determine the discriminant power of microbiota signatures in the identification of hypertension state, we applied random forest analysis. We obtained the discriminatory power of the area under the ROC curve (AUC) of 0,84 (Fig. 4a). 21 ASVs allowed to discriminate between HT patients and healthy subjects (p = 0,0017, Adonis test).

In line with LEfSe results, *B. coprocola* (HT-enriched) and *F. prausnitzii, Roseburia hominis*, Ruminococcaceae UCG-010, Lachnospiraceae FCS020_group and Ruminococcaceae NK4A214 group (HT-depleted), featured as microbiota members for the discrimination between groups (Fig. 4b). The results suggested that these specific bacterial taxa could have a strong relation with hypertension, however, more studies are needed to elucidate the mechanisms involved in this pathology.

Differences in microbiota metabolic functions. To explore functional hallmarks, we performed a metagenomic analysis in both groups. The sequencing of the metagenomes yielded a total of 4.76 Gb with an average of 78 Mb per sample. The functional composition of the microbiota was elucidated comparing all the ORFs with TIGRFAM database of prokaryotic protein family models, obtaining an assignment of 35,6% of the ORFs (82,269 genes per sample). TIGRFAM protein family model is a hierarchical classification entailing main roles, the highest functional levels, and subroles, which represent more specific functions within each main role. PCoA, based on Bray-Curtis dissimilarity index, indicated a similar functional composition at TIGRFAM protein family (TIGRFAM) and subrole level (**Additional file 5:** Fig. S3). In accordance with previous work[10,23], we detected, using LEfSe package, that NT group presented higher abundance of the subrole "Signal Transduction_Two Component Systems" (p = 0.015) (**Additional file 6:** Fig. S4). On the other hand, HT-associated microbiota was enriched in genes involved in the energy metabolism (subroles: Electron Transport, p = 0.04 and Anaerobic, p = 0.045), cellular processes (subrole DNA transformation, p = 0.03) and DNA metabolism (subrole DNA replication, recombination and repair, p = 0.042). Although the differential relative abundance in SCFA-producer bacteria reported, no significant differences have been detected in SCFA-producing pathways between NT and HT groups.

We assessed functional discrimination of hypertension status applying random forest model with subroles and TIGRFAM. The discriminatory model achieved an average AUC of 0.66 based on 18 functional subroles (Fig. 5a). However, the variable TIGRFAM was more powerful to distinguish NT and HT groups with an average AUC of 0.86 (Fig. 5b). We found that three TIGRFAMs involved in the pathway "DNA replication, recombination and repair" were more abundant in HT group and presented the highest discriminatory capacity. In contrast with Li *et al.* (2017)[10], the discriminatory TIGRFAMs, belonging to the functional categories related to secretion and transport systems as well as biosynthesis of lipopolysaccharides and transport were decreased in HT patients (Fig. 5b).

SCFA in feces and plasma. Recent studies have found that changes in BP often coordinate with changes in SCFA[32]. So in the present study, SCFA were reported separately in feces and plasma of HT and NT subjects

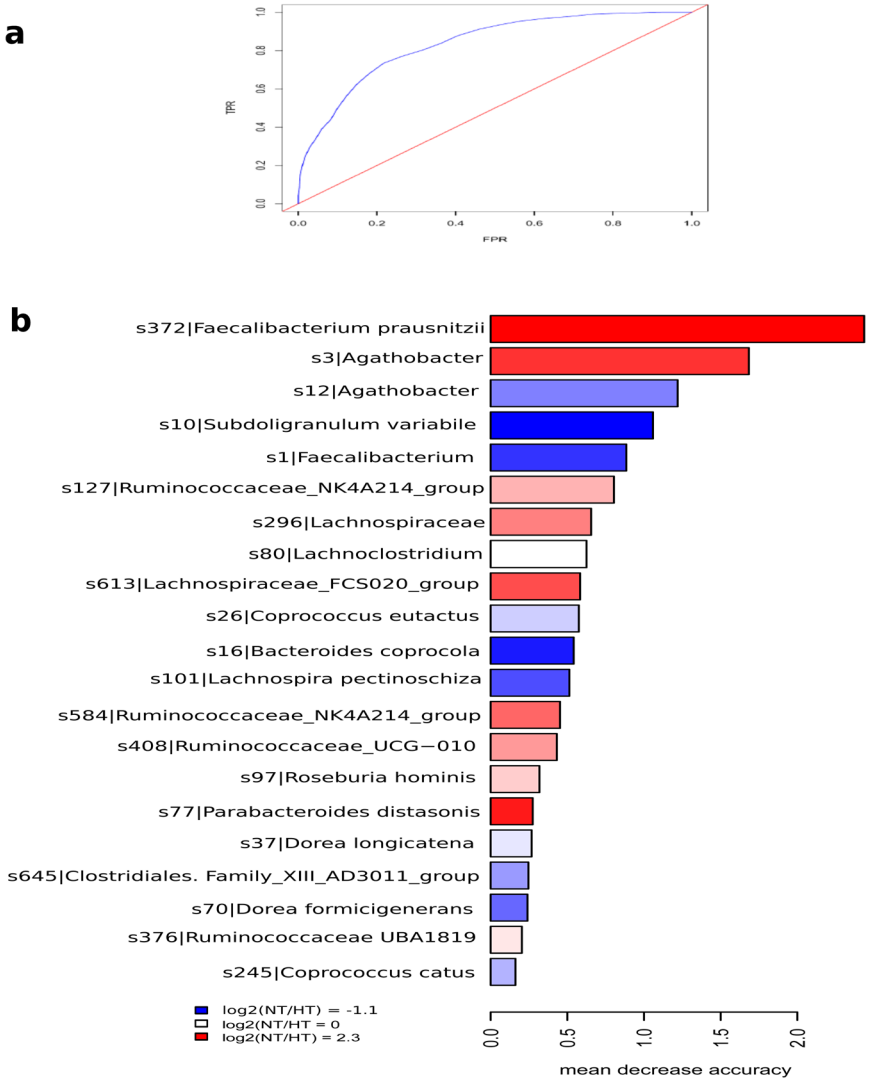


Figure 4. Random forest analysis at ASV level. **(a)** ROC curve using ASVs as variables. FPR, False Positive Rate, TPR, True Positive Rate. **(b)** The most discriminatory ASVs to classify individuals into NT or HT group. The colour indicates the enrichment in NT (red) or HT (blue) based on the log2 fold change between the relative abundance average of NT group and the relative abundance average of HT group. Length of bars represents the discriminatory power of the variables.

(Fig. 6). In feces, HT showed significantly higher concentrations of acetate ($p = 0.004$), propionate ($p = 0.005$), butyrate ($p = 0.002$) and valerate ($p = 0.003$) compared to NT subjects (Fig. 6a). Similarly to our findings, some animal studies found elevated levels of fecal acetate and propionate in HT rat model in contrast to control rats[33,34]. Another study conducted in rats, observed significant elevated levels of acetate in salt sensitive rats which received a microbial transplant that increased their BP[9]. In line with these results, de la Cuesta-Zuluaga *et al.* (2018)[15] examined in 441 subjects associations of fecal SCFA, gut microbiota and cardiometabolic outcomes, and they observed that higher fecal SCFA concentrations were associated with a measure of gut permeability and hypertension.

Previous cross-sectional studies have also reported higher fecal SCFA concentrations in overweight or obese individuals compared to lean individuals[35–38]. Our study corroborates these results, reporting also a significant positive correlation of SBP with fecal acetate (r 0,37; $p = 0,003$), propionate (r 0,35; $p = 0,005$) and butyrate (r 0,36; $p = 0,004$) (**Additional file 7:** Table S3).

SCFA are rapidly and efficiently absorbed in the colon with less than 5% being excreted in feces. Fecal SCFA concentrations have been used to determine SCFA production, however they are also a surrogate measure of SCFA absorption in the colon. Indeed, it has been suggested that fecal SCFA concentration could better represent SCFA absorption than its production[39]. So in the present study, we also analysed these fermentation products in plasma to study the SCFA absorption grade. To the best of our knowledge, no previous human studies have assessed both fecal and circulating SCFA in HT patients. Remarkably, plasma results showed an opposite trend compared to fecal results, reporting in HT group significant lower levels of circulating acetate ($p = 0.000$),

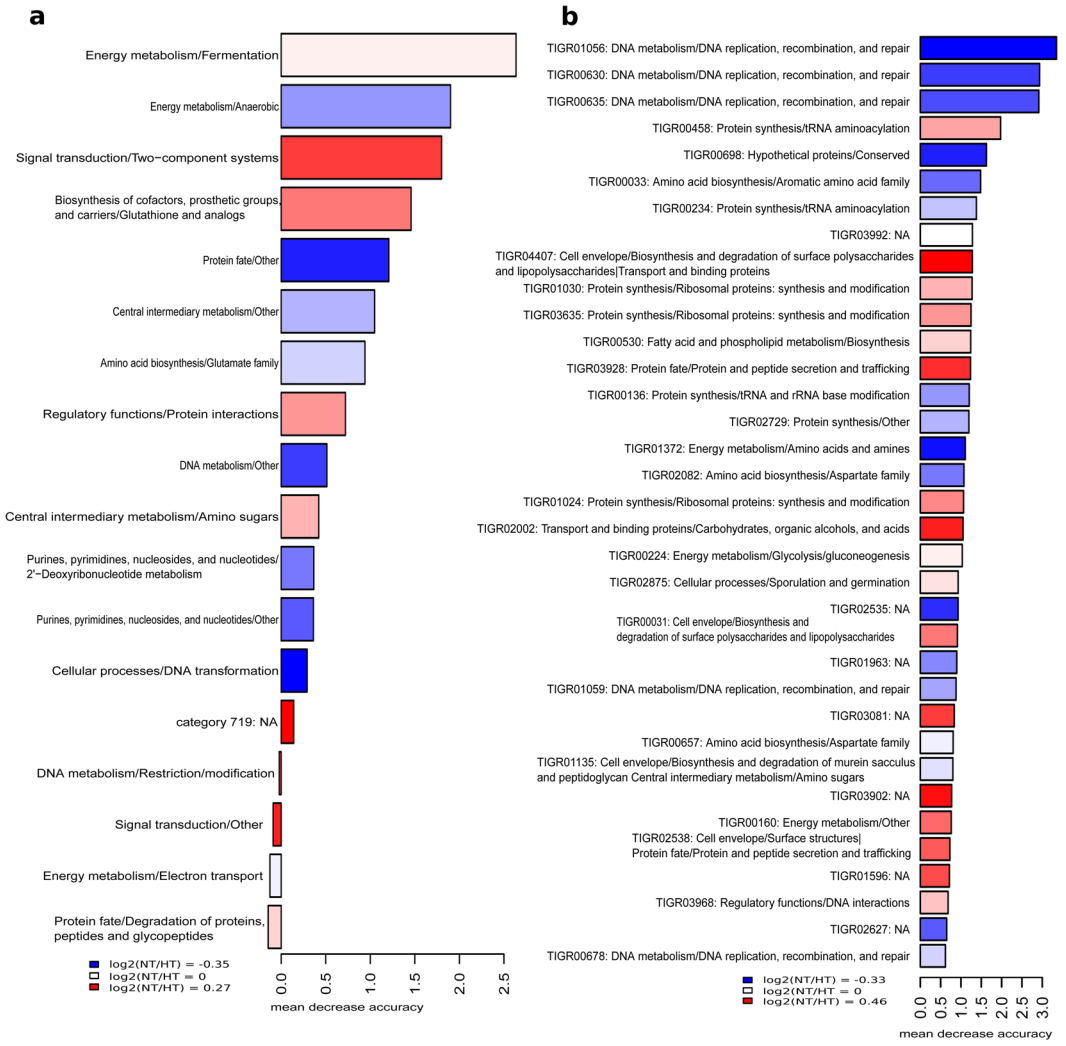


Figure 5. Random forest using **(a)** functional subroles and **(b)** TIGRFAM as discriminatory variables. The colour indicates the enrichment in NT (red) or HT (blue) based on the log2 fold change between the relative abundance average of NT group and the relative abundance average of HT group. Length of bars represents the discriminatory power of the variables.

isobutyrate ($p = 0.004$), butyrate ($p = 0.019$) and isovalerate ($p = 0.036$) compared to NT (Fig. 6b). A recent study performed in SHR, showed the same antagonistic results, reporting higher fecal butyrate levels in contrast to circulation levels[40]. They also reported lower expression levels of butyrate-sensing receptors in the hypothalamus of SHR and reduced expression of Slc5a8 transporter in the colon, so they concluded that a reduced availability of serum butyrate in the SHR is possibly due to a diminished absorption in the colon. Our study could corroborate this hypothesis in humans, and also extends these results, not only to butyrate, but also to other SCFA, such as acetate, the most abundant SCFA in plasma (Fig. 6).

Other recent studies may reinforce this hypothesis, reporting a strong relation between hypertension, gut mucosal permeability and altered inflammatory status in rats[11] and humans[41] with hypertension. Kim *et al.* reported significant increases in plasma of intestinal fatty acid binding protein, lipopolysaccharide, and augmented gut-targeting proinflammatory T helper 17 cells in high BP patients, which demonstrated increased intestinal inflammation and permeability in HT subjects[41]. Together with these results, they also observed significant lower levels of plasma butyrate in HT subjects.

Since our study demonstrates that HT subjects present significant depleted levels of butyrate-producing bacteria and plasma SCFA together with higher levels of SCFA in feces, our results strengthen the hypothesis that a lower efficiency in the absorption of SCFA could occur in HT subjects and that an imbalanced host-microbiome crosstalk may be an important cause of hypertension. Moreover, no significant differences were observed in bacterial pathways related to SCFA biosynthesis, which reinforces this hypothesis.

Another way that SCFA can influence host cells, especially acetate and propionate, is by cellular mechanisms related to host GPCRs involved in SCFA signalling[32]. Intriguingly, Gpr41 and Olfr78 play opposite roles in BP regulation. While Gpr41 null mice are hypertensive, the Olfr78 null mice are hypotensive[13,42], which points out the physiological importance of these SCFA signalling pathways on the BP control. Many studies support the

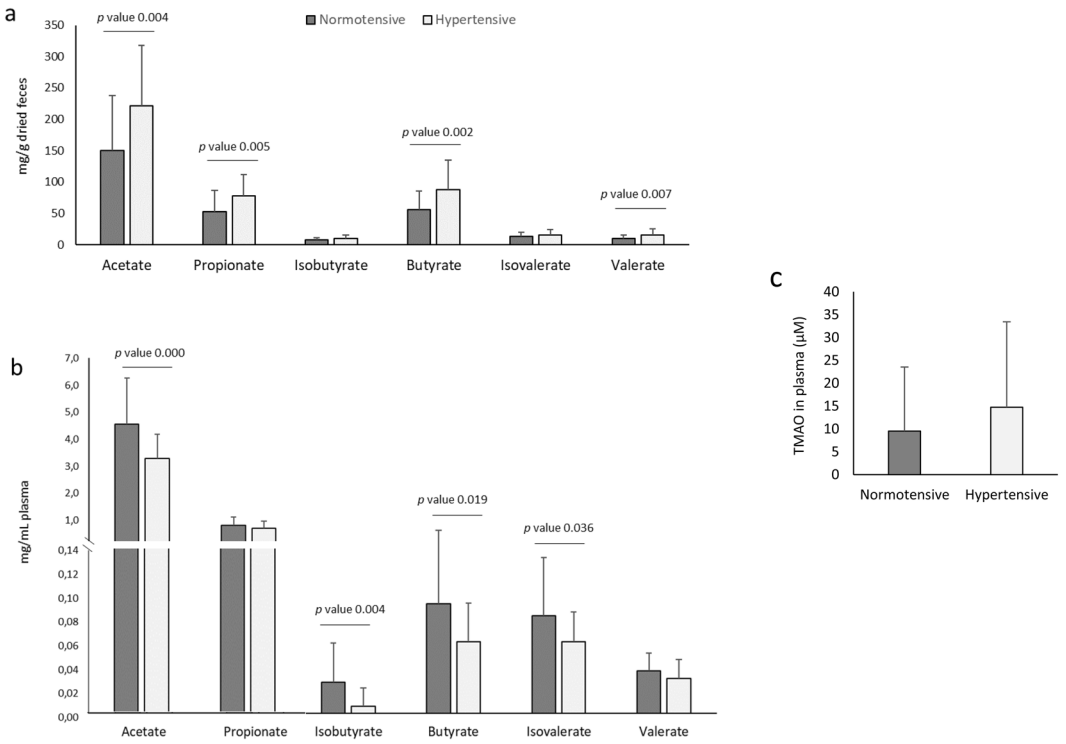


Figure 6. Mean values ± standard deviation of bacterial metabolites detected in plasma and feces. **(a)** Short chain fatty acids in feces expressed as mg/g dried feces (n = 61), (**b)** short chain fatty acids in plasma expressed as mg/mL (n = 61) and (**c)** trimethylamine N-oxide in plasma expressed as μmol/L (n = 59).

hypothesis that SCFA induce vasorelaxation[43,44], which may be due to an increase in vascular tone regulated by the action of SCFA on Gpr41 in the vascular endothelium. Our results provide human evidence that significant lower levels in circulation of SCFA, especially acetate, the most abundant SCFA in plasma, could be in part a causative factor for the higher BP levels in HT subjects.

Correlation of SCFA with clinical variables and diet. The relation between microbial metabolites, clinical variables and dietary parameters was also tested (**Additional file 7:** Table S3). As indicated, most of fecal SCFA detected in feces appeared to have a positive correlation with SBP and DBP. When we tested the relationships between plasma SCFA and BP, we only found a negative correlation between DBP and acetate ($r = -0{,}26$, $p = 0.045$), isobutyric ($r = -0{,}33$, $p = 0.009$) and isovaleric acid ($r = -0{,}30$, $p = 0.017$), and between SBP and isobutyric acid ($r = -0{,}42$, $p = 0.001$). However, other positive correlations were observed between anthropometrical parameters such as weight, BMI and waist circumference and fecal acetate, propionate, butyrate, valeric acid, isobutyrate and isovaleric acid, indicating that other variables could have influenced in the SCFA production or absorption efficiency.

In relation to diet, clinical studies have shown that a high intake of fruit and vegetables, considered sources of SCFA, is associated with reduced BP levels[45]. In the present study, the intake of fruits and vegetables was not different between both groups but the NT group presented a higher total dietary fiber intake, probably due to other foods rich in fiber such as whole-grain cereals **(Additional File 3:** Table S2). Despite that, a negative correlation was observed between fecal propionate, valeric and isobutyric acids and total dietary fiber intake (**Additional file 7:** Table S3), which could be related to the lower levels of fecal SCFA detected in NT compared to HT subjects. HT group also presented a higher intake of starch food sources such as potatoes, and starches presented a positive correlation with plasma propionate. These results could be related to the fermentation of resistant starches that contribute positively to colonic SCFA production as previously reported[46].

TMAO in plasma. No significant differences in fasting TMAO concentrations between HT and NT groups were found (Fig. 6c). Although a large body of evidence supports that TMAO is involved in atherosclerosis, the existing body of literature is sparse regarding TMAO and hypertension. Only in HT rats an increased permeability of the colonic gut-blood barrier to TMA, the main TMAO precursor, has been evidenced[47], but the role of TMAO in HT humans remains unclear.

Despite not reporting significant differences between groups, results from Pearson correlations showed significant positive relationships between TMAO and total cholesterol ($r = 0.28$, $p = 0.030$), LDL-cholesterol ($r = 0.30$, $p = 0.019$) and fasting blood glucose ($r = 0.27$, $p = 0.034$) (**Additional file 7:** Table S3). In fact, TMAO molecule has been identified as a novel biomarker of cardiovascular risk, and increased levels have been negatively associated with 'reverse cholesterol transport' and also with defects in cholesterol metabolic pathways[48,49]. Similarly to

our findings, a cross-sectional study performed with subjects at risk for type 2 diabetes found positive relationships of fasting TMAO concentrations with total- and LDL-cholesterol, and fasting glucose[50].

As observed (Fig. 6c), a great inter-individual variability was reported in fasting TMAO concentration. In accordance with a recent review, circulating TMAO levels are influenced by several factors including gut microbiota composition and activity, liver function and excretion, gut-blood barrier function and diet[51]. In the present study, we found no significant associations between TMAO and dietary food sources reported by 3-day dietary records. This may be explained in part by the short time elapsed between the intake of TMAO-rich food (such as fish) or its dietary precursors (choline and carnitine found in meat and eggs), and the appearance of the metabolite in plasma, as reported by Cho *et al.*[52]. As suggested by other authors[53], high inter-variation in TMAO plasma levels, may be attributed to differences in gut microbiota composition and function. Some families of bacteria from Firmicutes and Proteobacteria phyla are potent choline and carnitine consumers, and are able to synthetize TMA through the expression of specific enzymes. In addition, other biochemical factors such as hepatic FMO3 expression and activity can affect TMAO levels. Hence, the highly variable plasma levels in a disease versus non-disease state are influenced by differences in gut bacterial composition, and it does not necessarily have to be a marker in the disease process. Therefore, for future studies, further assessment of TMAO status is needed and it should include the collection of repeated samples at different times and the determination of the average levels.

Conclusions

The present study revealed that individuals with hypertension in a first disease grade prior to drug treatment possess a particular fecal bacterial signature. Our results provide a new approach to distinguish HT individuals from healthy subjects according to a specific bacterial and SCFA profile, despite the non-significant results in the overall composition of bacterial community. Specifically, HT subjects were characterized by lower abundance of SCFA producers *Faecalibacterium prausnitzii, Roseburia hominis*, Ruminococcaceae NK4A214, Ruminococcaceae_UCG-010, Christensenellaceae_R-7, and higher abundance of *Bacteroides coprocola, Bacteroides plebeius* and genera of *Lachnospiraceae*. Some of these bacterial taxa appeared to be positively and negatively associated with SBP and DBP after adjustment for confounders such as fiber intake, age and anthropometric variables. These results indicate that these taxa-specific differences detected in HT are not explained by the potential confounders and, therefore, they could be intrinsically related to hypertension. We also corroborate that SCFA fecal levels do not reflect SCFA levels in circulation, highlighting the importance of analysing SCFA in plasma, where they can be sensed by host GPCRs. As far as we are aware, this is the first time to show that higher fecal excretion of most of the SCFA (acetate, propionate, butyrate, valerate) together with lower plasmatic levels (acetate, isobutyrate, butyrate, isovalerate) is associated with hypertension in humans. In accordance with previous animal studies, we hypothesize that these opposed results could indicate a less efficient SCFA absorption in HT subjects. Further in-depth research must be done to elucidate how differ the production and metabolism of SCFA between HT and NT and to better understand the potential connection between hypertension and SCFA.

Despite the relevant results presented here, some limitations of the study must be noted. The number of subjects was low and not strictly homogenous in terms of age and several clinical variables. Also, the grade I hypertension in HT subjects could be influenced by the white-coat and masked hypertension. To minimize this effect, we monitored SBP and DBP by using multiple automated sphygmomanometer in two repeated readings, and with participants resting in a sitting position alone and unobserved in a quiet environment. By applying these practices the white-coat effect can be substantially reduced or eliminated[1].

Overall, our results present a disease classifier based on microbiota and bacterial metabolites to distinguish HT from NT individuals in a first disease grade prior to drug treatment. Moreover, it is newly reported that HT subjects showed a particular SCFA profile in feces and plasma that could indicate a less efficient SCFA absorption.

Materials and Methods

Subjects and study design. All individuals included in the present study were recruited between 9 June 2016 and 28 November 2017. Participants were recruited in Reus (Spain) by using tableaux advertisements in the Hospital Universitari Sant Joan (HUSJ), and using databases of volunteers who have previously participated in studies carried out in our research group. HT participants were included into the study if they were at grade I hypertension, defined as SBP between 140 and 159 mmHg and without major complications according to the ESC/ESH Guidelines (2018)[1], and were not using antihypertensive treatment. NT participants were included if presented optimal SBP < 120 mmHg. Additionally, SBP between 140 and 159 mmHg also correspond to stage 1 hypertension defined by the US -JNC7 report[54].

All participants fulfilled the following criteria: to be aged from 18 to 65, without family history for cardiovascular disease or evidence of chronic disease, and with willingness to provide informed consent before the initial screening visit. Subjects with BMI ≥ 30 kg/m^2; fasting glucose > 126 mg/dL; LDL-cholesterol > 190 mg/dL; triglycerides > 350 mg/dL, smoking, and suffering anemia or intestinal disorders were excluded. Individuals were also excluded if they had received antibiotics or probiotics within the last 3 months, and if they were following a vegetarian diet.

A total of 61 participants, 29 HT and 32 NT, were enrolled in the study. Volunteers attended to 2 visits at the HUSJ and Eurecat-Reus where the study was performed. In a first pre-selection visit, a clinical interview to verify that participants met all the eligibility criteria was done.

Study was approved by local ethics committee (Clinical Research Ethical Committee of HUSJ, Reus with 15-11-26/11obs4 reference) and informed consent was obtained from all subjects. The protocol and trial were conducted in accordance to the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization (ICH GCP).

Biological samples collection. Blood samples were collected at fasting state in serum and plasma blood collection tubes. Both samples were collected and kept at −80 °C until the further biochemical analysis.

Participants received detailed instructions to collect fecal samples. They were provided with a Protocult™ stool collection device (ABC, Minnesota, EEUU) and two different containers: a sterile pot and a specimen container with spoon containing 10 mL of RNAlater® storage solution (Sigma-Aldrich Quimica SL; Madrid, Spain). The volunteers were asked to transfer a small amount of fresh feces from the sterile pot to the other tube immediately after defecation and to freeze the samples. Volunteers transported the frozen samples to the laboratory with ice pack. Fecal samples from the sterile pot were lyophilized and stored at −80 °C until the chromatographic analysis to determinate SCFA. The feces preserved in RNAlater®, also stored at −80 °C, were used for the analysis of microbiota composition.

Clinical outcomes measurement. All clinical information was collected according to standard procedures and was measured in both visits. SBP and DBP was measured in a sitting position after 2–5 minutes participants respite by using an automatic sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain). Two readings were recorded with 1-min interval, and the average value was used for statistical analyses. Weight and body composition was measured by trained dietitians with a body composition analyzer (Tanita Leicester Portable; Tanita Corp., Barcelona, Spain). Waist circumference was measured at the umbilicus level using a 150 cm anthropometric steel measuring tape[55].

Lipid profile. A fasting blood sample was obtained in the second visit to determine lipid profile. Briefly, total cholesterol, LDL-cholesterol, high density lipoprotein cholesterol (HDL-cholesterol), triglycerides, Apolipoprotein A-1 (ApoA-1) and Apolipoprotein B-100 (ApoB-100) concentrations were measured in serum by standardized enzymatic automated methods in an autoanalyzer (Beckman Coulter-Synchron, Galway, Ireland).

Lifestyle outcomes measurement. Diet composition was assessed through a 3-day dietary record (2 labor days and 1 week-end day) and calculated by Spanish Food Composition Tables[56], and dietary habits were self-reported by volunteers through a validated, semi-quantitative, FFQ containing 137 food items related to the Mediterranean diet[57]. Physical activity was evaluated by completion of a validated questionnaire (Physical Activity Questionnaire Class AF)[58]. Usual sleep habits were assessed by Pittsburgh Sleep Quality Index (PSQI)[59].

Determination of fecal and plasma SCFA. For the sample extraction, 0.1 g of lyophilized feces were mixed with 1 mL of acidified aqueous solution (1% phosphoric acid) containing 4-methyl valeric acid (Sigma-Aldrich, St. Louis, MO, USA) as internal standard (IS, final concentration 500 μM). Samples were shaken for 15 min and centrifuged (10 min, 1800 × *g*, 4 °C). Before filtration (0.22 mm pore size filter), the supernatants were centrifuged (4 min, 8784 × *g*, 4 °C) once more. The analysis of acetic, propionic, butyric, isobutyric, isovaleric, and valeric acids was performed by gas chromatography (Agilent 7890 A Series) using a capillary BP-21 column (30 m, 0.25 mm, 0.25 μm; SGE, Cromlab SL, Barcelona, Spain), coupled to a flame ionization detector (GC-FID). The column temperature was programed at 90 °C, rising by 15 °C/min until it reached 150 °C, then 5 °C/min to 170 °C, and then 20 °C/min to 240 °C, and maintained 3 min (total run time 14.5 min). Helium was the carrier gas (1 mL/min). Injection was carried out with a split injector (1:100) at 220 °C, detector temperature was 250 °C, and 1 μL of the solution was injected into the GC-FID system. Identification of the SCFA was carried out according to the retention time of standard compounds (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid; Sigma-Aldrich) and their quantification was determined with reference to the peak side of IS (4-methyl valeric acid). All samples were analyzed in triplicate.

Quantification of SCFA in plasma was performed as described previously by Zhang *et al*. (2019)[60], with some modifications. Briefly, for sample extraction 200 μL of plasma was mixed with 200 μL of milliQ water containing 2-methyl valeric as IS and with 200 μL of diethyl ether (DE) in a 0.6 mL microtube containing a drop of hydrochloric acid (37%). Samples were shaken for 5 min at 4 °C and centrifuged (15 min, 9000 rpm, 4 °C). The DE layer (containing SCFA) was transferred to a new 0.6 mL microtube containing a small amount of anhydrous Na_2SO_4 (to remove the residual water). The remaining aqueous layer was further extracted with DE for two more times. The DE layers were pooled and mixed for further derivatization. For the derivatization procedure 210 μl of DE extract was accurately transferred into a glass insert in a GC vial and capped tightly after added 20 μl of (trimethylsilyl)-trifluoroacetamide (BSTFA). The mixture was kept in the GC vial and incubated with agitation at 37 °C for 2 h, 37 °C. The analysis of SCFA was performed by gas chromatography (Agilent 6890N-MSD 5973) using a DB5 MS-UI column (30 m, 0.25 mm, 0.25 μm; J&W, Agilent Tech), coupled to a mass detector using SIM mode. 1 μl of derivatized sample was injected with split ratio of 1:5. All the procedure was performed at low temperature using ice and cooling the sample carousel. The contents of SCFA were calculated with internal standard method. All samples were analyzed in duplicate, reporting coefficient of variability values lower than 10%.

TMAO in plasma. Quantification of TMAO in plasma samples was performed as described previously[61]. Briefly, for the sample extraction, 25 μL of plasma was mixed with 80 μL of methanol with labelled IS working solution (TMAO-d9; Cambridge Isotope Laboratories, Massachusetts, USA) and mixed 30 seconds to precipitate proteins. The samples were subjected to centrifugation at 9000 rpm for 5 min at room temperature, and the supernatants were diluted with 150 uL of MilliQ water. Diluted samples were filtered with PVDF filters 0.22 μm and transferred into HPLC vials for analysis. The analysis was performed by liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) (Waters, Milford, MA, USA) using a column Acquity UPLC BEH HILIC (1,7 μm 2,1 × 100 mm).

Fecal microbiota analysis. *DNA purification and sequencing.* Fecal samples stored in RNAlater® were diluted with PBS solution (dilution1:2). To remove fecal debris, the samples were centrifuged at 2000 rpm at 4 °C for 2 min. Total DNA was extracted from pelleted bacterial cells in the robotic workstation The MagNA Pure LC Instrument (Roche) using the MagNA Pure LC DNA isolation kit III (Bacteria, Fungi) (Roche) according to the manufacturer's instructions. The region V3-V4 of the 16S rRNA gene was amplified by PCR and used to amplicon library construction following Illumina instructions. Metagenomic libraries were constructed using NEXTERA XT kit according to the manufacturer's instructions (Illumina). Sequencing was performed with the Kit V3 (2 × 300 cycles) in MiSeq platform (Illumina, Eindhoven, Netherlands) in the Centre for Public Health Research (FISABIO-Salud Pública, Valencia, Spain). All sequences were deposited in the public European Nucleotide. Archive server under accession number PRJEB32411.

Sequence analysis. 16S rRNA gene reads with low-quality score and short read length as well as potential chimeras were removed using DADA2 pipeline in R package[62]. We used DADA2 pipeline to create the ASV. The taxonomic information of the 16S rDNA sequences was obtained by comparison with SILVA database (v.132)[63]. We considered only annotations that were obtained with a bootstrap value greater than 0.8, leaving the assignation at the last well-identified level and consecutive levels as unclassified.

For metagenome analysis, sequence trimming, filtering by quality and removal of host sequences were performed using a custom pipeline. Overlapping paired-end reads were joined using FLASH-1.2.11[64] applying default parameters. Next, we assembled the filtered sequences into contigs, using MEGAHIT v1.1.2[65]. To know the number of reads in each contig, the reads were mapped against the resulting contigs with bowtie2. Not assembled sequences were appended to the contigs. Subsequently, prediction of open reading frames (ORF) was implemented by the program Prodigal v2.6.3[66]. Functional assignment was carried out aligning the ORF dataset via HMMER (v3.1b2)[67] against the TIGRFAM database of prokaryotic protein family models (v9.0)[68]. After obtaining the functional annotation and the alignment coordinates for each obtained match, these coordinates were used to identify putative genes within the contigs. Finally, we aligned the filtered sequencing reads to the putative annotated genes in the contigs via megaBLAST and we quantified the abundace of each gene by counting the aligned reads, using in-house R scripts.

Statistical analysis and bioinformatics analysis. Statistical analysis of clinical parameters was performed using IBM SPSS version 23.0 (IBM SPSS, Inc, Chicago, IL, USA). The normality of variables was assessed by using the Kolmogorov-Smirnov test. The Mann-Whitney test was used for comparison of non-normally distributed variables. Student's t-test was used for comparison of normally distributed variables. Fisher's exact test was used for categorical variables comparisons.

Pearson correlation coefficients were calculated for relationships between bacterial metabolites, SCFA and TMAO, and clinical and dietary variables in both groups. Descriptive data were expressed as mean ± SD and percentages for categorical variables. The level of statistical significance was set at $p < 0.05$.

The alpha diversity (Chao1 richness estimator and the Shannon diversity index) was determined at ASV level using vegan library from the R package[62]. To analyze beta diversity, Bray-Curtis dissimilarity index was calculated on the base of the abundance matrix from the taxonomic and functional composition to quantify the compositional and functional dissimilarity between two different communities.

Box plots, Principal Coordinates analysis (PCoA), sample clustering Principal Component analysis (PCA) and heatmaps were generated with in-house R scripts. The pairwise comparisons of continuous variables were analyzed using Wilcoxon rank-sum test.

To statistically assess the effect of the environmental factors on the bacterial and functional composition, a multivariate analysis of variance based on dissimilarity tests (Adonis) was applied as implemented in the vegan library in R package (library "vegan" function "adonis"). To identify ASVs and metabolic pathways as biomarkers, we applied the linear discriminant analysis (LDA) effect size (LEfSe) algorithm[69]. We fixed an α-value <0.05 and the threshold used to consider a discriminative feature for the logarithmic LDA score was set at >2 or >2.5.

We assessed putative correlations between ASV biomarkers and clinical variables by means of a linear regression model setting ASV biomarkers as predictors and clinical variables as response variable. Moreover, we also computed a Spearman's test for each association. Thus, only pairs with statistically significant association ($p < 0.05$), based on both criteria, were selected to further analysis. To address potential confounding correlations, we adjusted the previous linear regression model by adding one by one the confounding variables as regressors in order to guarantee that, even in the presence of these confounders, the variance explained by the clinical variables (or metabolites) was still statistically significant. We adjusted for age, waist circumference (cm), LDL-cholesterol (mg/dL), BMI (kg/m^2), fat mass (%) and dietary fiber intake (g/day). All computations were performed using R base functionalities (R core package, 2014), in particular 'lm' and 'cor.test' functions.

To control the false discovery rate, we validated the statistical tests adjusting all p-values using the Benjamini-Hochberg correction[70] in R package (library "stats", function "p.adjust").

Random forest analysis. We applied random forest modeling[71] to find discriminant taxa (biomarkers) capable of correctly assigning the health status of our individuals. This algorithm is very flexible at capturing complex interactions between biomarkers as occur in our data. Random forest begins analyzing a first model considering the whole set of features and provides, from this first step, a measure of importance associated to each taxon. Then, we analyze successively additional models including lower and lower taxa with higher and higher associated importance as long as the prediction performance of the models increases. Since the number of parameters of those successive models decrease, we also get lower and lower overfitting. To compute the prediction performance of the model at each step, we perform 100-fold cross-validation at equal rates for training and test

to obtain false positive and true positive rates. Then, by varying the discrimination threshold for classification, we get a ROC curve for the model and score it by means of the Area Under this Curve (AUC), so higher AUC better prediction performance.

Received: 7 July 2019; Accepted: 24 March 2020;
Published online: 15 April 2020

References

1. Esh, H. *et al. 2018 ESC/ESH HYPERTENSION Guidelines*, https://doi.org/10.1097/HJH (2018).
2. Zhou, B. *et al.* Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19·1 million participants. *The Lancet* **389**, 37–55 (2017).
3. Mills, K. T. *et al.* HHS Public Access. *Pmc* **134**, 441–450 (2017).
4. Chow, C. K. *et al.* Prevalence, awareness, treatment, and control of hypertension in rural and urban communities in high-, middle-, and low-income countries. *JAMA - Journal of the American Medical Association* **310**, 959–968 (2013).
5. Rossier, B. C., Bochud, M. & Devuyst, O. The Hypertension Pandemic: An Evolutionary Perspective. *Physiology* **32**, 112–125 (2017).
6. Sandosh, P. & Mark, C. & F., D. A. Genetic and Molecular Aspects of Hypertension. *Circulation Research* **116**, 937–959 (2015).
7. Marques, F. Z., Mackay, C. R. & Kaye, D. M. Beyond gut feelings: How the gut microbiota regulates blood pressure. *Nature Reviews Cardiology* **15**, 20–32 (2018).
8. Yang, T. *et al.* Gut dysbiosis is linked to hypertension. *Hypertension (Dallas, Tex.: 1979)* **65**, 1331–1340 (2015).
9. Mell, B. *et al.* Evidence for a link between gut microbiota and hypertension in the Dahl rat. *Physiological Genomics* **47**, 187–197 (2015).
10. Li, J. *et al.* Gut microbiota dysbiosis contributes to the development of hypertension. *Microbiome* **5**, 1–19 (2017).
11. Santisteban, M. M. *et al.* Hypertension-Linked Pathophysiological Alterations in the Gut. *Circulation research* **120**, 312–323 (2017).
12. Koh, A., De Vadder, F., Kovatcheva-Datchary, P. & Bäckhed, F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell* **165**, 1332–1345 (2016).
13. Natarajan, N. *et al.* Microbial short chain fatty acid metabolites lower blood pressure via endothelial G protein-coupled receptor 41. *Physiological Genomics* **48**, 826–834 (2016).
14. Pluznick, J. L. *et al.* Olfactory receptor responding to gut microbiota-derived signals plays a role in renin secretion and blood pressure regulation. *Proceedings of the National Academy of Sciences* **110**, 4410–4415 (2013).
15. de la Cuesta-Zuluaga, J. *et al.* Higher Fecal Short-Chain Fatty Acid Levels Are Associated with Gut Microbiome Dysbiosis, Obesity, Hypertension and Cardiometabolic Disease Risk Factors. *Nutrients* **11**, 51 (2018).
16. Tang, W. H. W. & Hazen, S. L. The contributory role of gut microbiota in cardiovascular disease. *The Journal of clinical investigation* **124**, 4204–4211 (2014).
17. Wang, Z. *et al.* Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. *European Heart Journal* **35**, 904–910 (2014).
18. Koeth, R. A. *et al.* NIH Public Access. *HHS Public Access* **19**, 576–585 (2013).
19. Kalnins, G. *et al.* Structure and Function of CutC Choline Lyase from Human Microbiota Bacterium Klebsiella pneumoniae. *The Journal of biological chemistry* **290**, 21732–21740 (2015).
20. Senthong, V. *et al.* Plasma Trimethylamine N-Oxide, a Gut Microbe–Generated Phosphatidylcholine Metabolite, Is Associated With Atherosclerotic Burden. *Journal of the American College of Cardiology* **67**, 2620–2628 (2016).
21. de la Cuesta-Zuluaga, J. *et al.* Gut microbiota is associated with obesity and cardiometabolic disease in a population in the midst of Westernization. *Scientific Reports* **8**, 1–14 (2018).
22. Rogers, M. A. M. & Aronoff, D. M. The influence of non-steroidal anti-inflammatory drugs on the gut microbiome. *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **22**, 178. e1–178.e9 (2016).
23. Yan, Q. *et al.* Alterations of the Gut Microbiome in Hypertension. *Frontiers in Cellular and Infection Microbiology* **7**, 1–9 (2017).
24. Song, S. *et al.* Beneficial effects of a probiotic blend on gastrointestinal side effects induced by leflunomide and amlodipine in a rat model. 1–8, https://doi.org/10.3920/BM2016.0231 (2017).
25. Shoaie, S. *et al.* Quantifying Diet-Induced Metabolic Changes of the Human Gut Microbiome. *Cell Metabolism* **22**, 320–331 (2015).
26. Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R. & White, B. A. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. **6**, 121–131 (2008).
27. Chassard, C. & Bernalier-Donadille, A. H2 and acetate transfers during xylan fermentation between a butyrate-producing xylanolytic species and hydrogenotrophic microorganisms from the human gut. *FEMS Microbiology Letters* **254**, 116–122 (2006).
28. Bilen, M. *et al.* 'Pygmaiobacter massiliensis' sp. nov., a new bacterium isolated from the human gut of a Pygmy woman. *New microbes and new infections* **16**, 37–38 (2016).
29. Sakamoto, M., Iino, T. & Ohkuma, M. Faecalimonas umbilicata gen. nov., sp. nov., isolated from human faeces, and reclassification of Eubacterium contortum, Eubacterium fissicatena and Clostridium oroticum as faecalicatena contorta gen. nov., comb. nov., Faecalicatena fissicatena comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **67**, 1219–1227 (2017).
30. Zhang, M. *et al.* Faecalibacterium prausnitzii produces butyrate to decrease c-Myc-related metabolism and Th17 differentiation by inhibiting histone deacetylase 3. *International Immunology* **31**, 499–514 (2019).
31. Goodrich, J. K. *et al.* Human genetics shape the gut microbiome. *Cell* **159**, 789–799 (2014).
32. Pluznick, J. L. Microbial Short-Chain Fatty Acids and Blood Pressure Regulation. *Current Hypertension Reports* **19**, 1–9 (2017).
33. Marques, F. Z. *et al.* High-fiber diet and acetate supplementation change the gut microbiota and prevent the development of hypertension and heart failure in hypertensive mice. *Circulation* **135**, 964–977 (2017).
34. Bier, A. *et al.* A high salt diet modulates the gut microbiota and short chain fatty acids production in a salt-sensitive hypertension rat model. *Nutrients* **10**, 1–10 (2018).
35. Rahat-Rozenbloom, S., Fernandes, J., Gloor, G. B. & Wolever, T. M. S. Evidence for greater production of colonic short-chain fatty acids in overweight than lean humans. *International journal of obesity (2005)* **38**, 1525–1531 (2014).
36. Schwiertz, A. *et al.* Microbiota and SCFA in Lean and Overweight Healthy Subjects. **18** (2010).
37. Fernandes, J., Su, W., Rahat-Rozenbloom, S., Wolever, T. M. S. & Comelli, E. M. Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. *Nutrition & diabetes* **4**, e121–e121 (2014).
38. Teixeira, T. F. S. *et al.* Higher level of faecal SCFA in women correlates with metabolic syndrome risk factors. *British Journal of Nutrition* **109**, 914–919 (2013).
39. Vogt, J. A. & Wolever, T. M. S. Fecal Acetate Is Inversely Related to Acetate Absorption from the Human Rectum and Distal Colon. *The Journal of Nutrition* **133**, 3145–3148 (2003).
40. Yang, T. *et al.* Impaired butyrate absorption in the proximal colon, low serum butyrate and diminished central effects of butyrate on blood pressure in spontaneously hypertensive rats. *Acta Physiologica* **226**, e13256 (2019).
41. Kim, S. *et al.* Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure. *Clinical science (London, England: 1979)* **132**, 701–718 (2018).

42. Durgan, D. J. *et al.* Role of the Gut Microbiome in Obstructive Sleep Apnea-Induced Hypertension. *Hypertension (Dallas, Tex.: 1979)* **67**, 469–474 (2016).
43. Mortensen, F. V., Nielsen, H., Mulvany, M. J. & Hessov, I. Short chain fatty acids dilate isolated human colonic resistance arteries. *Gut* **31**, 1391–1394 (1990).
44. Knock, G., Psaroudakis, D., Abbot, S. & Aaronson, P. I. Propionate-induced relaxation in rat mesenteric arteries: a role for endothelium-derived hyperpolarising factor. *The Journal of physiology* **538**, 879–890 (2002).
45. Miura, K., Stamler, J., Liu, K., Daviglus, M. L. & Nakagawa, H. Relation of Vegetable, Fruit, and Meat Intake to 7-Year Blood Pressure Change in Middle-aged Men The Chicago Western Electric Study. **159**, 572–580 (2004).
46. Bird, A. R., Conlon, M. A., Christophersen, C. T. & Topping, D. L. Resistant starch, large bowel fermentation and a broader perspective of prebiotics and probiotics. *Beneficial Microbes* **1**, 423–431 (2010).
47. Jaworska, K. *et al.* Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite. *Plos One* **12**, e0189310–e0189310 (2017).
48. Warrier, M. *et al.* The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance. *Cell reports* **10**, 326–338 (2015).
49. Schiattarella, G. G. *et al.* Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: a systematic review and dose-response meta-analysis. 2948–2956, https://doi.org/10.1093/eurheartj/ehx342 (2018).
50. Randrianarisoa, E. *et al.* Relationship of Serum Trimethylamine N-Oxide (TMAO) Levels with early Atherosclerosis in Humans. *Nature Publishing Group*, 1–9, https://doi.org/10.1038/srep26745 (2016).
51. Nowiński, A. & Ufnal, M. AC SC. *Nutrition*, https://doi.org/10.1016/j.nut.2017.08.001 (2017).
52. Cho, C. E. *et al.* Trimethylamine- N -oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: A randomized controlled trial. 1600324, 1–12 (2017).
53. Canyelles, M. *et al.* Trimethylamine N-Oxide: A Link among Diet, Gut Microbiota, Gene Regulation of Liver and Intestine Cholesterol Homeostasis and HDL Function. *International journal of molecular sciences* **19**, 3228 (2018).
54. V., C. A. *et al.* Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension* **42**, 1206–1252 (2003).
55. McAuley, P. A. *et al.* Physical Activity, Measures of Obesity, and Cardiometabolic Risk: The Multi-Ethnic Study of Atherosclerosis (MESA). *Journal of physical activity & health* **11**, 831–837 (2013).
56. *Centro de Enseñanza superior de Nutrición Humana y Dietética. Tablas de Composición de Alimentos por Medidas Caseras de Consumo Habitual en España (Food composition tables in household measures commonly consumed in Spain)*. (2008).
57. Fernández-Ballart, J. D. *et al.* Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *British Journal of Nutrition* **103**, 1808–1816 (2010).
58. Vallbona Calbó, C., Roure Cuspinera, E., Violan Fors, M. & Alegre Martín, J. *Guia de prescripció d'exercici físic per a la salut (PEFS)*. (2007).
59. Buysse, D. J., Reynolds, C. F., Monk, T. H., Berman, S. R. & Kupfer, D. J. The Pittsburgh sleep quality index: A new instrument for psychiatric practice and research. *Psychiatry Research* **28**, 193–213 (1989).
60. Zhang, S., Wang, H. & Zhu, M.-J. A sensitive GC/MS detection method for analyzing microbial metabolites short chain fatty acids in fecal and serum samples. *Talanta* **196**, 249–254 (2019).
61. Zhao, X., Zeisel, S. H. & Zhang, S. Rapid LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine N-oxide, and creatinine in human plasma and urine. *ELECTROPHORESIS* **36**, 2207–2214 (2015).
62. R, R. D. C. T. & R Core Team. *R: A language and environment for statistical computing. R Found Stat Comput* 3, (2013).
63. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* **41**, D590–D596 (2013).
64. Magoč, T. & Salzberg, S. L. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics (Oxford, England)* **27**, 2957–2963 (2011).
65. Li, D., Liu, C.-M., Luo, R., Sadakane, K. & Lam, T.-W. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* **31**, 1674–1676 (2015).
66. Hyatt, D. *et al.* Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC bioinformatics* **11**, 119 (2010).
67. Durbin, R., R. Eddy, S., Krogh, A. & J. Mitchison, G. *Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids. Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids* **3** (1998).
68. Selengut, J. D. *et al.* TIGRFAMs and Genome Properties: tools for the assignment of molecular function and biological process in prokaryotic genomes. Nucleic acids research 35, D260–D264 (2007).
69. Segata, N. *et al.* Metagenomic biomarker discovery and explanation. *Genome biology* **12**, R60–R60 (2011).
70. Benjamini, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. *Series B (Methodological)* **57**, 289–300 (1995).
71. Liaw, A. & Wiener, M. Classification and Regression by RandomForest. *Forest* **23** (2001).

Acknowledgements

We wish to acknowledge the support of the Institut d'Investigació Sanitària Pere Virgili (IISPV) and the EURECAT-Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut (Reus, Spain), the University of Lleida through the SY grant, the LR Sara Borrell postdoctoral grant (CD14/00275), and AP Torres Quevedo contract (Subprograma Estatal de Incorporación, Plan Estatal de Investigación Científica y Técnica y de Innovación). LC was granted by Ayudas Para La Promoción De Empleo Joven E Implantación De La Garantía Juvenil En I + D + I (PEJ-2014-A-67028). NFOC-Salut group is a consolidated research group of Generalitat de Catalunya, Spain (2017 SGR522).

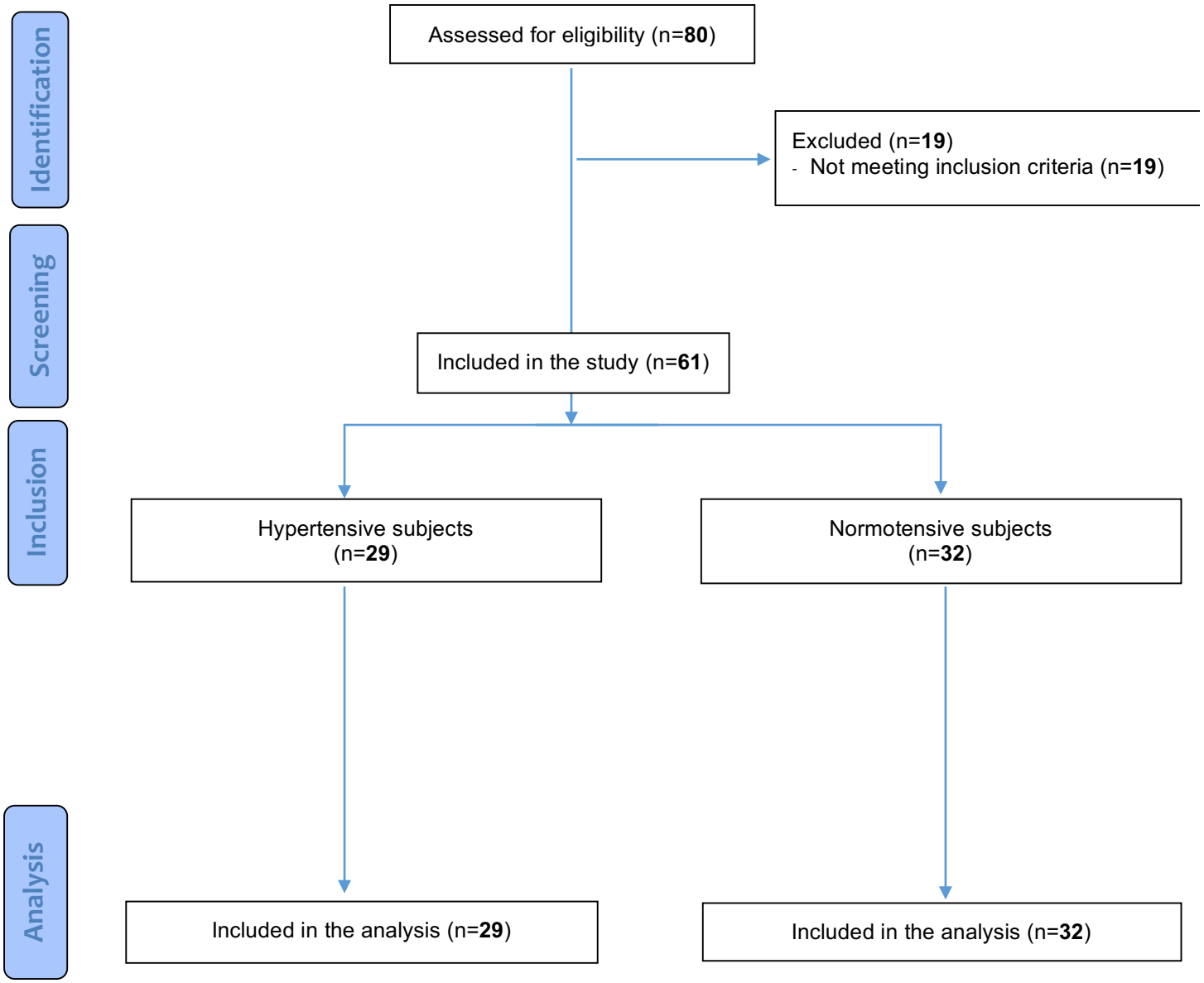
Author contributions

L.R. and R.S. conceived the study and directed the project. L.R., A.P., R.V., E.L., L.P., J.C. and L.C. executed the study, obtained the samples and clinical details. M.J.G., L.R. and N.J. performed fecal microbiota sequencing. A.A. and M.J.G. perfomed the computational metagenomic analysis. M.J.G., L.R. and N.J. assisted with microbiome sample processing. L.R., S.Y., I.L. and M.P.R. performed the analysis of fecal metabolites in plasma and feces. L.R., L.C. and M.J.G. interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional file 1. Figure S1. Flow diagram participants of study



Additional file 2: Table S1 | Mean energy and daily nutrients intake.

Nutrient	Hypertensive (n=28)	Normotensive (n=32)	P-value
Energy (Kcal)	2089.74 ± 543.43	2208.23 ± 654.06	0.452
Protein *(g)*	88.28 ± 21.28	94.10 ± 29.87	0.395
Total carbohydrates *(g)*	197.03 ± 61.41	212.65 ± 60.90	0.328
Complex carbohydrates	110.35 ± 42.29	116.00 ± 27.31	0.548
Added sugars	87.87 ± 29.64	96.62 ± 40.38	0.767
Total fat *(g)*	96.95 ± 31.67	102.70 ± 41.93	0.556
SFA	27.54 ± 10.54	27.75 ± 12.47	0.944
MUFA	45.40 ± 15.63	49.67 ± 21.92	0.395
PUFA	15.92 ± 7.29	17.22 ± 8.58	0.563
Total cholesterol *(mg)*	328.25 ± 130.77	358.22 ± 187.89	0.482
Dietary fiber *(g)*	20.95 ± 9.90	25.90 ± 11.63	**0.029**
Ethanol *(g)*	11.37 ± 13.04	7.76 ± 10.11	0.131
Sodium *(mg)*	2371.75 ± 858.61	2433.50 ± 824.65	0.778
Potassium *(mg)*	3409.72 ± 718.93	3619.49 ± 1110.46	0.383

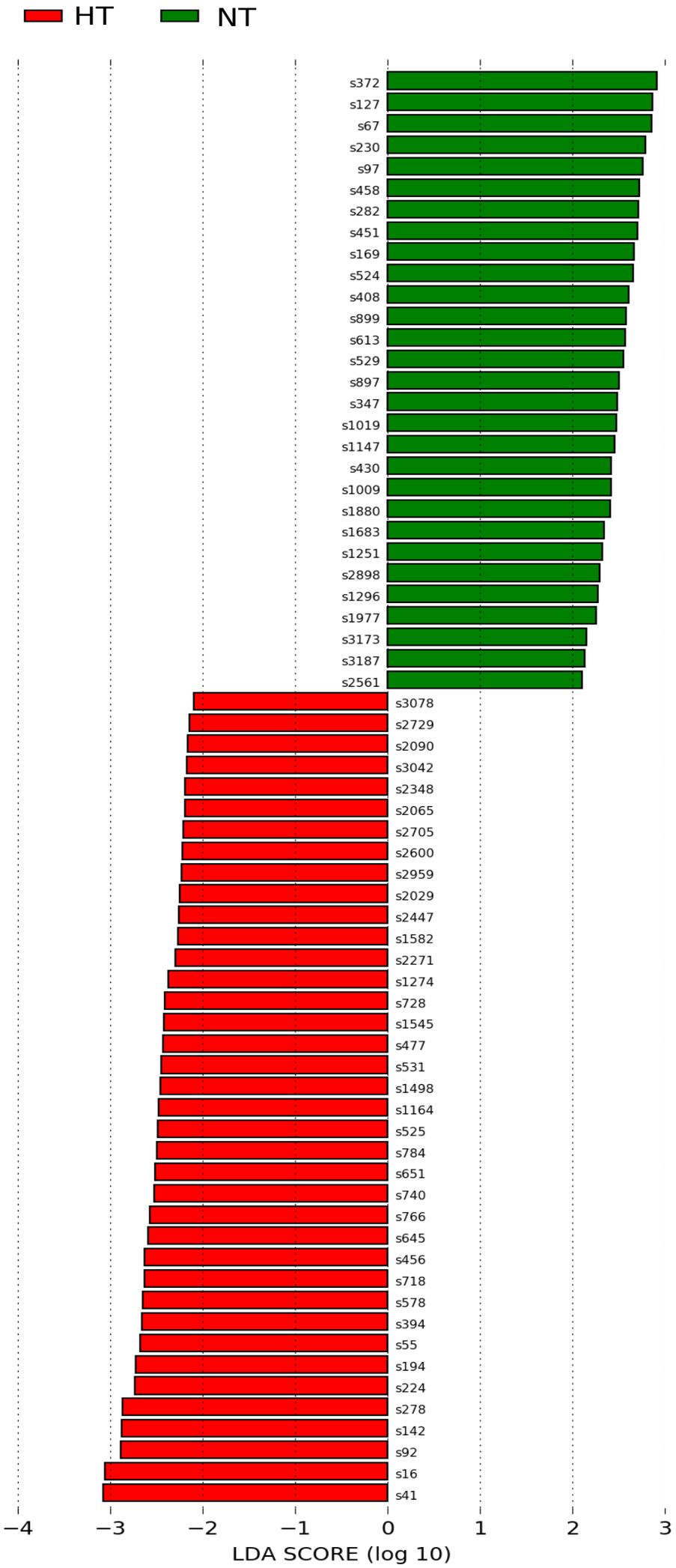
Data expressed as mean ± standard deviation. Daily mean nutrients intake was estimated from 3-day dietary record. SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. P-value estimated by Student's t-test and Mann-Whitney U test. Significant differences depicted in **bold.**

Additional file 3: Table S2 | Mean intake in g/day by food group.

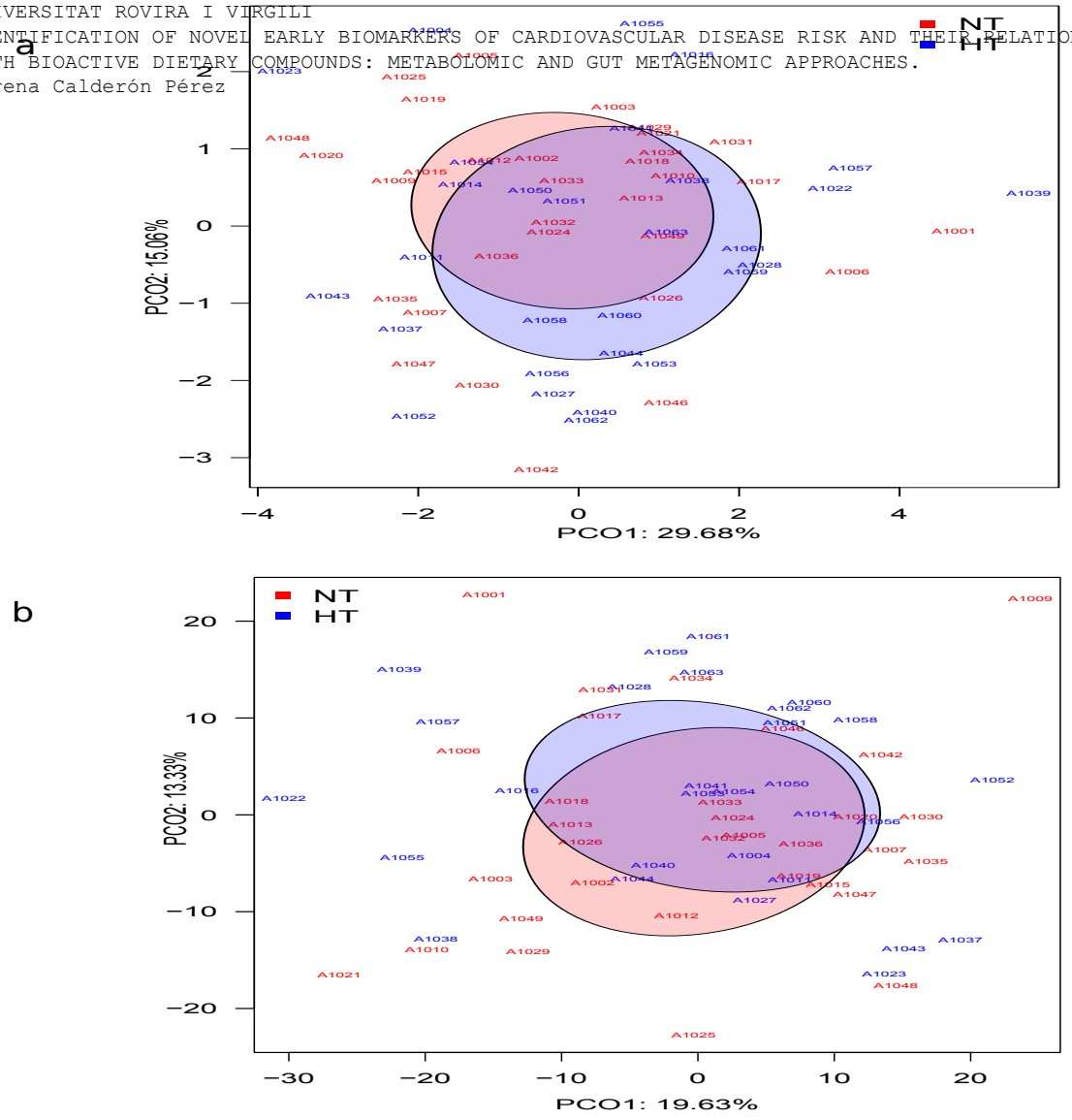
Food group intake *(g/day)*	**Hypertensive (n=27)**	**Normotensive (n=32)**	**P-value**
Dairy products	240.1 ± 150.4	329.3 ± 295.3	0.513
Whole dairy	65.62 ± 59.35	103.4 ± 154.1	0.610
Semi-skimmed dairy	86.28 ± 112.3	105.5 ± 151.7	0.766
Skimmed dairy	76.28 ± 115.2	85.78 ± 112.7	0.926
Eggs	25.25 ± 12.80	27.11 ± 28.71	0.537
Meats	193.1 ± 122.2	148.6 ± 106.3	0.140
Red meat	64.95 ± 44.70	53.30 ± 50.40	0.150
White meat	82.09 ± 81.11	65.71 ± 78.23	0.125
Processed meat	46.03 ± 28.52	29.54 ± 25.13	**0.016**
Fish and seafood	103.9 ± 57.60	107.8 ± 71.10	0.976
Whitefish	37.40 ± 26.50	36.46 ± 27.75	0.691
Bluefish	42.86 ± 23.09	48.13 ± 43.72	0.517
Seafood	23.72 ± 18.93	23.20 ± 16.14	0.770
Vegetables	467.1 ± 261.4	433.0 ± 217.7	0.533
Potatoes	66.22 ± 38.16	40.37 ± 32.58	**0.005**
Fresh fruit	399.9 ± 239.9	403.7 ± 338.5	0.420
Nuts	38.20 ± 36.09	42.46 ± 45.45	0.802
Legumes	27.72 ± 8.78	25.45 ± 11.92	0.417
Cereals	213.5 ± 176.8	238.9 ± 188.4	0.553
Refined cereals	121.4 ± 86.69	104.1 ± 85.13	0.312
Whole-grain cereals	26.51 ± 42.88	50.63 ± 68.66	**0.034**
Oils and fats	23.13 ± 14.96	27.70 ± 21.99	0.503
Olive oil	20.50 ± 14.61	25.30 ± 22.18	0.374
Sunflower oil	0.36 ± 0.89	1.17 ± 2.64	0.511
Butter	2.03 ± 4.58	1.21 ± 1.73	0.407

Pastries	14.52 ± 12.60	20.39 ± 19.76	0.583
Chocolate	7.03 ± 7.95	6.36 ± 8.40	0.590
Pre-cooked food	53.70 ± 40.00	39.53 ± 23.82	0.191
Non-alcoholic beverages	235.8 ± 172.1	144.8 ± 136.2	**0.004**
Sugary carbonated drinks	28.85 ± 45.34	9.46 ± 16.31	0.190
Soft drinks	12.66 ± 34.15	22.70 ± 56.08	0.713
Commercial juices	30.63 ± 96.65	18.83 ± 50.27	0.574
Natural juices	85.32 ± 81.47	27.88 ± 45.24	**0.008**
Coffee	60.11 ± 45.30	44.70 ± 57.45	**0.033**
Tea	18.23 ± 33.01	21.18 ± 36.97	0.906
Alcoholic beverages	160.8 ± 145.2	131.7 ± 162.2	0.108
Wine	57.60 ± 81.08	41.35 ± 61.76	0.278
Beer	101.9 ± 102.9	88.83 ± 112.1	0.213
Distilled spirits	1.28 ± 2.56	1.52 ± 4.05	0.756

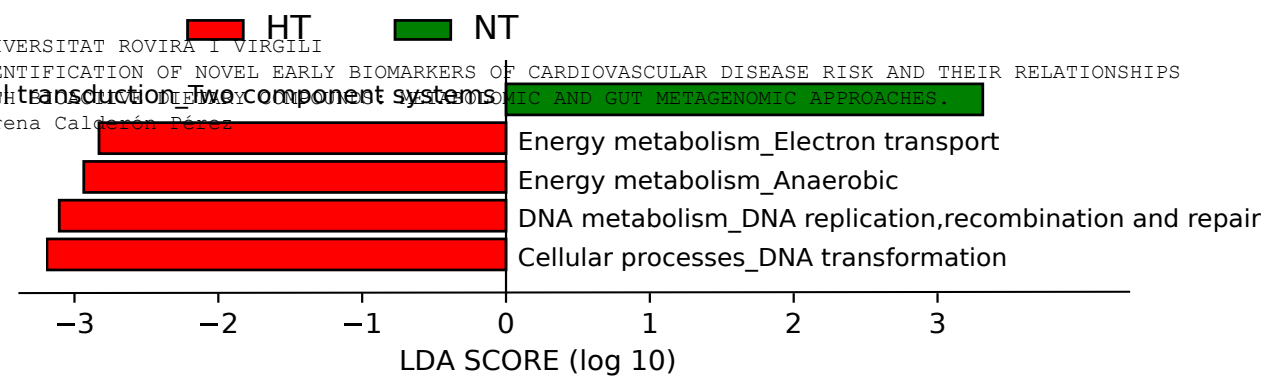
Data expressed as mean ± standard deviation. Daily mean intake by food groups was estimated from adapted Food Frequency Questionnaires performed in 59 subjects. P-value estimated by Student's t-test and Mann-Whitney U test. Significant differences depicted in **bold.**



Additional file 4: Figure S2: LefSe analysis of ASVs between NT (green) and HT (red). LDA scores (log10) for the most prevalent ASV in NT group are represented in positive scale whereas LDA-negative scores indicated enriched ASV in HT group.



Additional file 5: Figure S3: Beta diversity. Principal Coordinates Analyses (PCoA) based on Bray-Curtis dissimilarity index at functional level between N and HT groups. a Subrole level. b TIGRFAM level



Additional file 6: Figure S4: LEfSe analysis of subroles between NT (green) and HT (red). LDA scores (log10) for the most prevalent subrole in NT group is represented in positive scale whereas LDA-negative scores indicated enriched subroles in HT group.

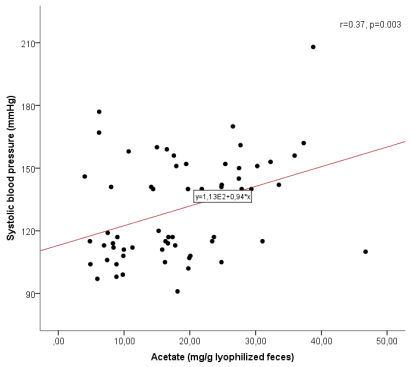
Additional file 7: Table S3 | Correlations between microbial metabolites (SCFA and TMAO) and clinical and dietary parameters.

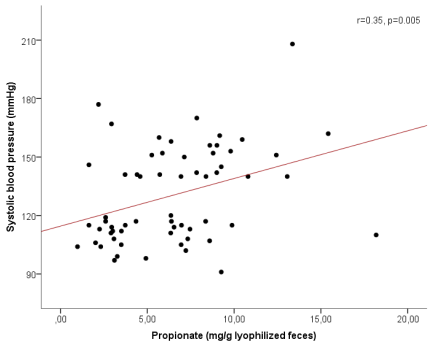
	Acetate				**Propionate**				**Butyrate**				**Valeric acid**				**Isobutyric acid**				**Isovaleric acid**				**TMAO**	
	r^a	**r^b**	**p^a**	**p^b**	**r^a**	**r^b**	**p^a**	**p^b**	**r^a**	**r^b**	**p^a**	**p^b**	**r^a**	**r^b**	**p^a**	**p^b**	**r^a**	**r^b**	**p^a**	**p^b**	**r^a**	**r^b**	**p^a**	**p^b**	**r^b**	**p^b**
Age	0.28	-0.07	**0.028**	0.574	0.27	-0.01	**0.034**	0.969	0.21	-0.01	0.101	0.923	0.23	0.07	**0.056**	0.571	0.32	-0.17	**0.010**	0.198	0.23	-0.20	0.069	0.126	0.05	0.682
Gender	0.14	-0.16	0.279	0.213	-0.13	-0.10	0.302	0.453	-0.08	0.12	0.526	0.348	-0.12	0.15	**0.040**	0.248	-0.10	0.21	0.446	0.115	-0.12	0.14	0.347	0.300	0.10	0.438
Weight	0.29	-0.07	**0.020**	0.572	0.28	-0.04	**0.028**	0.766	0.28	-0.25	**0.025**	0.059	0.26	-0.17	**0.002**	0.205	0.29	-0.20	**0.021**	0.121	0.26	-0.10	**0.039**	0.465	-0.08	0.514
BMI	0.30	-0.29	**0.018**	**0.023**	0.31	-0.10	**0.013**	0.432	0.28	-0.23	**0.026**	0.079	0.25	-0.09	**0.006**	0.495	0.32	-0.15	**0.011**	0.255	0.25	-0.15	**0.050**	0.267	0.02	0.990
Waist circumference	0.34	-0.11	**0.009**	0.428	0.32	-0.06	**0.015**	0.667	0.26	-0.07	**0.055**	0.629	0.25	-0.10	**0.003**	0.482	0.33	-0.22	**0.011**	0.103	0.25	-0.21	0.058	0.131	-0.11	0.394
Fasting blood glucose	0.00	-0.27	0.995	**0.037**	0.02	-0.06	0.874	0.629	0.04	-0.16	0.754	0.232	0.00	-0.10	0.336	0.455	0.05	-0.08	0.722	0.557	0.00	-0.05	0.979	0.696	0.27	**0.034**
Total cholesterol	0.22	-0.01	0.089	0.915	0.19	0.13	0.153	0.320	0.21	-0.02	0.112	0.880	0.04	0.05	0.284	0.685	0.12	-0.10	0.360	0.429	0.04	0.03	0.761	0.845	0.28	**0.030**
HDL-cholesterol	0.15	-0.07	0.254	0.580	-0.22	-0.07	0.088	0.580	-0.11	-0.06	0.386	0.646	-0.06	0.04	0.101	0.751	-0.08	0.11	0.525	0.423	-0.06	0.04	0.652	0.742	-0.02	0.848
LDL-cholesterol	0.24	0.01	0.065	0.939	0.28	0.11	**0.029**	0.414	0.18	-0.13	0.159	0.333	0.15	-0.03	0.087	0.842	0.22	-0.19	0.086	0.140	0.15	-0.10	0.252	0.436	0.30	**0.019**
Triglycerides	0.20	-0.05	0.117	0.710	0.29	0.11	**0.020**	0.410	0.32	-0.02	**0.011**	0.850	-0.04	-0.05	0.083	0.711	0.02	-0.22	0.887	0.093	-0.04	-0.02	0.750	0.889	0.03	0.785
SBP	0.37	-0.25	**0.003**	0.055	0.35	-0.10	**0.005**	0.443	0.36	-0.23	**0.004**	0.072	0.06	-0.18	**0.019**	0.176	0.17	-0.42	0.179	**0.001**	0.06	-0.23	0.667	0.075	0.11	0.371
DBP	0.39	-0.26	**0.002**	**0.045**	0.35	-0.11	**0.005**	0.405	0.44	-0.23	**0.000**	0.073	0.04	-0.10	**0.006**	0.441	0.15	-0.33	0.255	**0.009**	0.04	-0.30	0.761	**0.017**	-0.01	0.964
Physical activity	0.09	0.01	0.490	0.963	0.08	0.02	0.516	0.903	-0.12	0.08	0.345	0.548	-0.24	0.11	0.293	0.405	-0.19	0.25	0.152	**0.047**	-0.24	0.14	0.068	0.290	0.07	0.591
Carbohydrates	-0.20	0.18	0.117	0.170	-0.20	0.28	0.107	**0.029**	-0.22	0.15	0.079	0.261	-0.32	0.29	**0.026**	0.023	-0.33	0.19	**0.009**	0.148	-0.24	0.12	0.064	0.356	0.01	0.971
Starches	-0.22	0.15	0.086	0.259	-0.23	0.29	0.064	**0.020**	-0.26	-0.01	**0.041**	0.940	-0.34	0.12	**0.020**	0.346	-0.38	0.11	**0.002**	0.416	-0.29	0.12	**0.022**	0.365	0.08	0.507
Fats	-0.19	0.14	0.125	0.270	-0.20	-0.03	0.106	0.807	-0.20	0.04	0.119	0.785	-0.12	0.04	0.328	0.780	-0.17	0.14	0.200	0.285	-0.09	0.08	0.500	0.563	0.10	0.446
Protein	-0.15	0.11	0.221	0.399	-0.22	0.05	0.083	0.714	0.13	-0.05	0.204	0.704	-0.14	0.01	0.279	0.922	-0.20	0.15	0.130	0.262	-0.09	0.22	0.468	0.086	0.15	0.253
Dietary fiber	-0.18	0.25	0.151	0.058	-0.26	0.18	**0.038**	0.173	-0.21	0.08	0.103	0.522	-0.26	0.21	**0.015**	0.102	-0.25	0.20	**0.044**	0.128	-0.17	0.13	0.201	0.316	0.46	0.098
Ethanol	0.46	0.06	0.726	0.675	0.02	-0.02	0.865	0.857	0.03	-0.18	0.777	0.173	0.12	-0.24	0.330	0.059	0.25	-0.27	**0.044**	**0.037**	0.25	-0.18	**0.048**	0.179	0.32	0.808

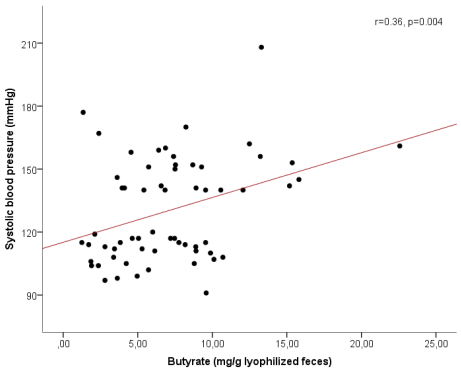
BMI, body mass index; LDL, low density lipoproteins; HDL, high density lipoproteins; SBP, systolic blood pressure; DBP, diastolic blood pressure. Significant correlations depicted in **bold**. N=61

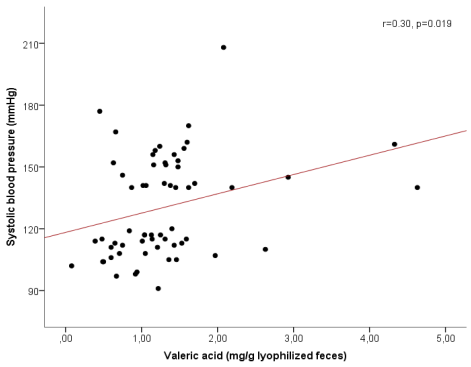
r^a: Pearson correlation coefficient between faecal SCFA and clinical and dietary variables; r^b: Pearson correlation coefficient between plasma SCFA and TMAO and clinical and dietary variables; p^a: Test of significance for faecal SCFA correlations; p^b: Test of significance for plasma SCFA and TMAO correlations

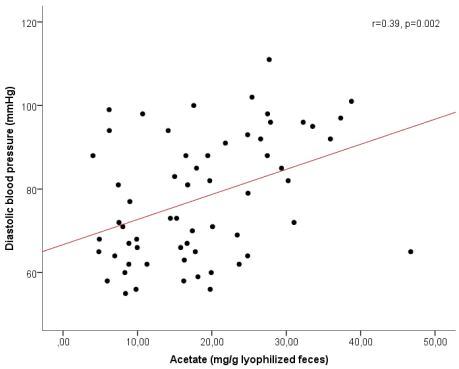
Relationship of SCFA concentrations with SBP and DBP. r. Pearson correlation coefficient and p-value (n=61)

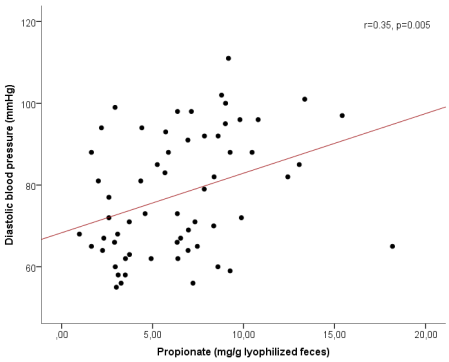


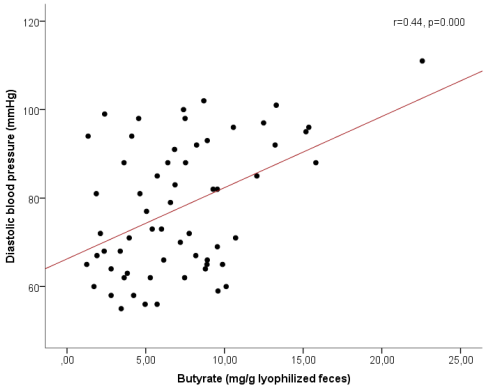


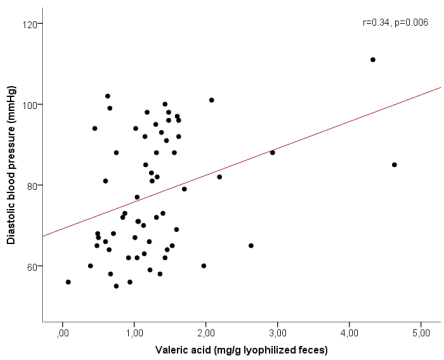












Article 2

Interplay between dietary phenolic compound intake and the human gut microbiome in hypertension: A cross-sectional study

Lorena Calderón-Pérez, Elisabet Llauradó, Judit Companys, Laura Pla-Pagà, Anna Pedret, Laura Rubió, Maria José Gosalbes, Silvia Yuste, Rosa Solà, and Rosa M Valls.

Food Chem. 2021 May 15; 344:128567

Food Chemistry 344 (2021) 128567

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Interplay between dietary phenolic compound intake and the human gut microbiome in hypertension: A cross-sectional study

Lorena Calderón-Pérez [a,b], Elisabet Llauradó [b], Judit Companys [a,b], Laura Pla-Pagà [a,b], Anna Pedret [a,b,*], Laura Rubió [c,*], Maria José Gosalbes [d,e], Silvia Yuste [c], Rosa Solà [a,b,f,1], Rosa M Valls [a,b,1]

[a] Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain
[b] Universitat Rovira i Virgili, Facultat de Medicina i Ciències de la Salut, Functional Nutrition, Oxidation, and Cardiovascular Diseases Group (NFOC-Salut), Reus, Spain
[c] Food Technology Department, XaRTA-TPV, Agrotecnio Center, Escola Tècnica Superior d'Enginyeria Agrària, University of Lleida. Lleida, Catalonia, Spain
[d] Fundación para el Fomento de la Investigación Sanitaria y Biomédica, Valencia, Spain
[e] CIBER en Epidemiología y Salud Pública (CIBEResp) Madrid, Spain
[f] Hospital Universitari Sant Joan de Reus, Reus, Spain

ARTICLE INFO

Keywords:
Phenolic compound
Coffee polyphenols
Olive polyphenols
Hypertension
Blood pressure
Gut microbiota
Short-chain fatty acids

ABSTRACT

In the present study, potential associations between dietary phenolic compounds (PCs), gut microbiota composition and targeted faecal metabolites were identified in a cross-sectional study including grade 1 hypertensive (HT) and normotensive (NT) subjects. We performed comprehensive quantification of PC intake, together with 16S rRNA gene sequencing of the gut microbiota, and faecal and plasma short-chain fatty acids (SCFAs) determination. The results showed multiple-way relationships between PCs from several plant-based foods and 25 bacterial taxa previously defined as discriminant biomarkers among groups. Remarkably, coffee PCs were positively associated with systolic and diastolic blood pressure, faecal SCFAs, *Bacteroides plebeius* and *Bacteroides coprocola* in HT and negatively associated with *Faecalibacterium prausnitzii* and Christensenellaceae R-7 in NT. Olive fruit PCs were positively associated with Ruminococcaceae UCG-010, Christensenellaceae R-7 and plasma SCFAs in NT. These interplays with discriminant bacterial taxa in HT and NT subjects highlight the potential role of specific PCs as gut microbiome modulators in either the pathogenesis or prevention of hypertension.

1. Introduction

Diet can modulate the composition and functional capacity of the human gut microbiota, an effect that depends largely on individual dietary choices (Danneskiold-Samsøe, de Freitas, Queiroz Barros, Santos, Bicas, Cazarin, Madsen, & Maróstica Júnior, 2019). The consumption of different food components, such as animal protein, digestible and non-digestible carbohydrates, fats, and probiotics, induces shifts in host microbial diversity, with secondary effects on immunological, biochemical and metabolic markers (Danneskiold-Samsøe et al., 2019).

Phenolic compounds (PCs) are phytochemicals mainly present in fruits, vegetables, olive oil, cocoa products and wine, and their regular dietary intake has protective effects against several chronic diseases, such as cardiovascular diseases (CVDs); in addition, PCs have anti-carcinogenic and anti-inflammatory properties (Costa et al., 2017). Recent findings suggest that dietary PCs could help alleviate CVDs biomarkers by altering the gut microbiota (Moorthy, Chaiyakunapruk, Jacob, & Palanisamy, 2020). It is estimated that 90–95% of total PC intake is not absorbed in the small intestine and accumulates in the large intestinal lumen, where the gut microbiota has the metabolizing capacity to convert the PCs into active metabolites, which could be responsible for the observed health effects (Selma, Espín, & Tomás-Barberán, 2009). In turn, PCs can modulate gut microbial composition through a 'prebiotic-like effect', in which they are able to promote the growth of certain beneficial bacterial taxa (Tomas-Barberan, Selma, & Espin, 2016). Other beneficial commensal bacteria, such as mucin-

* Corresponding authors at: Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut., Av. de la Universitat, 1, 43204 Reus, Spain (A. Pedret). University of Lleida, Food Technology Department, Av. de l'Alcalde Rovira Roure, 191, 25198 Lleida, Spain (L. Rubió).
E-mail addresses: anna.pedret@eurecat.org (A. Pedret), laura.rubio@udl.cat (L. Rubió).
[1] Senior authors.

https://doi.org/10.1016/j.foodchem.2020.128567
Received 11 August 2020; Received in revised form 23 October 2020; Accepted 2 November 2020
Available online 7 November 2020

degrading *Akkermansia* spp. and butyrate-producing *Faecalibacterium* spp., were recently shown to increase after administration of proanthocyanidin-rich extracts and foods containing dihydroflavonols in animal models (Tomas-Barberan et al., 2016).

Preclinical and clinical human studies have also shown a positive influence of dietary PC intake on the relative abundance of gut microbes. A reduction in the number of potential pathogens, including *Clostridium perfringens* and *Clostridium histolyticum* and certain gram-negative *Bacteroides* spp., and an increase in the growth of beneficial Clostridia, Bifidobacteria and Lactobacilli have been detected after PC intake (Duenas et al., 2015).

In most cases, human studies have focused on the administration of PC-enriched diets and controlled supplementation with PC-rich extracts or nutraceuticals with high doses of PCs (Most, Penders, Lucchesi, Goossens, & Blaak, 2017; Nash et al., 2018; Xie et al., 2016). However, none of these studies have assessed the interplay between the gut microbiome and the individual PC intake provided by foods in the context of habitual diet.

Hypertension is one of the most common chronic diseases and remains the major preventable cause of CVD globally (Esh, Agabiti, France, Uk, Germany, Kerins, & France, 2018). Dietary intake of fruit, vegetables and fibre has been inversely associated with blood pressure (BP) levels (Marques, Mackay, & Kaye, 2018), and has shown a long-term effect of reducing systolic and diastolic BP in subjects with normal BP or mild hypertension (Nissensohn, Roman-Vinas, Sanchez-Villegas, Piscopo, & Serra-Majem, 2016). Likewise, the Mediterranean diet has emerged as an optimal strategy for promoting gut microbial diversity and stability and preventing dysbiosis (Rinninella et al., 2019). Negative associations of total polyphenol intake (TPI), assessed by urinary total polyphenol excretion (TPE), with BP levels and the prevalence of hypertension in a Mediterranean population at high cardiovascular risk were also reported (Alexander Medina-Remon et al., 2011). Recently, the role of gut microbiota-derived metabolites in the regulation of BP has been suggested (Marques et al., 2018). For instance, SCFAs, which are the major end products of faecal bacterial activity after dietary fibre fermentation, can help lower BP levels (Natarajan et al., 2016). Moreover, our previous results supported the hypothesis that greater faecal excretion together with lower circulating levels of SCFAs could be a marker of a hypertensive state due to poor gut health (Calderón-Pérez et al., 2020). Dietary intake of PCs such as anthocyanins and lignans has been positively associated with faecal concentrations of SCFAs (Fernandez-Navarro et al., 2018), which highlights the importance of investigating the bacterial modulatory effects of PCs and their links with bacterially derived metabolites.

Thus, dietary PCs may represent promising candidates for preventing or delaying the onset of hypertension, and the investigation of whether dietary PC intake attenuates BP through a beneficial impact on the gut microbiome is a new developing field (de Brito Alves et al., 2016). On the basis of our previous study (Calderón-Pérez et al., 2020) reporting a particular bacterial signature in the guts of non-treated grade 1 hypertensive (HT) and normotensive (NT) subjects, the present study provides further knowledge of the relation of this effect with diet. Specifically, we hypothesize that specific dietary PCs from habitual diet could be precursors for the occurrence of particular bacterial taxa that differ between HT and NT subjects. Therefore, the main purpose of the present study is to identify potential associations between dietary PC intake, faecal microbiota composition and SCFAs as target faecal metabolites in non-treated grade 1 HT subjects compared to NT subjects.

2. Material and methods

2.1. Subjects and study design

All the individuals included in the present study were recruited between 9 June 2016 and 28 November 2017. Participants were recruited by using tableaux advertisements in the *Hospital Universitari Sant Joan de Reus* (HUSJ, Spain) and databases of volunteers who had previously participated in studies carried out by our research group. HT participants were included in the study if they exhibited grade 1 hypertension, defined as systolic BP between 140 and 159 mm Hg and without major complications, according to the *ESC/ESC Guidelines (2018)* (Esh et al., 2018), and not using antihypertensive medication. NT participants presented optimal systolic BP below 120 mm Hg. All subjects fulfilled the following criteria: aged from 18 to 65 years, without a family history of cardiovascular disease or evidence of chronic disease, and willing to provide informed consent before the initial screening visit. Subjects with BMI $\geq$ 30 kg/m^2, fasting glucose $>$ 126 mg/dL, low-density lipoprotein (LDL) cholesterol $\geq$ 190 mg/dL, triglycerides $>$ 350 mg/dL, a history of smoking, and anaemia or intestinal disorders were excluded. Individuals were also excluded if they were using anti-hypertensive or lipid-lowering medication, as well as if they were menopausal, pregnant or breastfeeding. Further, subjects with chronic alcohol consumption, use of either antibiotics or probiotics, or a vegetarian or vegan diet at the time of inclusion were also excluded.

Participants visited the HUSJ and Eurecat-Reus, where the study was performed, twice. In a first pre-selection visit, a clinical interview verifying that participants met all the eligibility criteria was performed. In addition, blood extraction was performed, BP was measured, and anthropometric and physical activity data were collected. In the second visit, in addition to the previous measures, urine and stool samples were collected. A 3-day dietary record and a food-frequency questionnaire (FFQ) were provided to assess dietary habits.

The study protocol was approved by the local ethics committee (Clinical Research Ethical Committee of HUSJ, Reus with the 15–11-26/11obs4 reference) prior to the study beginning, and informed consent was obtained from all subjects. The protocol was conducted in accordance to the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization (ICH GCP).

2.2. Clinical and nutritional data collection

All clinical information was collected according to standard procedures. BP was monitored with participants in a seated position after resting for 2–5 min by a using multiple automated sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain). Two repeated readings were recorded with a 1-min interval, and the average value was used for statistical analyses. To minimize white-coat/masked hypertension, it was ensured that participants rested alone and were unobserved in a quiet environment during the monitoring (Esh et al., 2018). Anthropometric parameters, including weight, height and body composition, were measured with a body composition analyser (Tanita SC 330-S; Tanita Corp., Barcelona, Spain). Waist circumference was measured at the umbilicus level using a 150-cm anthropometric steel measuring tape. Physical activity was evaluated by completion of the "Physical Activity Questionnaire Class AF" validated questionnaire (Vallbona Calbó, Roure Cuspinera, Violan Fors, & Alegre Martín, 2007).

Habitual dietary intake was assessed by using a validated, semi-quantitative, food-frequency questionnaire (FFQ) including 137 food items related to the Mediterranean diet (Fernández-Ballart et al., 2010). Mean daily energy and nutrient intakes were assessed through a 3-day dietary record (2 labour days and 1 week-end day) and calculated by Spanish food composition tables (Tablas de composición de alimentos del Cesnid, 2008)(Interamericana, M., 2008).

2.3. Biological sample collection

Fasting blood samples were obtained in the second visit to determine lipid profiles. Total cholesterol, LDL cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride concentrations were measured in serum by standardized, automated enzymatic methods in an auto-analyzer (Beckman Coulter-Synchron, Galway, Ireland).

Faecal samples were obtained with a Protocult^{TM} stool collection

device (ABC, Minnesota, EEUU) with two different containers: a sterile pot and a specimen container with a spoon containing 7 mL of RNAlater® storage solution (Sigma-Aldrich Quimica SL; Madrid, Spain). The samples were frozen at home and transported to the laboratory with an ice pack. The samples were stored then at −80 °C until the analysis of gut microbiota composition and SCFAs determination.

Urine samples were collected in a special container over a 24-hour period, and the supernatants of centrifuged samples were kept at −80 °C.

All samples were stored in the central laboratory's biobank at HUSJ-Eurecat (biobanc.reus@iispv.cat) until they were required for batch analyses.

2.4. Estimation of dietary phenolic compound intake

Three-day dietary records provided by participants were used to obtain information about the consumption of PC-rich foods and ingredients. Dietary PC intake was estimated according to the phenolic content of the foods listed in the Phenol-Explorer database (Neveu, Perez-Jimenez, Vos, Crespy, du Chaffaut, Mennen, & Scalbert, 2010) by using individual PC values determined by high-performance liquid chromatography (HPLC).

The daily PC intake values were expressed as mg/day in two distinct ways: i) individually by phenolic class and subclass and ii) by the sum of total PCs from major food sources. To establish associations, the main PC-rich food sources were selected and grouped into categories (tubers, cereals, vegetables, legumes, fresh fruits, nuts, oils, sweets, non-alcoholic beverages, alcoholic beverages, and seasonings) and subcategories (refined cereals, whole grains, berries, olive fruits, olive oil, chocolate, natural juices, coffee, tea, and wine).

TPI was calculated by matching food consumption data and the total polyphenol content quantified by the Folin assay method from Phenol-Explorer.

2.5. Total polyphenol excretion in urine

TPE was determined in 24 h urine samples by means of the Folin-Ciocalteu method using an Oasis® MAX 96-well plate cartridge for solid-phase extraction, as described by *Medina-Remón et al., 2009* (Alexander Medina-Remon et al., 2009). Creatinine measurements were used to adjust for variations in analyte concentration in urine. To analyze creatinine in urine, a reaction method in plates was applied with picric acid as the reagent, followed by spectrophotometric measurement. TPE was expressed as mg gallic acid equivalent (GAE)/g of creatinine.

2.6. Determination of faecal and plasma SCFAs

Faecal SCFAs were analyzed in lyophilized feces by using an integrated system including a gas chromatographer coupled to a flame ionization detector (GC-FID), as previously described (Calderón-Pérez et al., 2020). Identification of the SCFAs was carried out according to the retention time of standard compounds (acetic acid, propionic acid, butyric acid, and valeric acid; Sigma-Aldrich), and their quantification was determined with reference to the peak side of the internal standard (4-methyl valeric acid). All samples were analysed in triplicate.

Plasma SCFAs were quantified by a derivatization procedure and analysed by gas chromatography (Agilent 6890*N*-MSD 5973) using a DB5 MS-UI column (30 m, 0.25 mm, 0.25 μm; J&W, Agilent Tech), coupled to a mass detector using SIM mode, as previously described (Calderón-Pérez et al., 2020). The contents of SCFAs were calculated with internal standard method. All samples were analyzed in duplicate, reporting coefficient of variability values lower than 10%.

2.7. Faecal microbiota composition analysis

2.7.1. DNA purification and sequencing

DNA was extracted from faecal samples stored in RNAlater® as previously described (Calderón-Pérez et al., 2020). The V3-V4 region of the 16S rRNA gene was amplified, and amplicon libraries were constructed according to the manufacturer's instructions (Illumina). Sequencing was performed with the MiSeq V3 Kit (2x230 cycles) (Illumina, Eindhoven, Netherlands) at the Centre for Public Health Research (FISABIO-Salud Pública, Valencia, Spain). All sequences were deposited on the public European Nucleotide. Archive server under accession number PRJEB32411.

2.7.2. Sequence analysis

16S rRNA gene reads with low-quality scores (<30) and reads shorter than 100 nucleotides as well as potential chimeras and human sequences were removed using the DADA2 (v1.8.0) pipeline (Callahan et al., 2016) in R package. The error rates for each base transition were estimated, and dereplication was carried out to combine all identical reads into unique sequences, with abundance equal to the number of reads combined. Taking the dereplicated reads and the error estimates, amplicon sequence variants (ASVs) were inferred. The SILVA database (v.132) (Quast et al., 2013) was set as the reference for taxonomic classification of each ASV. This ASVs were classified to the genus level, applying exact matching (100% identity) to assign a unique species to each ASV sequence. ASVs with an assigned genus but without exact matching at the species level, were mapped against the same reference database with a minimum identity of 97%.

2.8. Statistical analysis

Statistical analysis was performed using IBM SPSS version 25.0 (IBM SPSS, Inc, Chicago, IL, USA). The normality of variables was analysed by means of the Kolmogorov-Smirnov test. The Mann-Whitney test was used to compare non-normally distributed variables. Student's *t*-test was used to compare normally distributed variables. Fisher's exact test was used for categorical variable comparisons. Descriptive data are expressed as the mean ± SD, with percentages for categorical variables.

In the first step, we analysed the Pearson correlations of TPI, urinary TPE and PC intake from major dietary sources (mg/day) and their classes and subclasses (mg/day) with systolic and diastolic BP values in all subjects. Stepwise multiple linear regression analysis was subsequently used to predict the strength of the associations. Given the possible confounding effect of dietary fibre and assuming that it is usually present in the same food matrix of PCs, this component was controlled for in the regression analysis. Energy intake and age were also controlled to ensure that the identified interactions between PCs and the gut microbiota were attributable only to the intrinsic effect of PCs.

In a second step, to identify specific bacterial taxa as biomarkers in both the HT and NT groups, the linear discriminant analysis (LDA) effect size (LEfSe) method was performed at the ASV level. The associations of ASVs with an LDA score > 2 with TPI, TPE, PC dietary sources and the main phenolic classes were analysed. Correlations were first tested with Pearson correlation analysis. Then, multivariate regression analysis was used, setting ASV biomarkers as predictors and PC variables as response variables. We also adjusted for potential confounding factors. The statistical parameters employed were β (standardized regression coefficient) and R^2 (coefficient of multiple determination). Additionally, linear regression analysis was used for graphical representation of significant relationships. Only significant results are presented. The conventional probability value for significance of 0.05 was used in the interpretation of the results.

Sample size was estimated based on a study by *Yang T et al.* (Yang, Santisteban, Rodriguez, Li, Ahmari, Carvajal, & Mohamadzadeh, 2015) where significant differences were observed in the gut microbiota composition between 7 hypertensive patients and 10 healthy controls.

Thus, we assumed that an expanded sample size of 30 untreated grade 1 HT subjects and their respective healthy controls (1:1 ratio) was enough to achieve significant power.

3. Results

3.1. Clinical and lifestyle characteristics of the study participants

A total of 61 participants, including 29 HT and 32 NT , were enrolled in the study (Supplementary Fig. 1). The general characteristics of the HT and NT subjects are shown in Table 1. The HT subjects were older ($p < 0.001$) and presented a higher BMI ($p < 0.001$), waist circumference ($p < 0.001$) and fat mass ($p = 0.037$) than the NT subjects. Systolic BP and diastolic BP expressed as the mean ± SD were 153.1 ± 14.6 and 91.0 ± 8.8 mm Hg in HT subjects and 109.7 ± 7.1 and 65.7 ± 6.7 mm Hg in NT subjects, respectively. With respect to biochemical parameters, HT subjects showed higher fasting blood glucose ($p = 0.001$), total cholesterol ($p = 0.017$) and LDL cholesterol ($p = 0.002$) levels.

In relation to faecal SCFAs, HT subjects showed higher concentrations of acetate ($p = 0.004$), propionate ($p = 0.005$), butyrate ($p = 0.002$) and valerate ($p = 0.003$) than NT subjects (Table 1).

Daily energy and nutrient intakes were similar between the two groups, with the exception of total dietary fibre intake, which was greater in NT subjects ($p = 0.029$) (Table 1). In relation to habitual dietary intake, data reported via the FFQ revealed significantly greater daily mean intakes of processed meat, tubers, natural juices and coffee and a significantly lower intake of whole-grain in HT subjects than in NT (Supplementary Table 1). No differences were found in other lifestyle factors, such as physical activity.

Table 1
Baseline characteristics of study participants.

Variables	Hypertensive	Normotensive	P-value
N	29	32	
Age, *y*	53.7 ± 9.6	41.1 ± 9.1	<0.001
Gender, *(F/M)*	(10/19)	(16/16)	0.301
Anthropometry			
Weight, *kg*	75.3 ± 9.3	68.9 ± 10.8	0.017
BMI, kg/m^2	26.2 ± 2.5	23.8 ± 2.7	<0.001
Fat mass, *%*	26.6 ± 7.9	22.1 ± 7.8	0.037
Waist circumference, *cm*	94.4 ± 8.3	84.0 ± 9.0	<0.001
Blood chemistry *(mg/dL)*			
Total cholesterol	199.6 ± 43.9	181.7 ± 34.7	0.017
LDL cholesterol	123.7 ± 21.3	100.7 ± 33.2	0.002
HDL cholesterol	62.6 ± 14.0	64.9 ± 18.0	0.580
Triglycerides	97.3 ± 38.8	80.7 ± 42.6	0.067
FBG	91.2 ± 11.3	81.1 ± 7.5	0.001
Blood pressure *(mm Hg)*			
Systolic BP	153.1 ± 14.6	109.7 ± 7.1	<0.001
Diastolic BP	91.0 ± 8.8	65.7 ± 6.7	<0.001
Faecal SCFAs concentration *(mg/g feces)*			
Acetate	22.11 ± 9.70	15.05 ± 8.74	0.004
Propionate	7.78 ± 3.38	5.26 ± 3.41	0.005
Butyrate	8.80 ± 4.71	5.62 ± 2.93	0.002
Valerate	1.57 ± 0.94	1.04 ± 0.50	0.003
Physical activity *(%)*			
Inactive	6.9	0.0	
Very low activity	10.3	10.0	
Low activity	10.3	6.7	0.729
Moderate activity	20.7	20.0	
High activity	51.7	63.3	
Diet			
Energy intake, *Kcal/day*	2089.74 ± 543.43	2208.23 ± 654.06	0.452
Protein, *g/day*	88.28 ± 21.28	94.10 ± 29.87	0.395
(% DEI)	*(17.2)*	*(17.5)*	0.741
Total carbohydrates, *g/day*	197.03 ± 61.41	212.65 ± 60.90	0.328
(% DEI)	*(38)*	*(39.3)*	0.441
Complex carbohydrates, *g/day*	110.35 ± 42.29	116.00 ± 27.31	0.548
(% DEI)	*(21)*	*(21.8)*	0.917
Simple carbohydrates, *g/day*	87.87 ± 29.64	96.62 ± 40.38	0.767
(% DEI)	*(17)*	*(17.6)*	0.611
Total fat, *g/day*	96.95 ± 31.67	102.70 ± 41.93	0.556
(% DEI)	*(41.3)*	*(40.8)*	0.782
SFA, *g/day*	27.54 ± 10.54	27.75 ± 12.47	0.944
(% DEI)	*(11.5)*	*(11.4)*	0.843
MUFA, *g/day*	45.40 ± 15.63	49.67 ± 21.92	0.395
(% DEI)	*(19.4)*	*(19.7)*	0.779
PUFA, *g/day*	15.92 ± 7.29	17.22 ± 8.58	0.563
(% DEI)	*(6.8)*	*(6.9)*	0.711
Total cholesterol, *mg/day*	328.25 ± 130.77	358.22 ± 187.89	0.482
Dietary fibre, *g/day*	20.95 ± 9.90	25.90 ± 11.63	0.029
Ethanol, *g/day*	11.37 ± 13.04	7.76 ± 10.11	0.131

Data expressed as mean ± standard deviation or percentage. Abbreviations: BP, blood pressure; BMI, body mass index; FBG, fasting blood glucose; LDL. low density lipoproteins; HDL, high density lipoproteins; SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. % DEI, mean percentage of daily energy intake. P-value for gender and physical activity was calculated by Fisher's exact test. P-value for the rest of the variables was calculated by Student's *t*-test and Mann-Whitney *U* test.

3.2. Phenolic compound intake and its association with systolic and diastolic blood pressure

Daily PC intake expressed by dietary sources and by phenolic classes and subclasses, together with TPI and TPE in urine, is reported separately for the HT and NT groups in Table 2. PCs from vegetables, legumes, fresh fruits, chocolate, coffee and wine, together with flavonoids and phenolic acids, were the major contributors to TPI in both groups. We found no significant differences between groups in terms of TPI and urinary TPE. However, HT subjects had a significantly higher mean daily PC intake from non-alcoholic beverages, mainly coffee, and from alcoholic fermented beverages, such as wine and beer, and a lower PC intake from vegetables and whole-grain cereals than NT subjects ($p < 0.05$). With respect to phenolic classes, HT subjects consumed more hydroxycinnamic acids, alkylmethoxyphenols and methoxyphenols than NT subjects ($p < 0.05$) (Table 2).

To test the relationships between PCs that could contribute to the modulation of systolic and diastolic BP, we performed Pearson correlations in all populations (Supplementary Table 2). Based on these correlations, we subsequently conducted stepwise multiple linear regression analysis to assess major associations independent of age, dietary fibre intake and energy intake (Table 3). PCs from coffee and hydroxycinnamic acids showed a positive association with systolic BP (Supplementary Table 2), which was reinforced by multiple linear regression analysis ($\beta = 0.278$ and $\beta = 0.310$, respectively) ($p < 0.05$). Coffee and beer PCs and alkylmethoxyphenols and chalcones were also positively related to diastolic BP ($\beta = 0.259$, $\beta = 0.259$, $\beta = 0.274$ and $\beta = 0.268$, respectively) ($p < 0.05$) (Table 3).

3.3. Associations between phenolic compound intake and gut microbiota composition in hypertensive and normotensive subjects

With the LEfSe approach and an LDA score > 2, we detected a total of 67 ASV biomarkers that had significantly different abundances between HT and NT subjects (Fig. 1-*A*) (Calderón-Pérez et al., 2020). Of these ASVs, 14 were selected in the NT group, and 11 were selected in the HT group, showing the strongest Pearson correlations ($r \geq 0.30$) with PC dietary sources and classes (data not shown). Stepwise multiple linear regression analysis was conducted to corroborate major associations between PC dietary sources and the bacterial abundance of selected bacterial ASVs in both groups (Table 4). In addition, the most significant associations from the multiple linear regression analysis are shown in Fig. 1-*B* (NT) and *1-C* (HT).

In the NT group, several positive associations were observed regarding olive phenols. Specifically, PCs from olive fruits were positively associated with Ruminococcaceae UCG-010 (s408 and s1019),

Table 2
Daily intake of phenolic compounds expressed by major dietary food sources and by phenolic classes and subclasses in hypertensive and normotensive subjects.

	Hypertensive (n = 29)	Normotensive (n = 32)	P-value
PC intake by dietary source (mg/day)			
Tubers	57.45 ± 33.03	43.95 ± 37.52	0.147
Cereals	92.07 ± 109.05	113.98 ± 142.13	0.224
Whole-grain cereals	29.08 ± 45.23	44.91 ± 41.71	**0.012**
Vegetables	244.56 ± 253.40	380.66 ± 362.88	**0.042**
Legumes	168.36 ± 431,38	248.23 ± 464.33	0.352
Fresh fruits	319.09 ± 249.22	417.63 ± 365.33	0.237
Berries	38.80 ± 54.72	42.59 ± 81.43	0.525
Dried fruit	30.40 ± 130.10	27.84 ± 69.38	0.631
Nuts	98.33 ± 135.08	112.39 ± 179.49	0.812
Olive fruit	13.95 ± 21.71	24.79 ± 53.68	0.928
Oils	6.88 ± 3.15	7.49 ± 4.12	0.527
Olive oil	6.44 ± 3.48	7.68 ± 3.79	0.239
Sweets	115.35 ± 149.24	222.83 ± 335.53	0.440
Chocolate	113.82 ± 149.13	220.16 ± 333.91	0.300
Non-alcoholic beverages	254.17 ± 151.61	159.45 ± 150.98	**0.006**
Natural fruit juice	22.90 ± 38.38	22.60 ± 52.34	0.577
Coffee	203.45 ± 150.12	115.52 ± 128.44	**0.013**
Tea	24.74 ± 51.04	29.23 ± 82.87	0.889
Alcoholic beverages	163.92 ± 169.88	96.32 ± 151.14	**0.040**
Wine	127.51 ± 153.42	74.10 ± 132.40	0.074
Beer	20.30 ± 31.34	32.58 ± 40.27	0.120
Seasonings	6.85 ± 15.01	8.63 ± 23.27	0.683
PC intake by class and subclass (mg/day)			
Total flavonoids	224.32 ± 137.94	209.68 ± 117.86	0.824
Anthocyanins	30.11 ± 28.05	20.82 ± 31.77	0.112
Chalcones	0.002 ± 0.005	0.0007 ± 0.011	0.349
Dihydrochalcones	0.88 ± 1.73	2.10 ± 3.59	0.369
Dihydroflavonols	3.20 ± 3.87	1.84 ± 3.34	0.074
Flavanols	82.66 ± 70.08	88.42 ± 73.08	0.614
Flavanones	51.66 ± 58.11	30.08 ± 38.43	0.208
Flavones	15.18 ± 11.09	23.03 ± 19.35	0.159
Flavonols	28.66 ± 18.33	32.18 ± 21.25	0.604
Isoflavonoids	11.95 ± 31.55	11.18 ± 21.52	0.233
Total phenolic acids	319.66 ± 120.44	262.83 ± 131.28	0.088
Hydroxibenzoic acids	38.72 ± 43.89	38.88 ± 49.51	0.882
Hydroxycinnamic acids	277.35 ± 115.29	220.25 ± 104.95	**0.042**
Stilbenes	2.07 ± 2.43	1.21 ± 2.09	0.120
Lignans	45.51 ± 65.32	47.37 ± 41.08	0.279
Other polyphenols	61.75 ± 48.72	67.82 ± 46.51	0.424
Alkylmethoxyphenols	1.03 ± 0.63	0.61 ± 0.61	**0.012**
Alkylphenols	21.87 ± 41.09	29.33 ± 40.34	0.106
Curcuminoids	0.77 ± 1.80	0.62 ± 2.18	0.373
Methoxyphenols	0.12 ± 0.08	0.07 ± 0.07	**0.010**
Tyrosols	34.16 ± 23.28	33.98 ± 29.84	0.625
Total polyphenol intake	1616.49 ± 768.10	1634.91 ± 641.37	0.922
Total polyphenol excretion	156.74 ± 43.41	154.30 ± 33.02	0.822

Data expressed as mean ± standard deviation. Total polyphenol intake from PC dietary food sources and classes expressed as mg/day. Total polyphenol excretion expressed as mg GAE/g creatinine. P-value was calculated by Student's *t*-test and Mann-Whitney *U* test.

Christensenellaceae R-7 (s430) and *Bilophila wadsworthia* from the Desulfovibionaceae family (s1147) ($p < 0.001$) (Table 4; Fig. 1-*B*), whereas olive oil PC intake was associated with taxa s347 and s529 from the Ruminococcaceae and Rikenellaceae families, respectively ($p < 0.05$). Additionally, a strong positive association was found between some of the aforementioned taxa (s1019 and s430) and anthocyanins present in olive fruits ($p < 0.001$) (Table 4). Moreover, PCs from dried fruits (including dried apricots, plums, dates, figs and raisins) showed a positive correlation with the abundance of Ruminococcaceae NK4A214 (s127) ($p = 0.002$) and Christensenellaceae R-7 (s3173) ($p < 0.001$), both of which were strongly associated with hydroxybenzoic acids ($p < 0.001$) (Table 4). Other minor positive associations were found for PCs from fresh fruit, natural fruit juices and nuts with taxa s408, s1019, s1147, s430, s897 and s3187 in the Ruminococcaceae, Christensenellaceae, Tannerellaceae and Erysipelotrichaceae families ($p < 0.05$) (Table 4; Fig. 1-*B*). PC intake from tubers and lignans was associated with *Bacteroides xylanisolvens* (s230) ($p = 0.020$) from the Bacteroidetes phylum (Table 4). Conversely, coffee PCs and hydroxycinnamic acids presented negative associations with the abundances of *Faecalibacterium prausnitzii* (s372) and Christensenellaceae R-7 (s3173) ($p < 0.05$) (Table 4, Fig. 1-*B*).

In the HT group, PCs from vegetables and lignans contributed positively to *Ruminococcaceae* taxa abundance (s728) ($p < 0.001$ and $p = 0.003$, respectively) (Table 4, Fig. 1-*C*). In addition, PC intake from fresh fruit and dried fruits, and flavanones, the major phenolic class, showed positive associations with Ruminiclostridium 5 (s2600), *Streptococcus thermophilus* (s224) and Lachnospiraceae (s142) ($p < 0.05$), all from the Firmicutes phylum (Table 4, Fig. 1-*C*). PC intake from whole-grain cereals and flavanols was positively related to the abundance of *Bacteroides vulgatus* (s651) ($p < 0.001$) (Fig. 1-*C*). Other positive associations were found between PCs from wine and taxa s3042 in the Clostridiaceae family and between PC intake from berries and taxa s278 in the Muribaculaceae family ($p < 0.05$). Coffee PCs, together with alkylmethoxyphenols and methoxyphenols, were identified as positive contributors to *Bacteroides plebeius* (s92) and *Bacteroides coprocola* (s16 and s41) abundance ($p < 0.05$) (Fig. 1-*C*), both defined in our previous work (Calderón-Pérez et al., 2020) as specific taxa with higher discriminatory power in the HT group.

3.4. Associations between phenolic compound intake and faecal and plasma short-chain fatty acids in hypertensive and normotensive subjects

Additionally, possible associations between PC intake and faecal and plasma SCFA concentrations were tested by Pearson correlation (Supplementary Tables 3 and 4). Interestingly, in the HT group, coffee PCs and their main classes (hydroxycinnamic acids, alkylmethoxyphenols and methoxyphenols) were positively correlated with faecal levels of propionate ($p = 0.006$), acetate ($p = 0.023$), and valerate ($p = 0.003$), whereas dried fruit PCs also showed a positive correlation with faecal propionate and acetate in this group ($p = 0.049$ and 0.043, respectively).

Table 3
Multiple stepwise linear regression results for associations of phenolic compound dietary sources and *classes* with systolic and diastolic blood pressure levels (mm Hg) for all subjects.

Variables	Systolic Blood Pressure			Variables	Diastolic Blood Pressure		
	R^2	β	p		R^2	β	p
Coffee	0.077	0.278	0.032*	Coffee	0.153	0.259	0.040*
Hydroxycinnamic Acids	0.096	0.310	0.016*	Beer		0.259	0.040*
				Alkylmethoxyphenols	0.187	0.274	0.032*
				Chalcones		0.268	0.035*

R^2, coefficient of multiple determination; percentage of the variance in the dependent variable explained by the independent variables in the model.
β, standardized regression coefficient.
p, two-sided test of significance.* p value ≤ 0.05.
Independent variables included in the model: only phenolic compound significant correlations from Pearson analysis.
Analysis was adjusted for age, total fibre and energy intake. Only statistically significant results are shown.

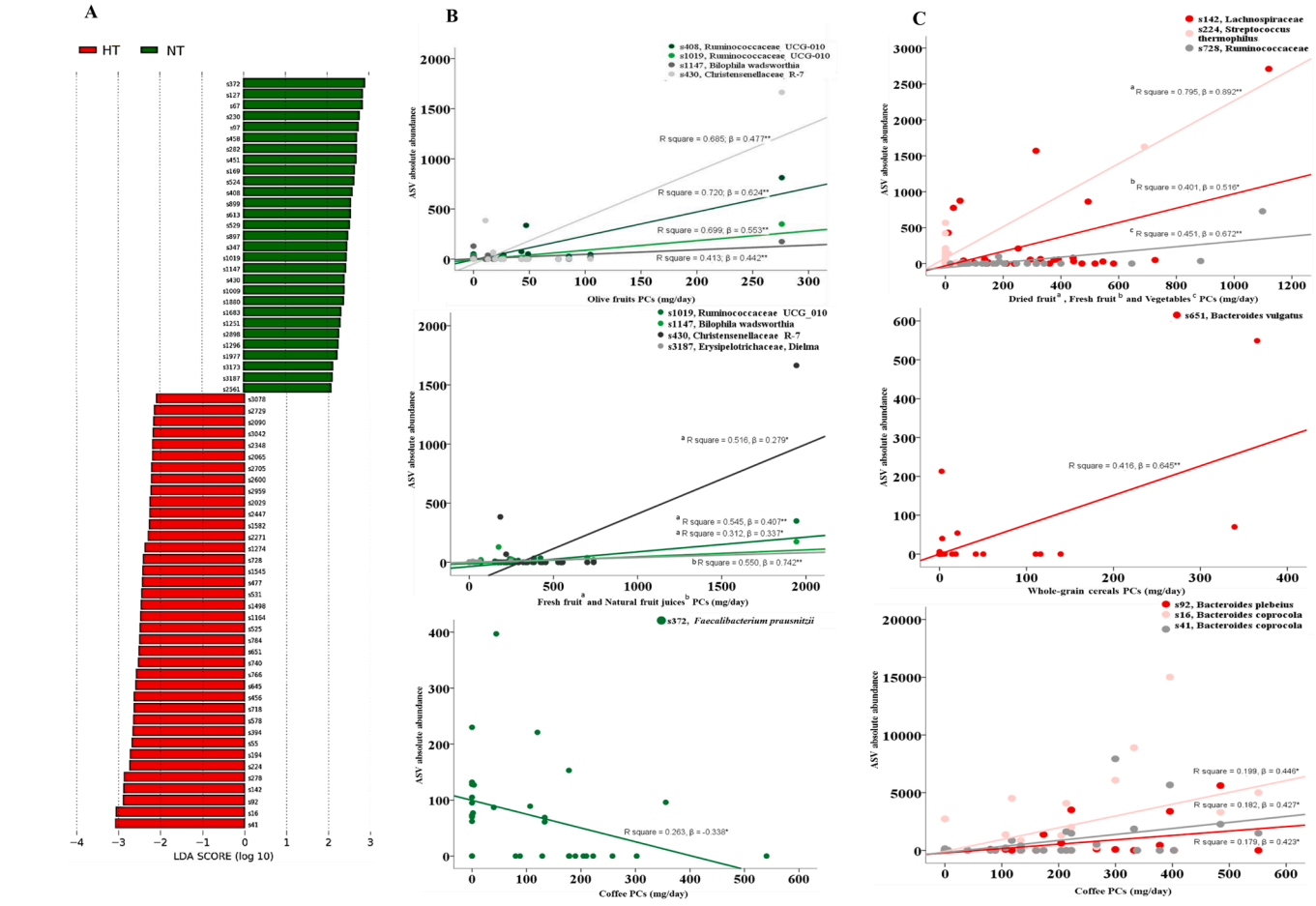


Fig. 1. Relationships between phenolic compound intake from major food sources and gut microbial signatures in hypertensive (HT) and normotensive (NT) subjects. (A) LefSe analysis of ASVs between NT (green) and HT (red) subjects. LDA scores (log10) for the most prevalent ASVs in the NT group are represented on a positive scale, whereas negative LDA scores indicate enriched ASVs in the HT group. (B) Multiple linear regression analyses representing major PC food sources associated with the absolute abundance of faecal microbial ASVs in the NT group (green) and (C) HT group (red); R^2, coefficient of multiple determination; β, standardized regression coefficient; [a, b, c] indexes representing regressions for individual food sources in food categories; *p value $\leq$ *0.05;* ** p value $\leq$ *0.001*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Additionally, positive correlations were found for chalcones and dihydrochalcones with faecal valerate in NT ($p = 0.003$ and 0.039, respectively). On the other hand, in the NT group, significant positive correlations were noted for fresh fruit and olive fruit PCs with plasma butyrate ($p = 0.042$ and 0.049, respectively) and valerate ($p = 0.001$ and 0.020, respectively) levels. Additionally, anthocyanins were positively correlated with plasma valerate ($p = 0.047$).

A schematic of major interrelationships between PC intake by food source and phenolic class, gut bacterial taxa at the family and ASV levels and SCFAs in both groups is provided in Fig. 2.

4. Discussion

The present study provides new insights into the interplay of dietary PCs with human gut microbiota composition and its derived metabolites in grade 1 hypertension. Our results confirm the hypothesis that specific dietary PCs could be precursors for the occurrence of particular bacterial taxa that differs in the guts of non-treated grade 1 HT and NT subjects. We identified potential associations between PCs from plant-based foods, such as vegetables, fresh fruits, dried fruits, whole-grain cereals, tubers, olive oil, olive fruits, natural fruit juices and coffee, and the abundance of several bacterial taxa in the Ruminococcaceae, Bacteroidaceae, Lachnospiraceae, Christensenellaceae, Streptococcaceae, Erysipelotrichaceae and Clostridiaceae families in HT and NT subjects. In addition, we found interesting associations of PCs with faecal and plasma SCFA concentrations, which suggested a concomitant involvement of these colonic metabolites in the PC-mediated changes in gut bacterial composition.

A novel finding of our study was the multiple-way relationship among coffee PCs, discriminant bacterial taxa in HT and NT subjects, faecal SCFAs, and BP. Initially, in HT subjects, we observed positive associations between coffee PC intake and its chlorogenic acids, such as hydroxycinnamic acids and alkylmethoxyphenols, with particular *Bacteroides* spp. such as *B. plebeius* and *B. coprocola*. Previous studies reported an impact of moderate coffee consumption, defined as three cups of coffee/day, on particular members of the human colonic microbiota, principally species of *Bifidobacterium*, a genus with reputed beneficial effects for human health (Jaquet, Rochat, Moulin, Cavin, & Bibiloni, 2009). However, the impact of coffee intake on other dominant bacterial genera, such as *Bacteroides,* has not yet been reported, and whether the modulatory action of coffee on the gut microbiota can be attributed to PC content is still not clear. Moreover, our present data revealed positive associations of the hydroxycinnamic acid and alkylmethoxyphenol contents in coffee with systolic and diastolic BP in all subjects, even when multiple linear regression was applied. These results highlight the overall predictive capacity of coffee PCs for systolic and diastolic BP levels and, therefore, the potential involvement of specific coffee PCs in BP regulation. Additionally, in HT subjects, we found concomitant positive correlations of coffee PCs with faecal propionate, acetate and valerate levels ($r > 0.4$), which suggested the modulatory capacity of

Table 4
Multivariate linear regression analysis with differential faecal ASV biomarkers in normotensive and hypertensive subjects and the intake of phenolic compounds (mg/day) from major dietary sources and classes.

ASV	Bacterial group	Phenolic intake by dietary source				Phenolic intake by class			
		Variables	R^2	β	p	Variables	R^2	β	p
Normotensive (n = 32)									
s372†	Ruminococcaceae, *Faecalibacterium prausnitzii*	Coffee	0.263	−0.338	0.047	Hydroxycinnamic acids	0.149	−0.385	0.029
s127†	Ruminococcaceae NK4A214	Dried fruit	0.285	0.534	0.002	Hydroxybenzoic acids	0.393	0.627	<0.001*
s230	Bacteroidaceae, *Bacteroides xylanisolvens*	Tubers	0.172	0.415	0.020	Lignans	0.168	0.410	0.020
s408	Ruminococcaceae UCG-010	Olive fruit	0.793	0.624	<0.001*	Anthocyanins	0.630	0.366	0.032
		Nuts		0.346	0.005	Hydroxybenzoic acids		0.490	0.005
s529	Rikenellaceae_RC9_gut_group	Olive oil	0.187	0.432	0.015	Flavones	0.178	0.422	0.016
s897	Tannerellaceae, *Parabacteroides distasonis*	Natural juices	0.444	0.326	0.032	Flavanones	0.157	0.396	0.025
		Fresh fruits		0.312	0.039				
s347	Ruminococcaceae	Olive oil	0.170	0.413	0.021	Flavones	0.161	0.401	0.023
S1019	Ruminococcaceae UCG-010	Olive fruit	0.837	0.553	<0.001*	Anthocyanins	0.766	0.932	<0.001*
		Fresh fruits		0.407	<0.001*	Stilbenes		−0.385	<0.001*
		Olive oil		−0.239	0.006				
s1147	Desulfovibrionaceae, *Bilophila wadsworthia*	Olive fruit	0.842	0.442	<0.001*	Anthocyanins	0.821	0.735	<0.001*
		Fresh fruits		0.337	0.002	Hydroxybenzoketones		0.425	<0.001*
		Nuts		0.285	0.011				
s430	Christensenellaceae R-7	Olive fruit	0.799	0.477	0.001*	Anthocyanins	0.765	0.927	<0.001*
		Fresh fruits		0.279	0.019	Stilbenes		−0.426	<0.001*
		Nuts		0.276	0.027				
s1683	Ruminococcaceae, *Butyricicoccus faecihominis*	Tea	0.525	0.724	<0.001*	Flavonols	0.177	0.421	0.017
s1296	Clostridiales vadin	Olive fruit	0.226	0.475	0.007	Anthocyanins	0.173	0.416	0.018
s3173	Christensenellaceae R-7	Dried fruit	0.762	0.825	<0.001*	Hydroxybenzoic acids	0.678	0.603	<0.001*
		Coffee		−0.259	0.009	Tyrosols		0.391	0.002
s3187	Erysipelotrichaceae, *Dielma*	Natural juices	0.550	0.742	<0.001*	Hydroxybenzoic acids	0.175	0.418	0.017
Hypertensive (n = 29)									
s3042	Clostridiales, Family_XIII	Wine	0.150	0.387	0.042	Stilbenes	0.156	0.395	0.037
s2600	Ruminococcaceae, Ruminiclostridium 5	Fresh fruits	0.483	0.466	0.005	Flavanones	0.510	0.714	<0.001*
		Wine		0.403	0.012				
s728	Ruminococcaceae	Vegetables	0.451	0.672	<0.001*	Lignans	0.289	0.538	0.003
s651	Bacteroidaceae, *Bacteroides vulgatus*	Whole grain cereals	0.416	0.645	<0.001*	Flavanols	0.309	0.555	0.002
s740	Lachnospiraceae, *Lachnoclostridium pacaense*	Coffee	0.148	0.385	0.043	Methoxyphenols	0.184	0.429	0.023
s224	Streptococcaceae, *Streptococcus thermophilus*	Dried fruit	0.795	0.892	<0.001*	Isoflavonoids	0.249	0.499	0.007
s278	Muribaculaceae	Berries	0.159	0.399	0.035	Anthocyanins	0.162	0.403	0.033
s142	Lachnospiraceae	Fresh fruits	0.401	0.516	0.003	Flavanones	0.288	0.537	0.003
		Olive oil		−0.490	0.005				
s92†	Bacteroidaceae, *Bacteroides plebeius*	Coffee	0.179	0.423	0.025	Alkymethoxyphenols	0.227	0.476	0.010
s16†	Bacteroidaceae, *Bacteroides coprocola*	Coffee	0.199	0.446	0.017	Methoxyphenols	0.395	0.356	0.033
s41†	Bacteroidaceae, *Bacteroides coprocola*	Coffee	0.182	0.427	0.023	Methoxyphenols	0.169	0.411	0.030

R^2, coefficient of multiple determination; β, standardized regression coefficient; p, two-sided test of significance.
Values from multiple stepwise regression analysis. Analysis adjusted by age, daily energy intake and total fibre intake.
Only statistically significant results are shown.
* p value ≤ 0.001.
† ASVs with the highest discriminant power in NT and HT group (LDA score > 2.9).

coffee PCs on SCFA-producing bacteria (Jaquet et al., 2009). Accordingly, we hypothesize that an increase in the growth of the species *B. plebeius* and *B. coprocola* induced by coffee PCs could contribute to the weakening of the intestinal absorption of SCFAs, leading to higher faecal levels in HT subjects, as reported in our previous study (Calderón-Pérez et al., 2020). Conversely, the negative associations found in NT subjects for coffee PCs with *F. prausnitzii* and Christensenellaceae R-7, among the most discriminant ASV biomarkers in NT subjects, indicated that a decrease in these specific bacteria could precede the alteration of BP. Both *F. prausnitzii* and Christensenellaceae R-7, are well-known butyrate producers in the human gut with potent anti-inflammatory effects (Zhang et al., 2019), so depletion of these specific bacteria may have functional consequences on SCFA production in the gut and, therefore, on the host's ability to repair epithelium and regulate inflammation, a risk factor for hypertension (Yan et al., 2017). Although the habitual coffee intake reported in the HT group (average of 60.11 mL/day) was significantly higher than that in the NT group (average of 45.30 mL/day), the observed opposite relationships between coffee PCs and discriminant ASV biomarkers in HT and NT subjects may not be explained only by the amount ingested. Therefore, we hypothesize that specific coffee PCs could also modulate the gut microbiome in different ways depending on the stage of disease, either increasing particular bacterial taxa in HT subjects, or decreasing beneficial bacteria in NT subjects. Thus, given the overall negative impact of coffee PCs on the gut microbiome in both groups, our results suggest a new pattern in which coffee PCs could precede the rise in BP.

Another remarkable finding in the present study was the strong positive associations reported in NT subjects between PCs from olive fruits, including those in the anthocyanin class, discriminant ASV biomarkers, such as Ruminococcaceae UCG-010 and Christensenellaceae R-7, and plasma butyrate and valerate. These interrelationships could reflect a favoured butyrate and valerate production and absorption in the colon of NT group mediated by olive fruit PCs. Commercial table olive fruits are the most widely consumed fermented food in Mediterranean countries and are particularly rich in hydroxytyrosol which is found at a high abundance in Greek-style natural black olives and Spanish-style green olives (approximately 250–760 mg hydroxytyrosol/kg) (Kiritsakis & Shahidi, 2017). In our study, the total intake of PCs from olive fruits ranged from 13.95 mg/day in HT subjects to 24.79 mg/day in NT subjects. This intake was specifically provided by the high average consumption of olive fruits in NT (25 g/day), which was about three times over the median consumption of 7 g/capita/day reported in the Spanish population. In addition, the negative correlations found in our previous work for Christensenellaceae R-7 with systolic and diastolic

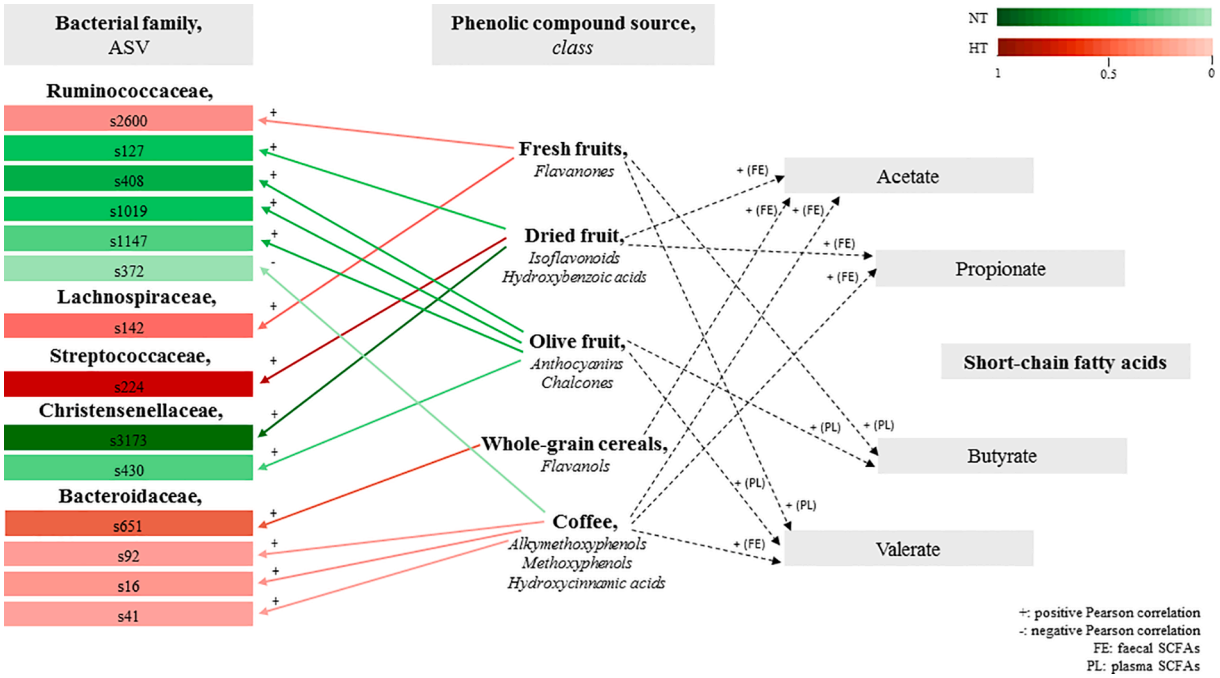


Fig. 2. Schematic of the major interrelationships between PC intake by food sources and phenolic classes, microbial taxa (expressed at the family and ASV levels) and SCFAs in hypertensive (HT) and normotensive (NT) subjects. The colour key in green (NT) and red (HT) represents the strength of the relation. Color arrows represent the relation among PCs and microbial taxa. Doted arrows represent the relation among PCs and faecal or plasma SCFA levels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

BP (Calderón-Pérez et al., 2020), highlight the possible interplay of olive fruit PCs with the gut microbiota and reinforce the role of these compounds in the prevention of hypertension.

In addition to olive fruit PCs, positive relations were detected in NT subjects between PCs from olive oil, including flavones, the main flavonoids present in virgin olive oil, and specific taxa in the Ruminococcaceae and Rikenellaceae families. Recently, the intake of extra-virgin olive oil was reported to be associated with greater promotion of gut bacterial diversity, mediated by an increase in the Firmicutes/Bacteroidetes ratio, in spontaneously hypertensive rats (Hidalgo et al., 2018). A similar effect has also been found in humans, in which PC-enriched olive oils are able to induce antioxidant activities and, consequently, decrease BP (Martin-Pelaez et al., 2017; Moreno-Luna et al., 2012).

Secondarily, from our data, positive associations between PCs from fresh fruits and vegetables, including flavanones and lignans, and several species of the Ruminococcaceae family were observed in both HT and NT subjects. The Ruminococcaceae family is mainly composed of numerous species able to act as primary degraders of insoluble plant cell walls in the lower gastrointestinal tract (Flint, Bayer, Rincon, Lamed, & White, 2008). Members of this family also consume hydrogen and produce acetate that can be utilized by other butyrate-producing xylanolytic species (Chassard & Bernalier-Donadille, 2006). Similarly, the positive associations found between PCs from whole-grain cereals and *B. vulgatus* abundance in HT subjects and between PCs from tubers and *B. xylanisolvens* abundance in NT subjects support the role of these bacterial strains in the colonic catabolism of soluble polysaccharides, such as amylose, amylopectin and xylans, contained in whole grains and tubers. All these positive associations of whole-grain cereals and tubers with *Bacteroides* spp. abundance remained significant after adjusting for dietary fibre intake, suggesting an independent contribution of dietary PCs contained in the same polysaccharide-rich food matrix to the growth of *Bacteroides* spp. Moreover, the weak positive correlation found between whole-grain cereal PCs and faecal acetate in HT subjects reinforces their interplay with the gut microbiota.

On the other hand, in NT subjects, we observed significant positive relationships of PCs from dried fruits and their main phenolic classes, such as phenolic acids and flavonoids, with Ruminococcaceae NK4A214 and Christensenellaceae R-7, which are among the most discriminant bacteria in NT subjects. In contrast, in HT subjects, dried fruit PCs were significantly positively related to *S. thermophiles*, pointing out the role of dried fruit PCs in gut microbial modulation, probably due to their coexistence with lactic acid probiotic bacteria (Kumar Singh et al., 2019). Additionally, the positive correlations reported in HT subjects for dried fruit PCs with faecal propionate and acetate levels led us to propose dried fruit PCs as possible precursors to SCFAs synthesis with a metabolic fate mediated by the gut microbial community. Nevertheless, given the low daily intake of dried fruits in both groups (approximately 11 g/day), we cannot verify their impact on SCFAs synthesis, so more studies are needed to confirm the possible mechanism linking dried fruit sources of PCs with gut bacteria involved in SCFAs synthesis and the connection of this relationship with hypertension.

Interestingly, in the NT group, we also found PCs from natural fruit juices, mainly citrus juices, as possible contributors to the abundance of genus *Dielma* in the Erysipelotrichaceae family, which is able to acidify glucose and other dietary sugars and has been recently associated with inflammation-related gastrointestinal diseases and metabolic disorders in humans (Kaakoush, 2015). Moreover, a positive correlation was found between natural fruit juice PCs, particularly flavanones, and systolic BP in all subjects. Given previous evidence concerning the beneficial effect of citrus juice PCs on BP (Valls et al., 2020), we speculate that the increase in the abundance of the *Dielma* genus after citrus juice intake would be strongly related to the sugar content of the juice. In this sense, an increase in this particular bacterial genus could induce a gut inflammatory response being as a precursor to the hypertensive state (Yan et al., 2017).

It should be noted that the correlations found in NT subjects between the nuts, dried fruits, natural juices PCs, and hydroxybenzoic acids class with Ruminococcaceae and Christensenellaceae families could explain a protective effect of ellagitannins on BP (Wang et al., 2018), as they appear to be included in this phenol class on the Phenol Explorer data base. However, given the lack of correlations with SCFAs, we cannot suggest that the supposed ellagitannin hypotensive effect is mediated by

SCFA action.

In all participants, positive correlations were observed for fermented alcoholic beverage PCs, such as wine and beer PCs, with systolic and diastolic BP. This finding reinforces the observed dose–response alcohol-induced increases in BP levels of approximately 5 to 10 mm Hg after regular alcohol consumption at a threshold of three drinks per day (30 g of ethanol/day), with systolic increases nearly always greater than diastolic increases (Husain, Ansari, & Ferder, 2014). We specifically noted a positive contribution of beer PCs to diastolic BP levels after linear regression. Nevertheless, given the high ethanol intake in the HT group, at approximately 11 g/day, we suppose that the positive association with diastolic BP was related to the alcohol content in beer. Moreover, in a previous dietary study (Chiva-Blanch et al., 2015), after 4 weeks of moderate administration of a non-alcoholic beer rich in PCs, a decrease in BP levels and improvements in other cardiovascular risk factors were observed compared to those under the administration of a standard alcoholic beer, suggesting an alcohol-independent effect on BP of the PCs contained in the non-alcoholic fraction of the beer.

In the present work, the fact that no significant associations were reported for TPI and urinary TPE, an objective marker of exposure to PC intake, with BP values could be attributed to both the small sample size and the high inter-individual variations in gut microbiota affecting PC intrinsic activity, metabolization and absorption through the gut barrier (Tomas-Barberan et al., 2016).

The relationship between the gut microbiota and BP is complex, and current evidence is controversial in relation to the mechanisms involved. Most of the hypotheses point to dysbiosis as a precipitant factor for BP alteration (Al Khodor, Reichert, & Shatat, 2017). However, the observed interplays in the present study of plant-derived dietary PCs consumed voluntarily could explain their role as precursors for gut bacterial modifications and concomitant associations with faecal and plasma SCFA levels. Our findings extend previous knowledge on the correlation between the gut microbiota and hypertension in humans (Calderón-Pérez et al., 2020) and provide a new way in which dietary PCs could induce changes in gut bacterial composition to affect SCFA production and absorption. Furthermore, the opposite trends observed in associations among faecal and plasma SCFAs with particular dietary PCs in HT and NT subjects could explain different absorption modes depending on the hypertension stage. In addition, it will be helpful to better understand the dynamics of SCFAs in the human body (Yamamura et al., 2020). Specifically, fresh fruit and olive fruit PCs seem to favour SCFA absorption, leading to higher plasma concentrations of butyrate and valerate in NT, while coffee PCs are related to higher faecal SCFA levels in HT, suggesting a lower efficiency in their intestinal absorption, which could be a causative factor for the higher BP, as hypothesized in our previous work (Calderón-Pérez et al., 2020).

One distinctive point of the present study was the application of suitable tools to report PC intake, such as the FFQ and 3-day dietary records, which provided a complete picture of participants' habitual dietary PC intake. Furthermore, the large dataset on diet composition allowed us to control for potential confounders such as fibre intake. Nonetheless, the study had some limitations. The small sample size and the heterogeneity in clinical variables may have hampered the detection of other significant associations. Additionally, the cross-sectional design may have limited the establishment of causal relationships, and the existence of unmeasured human factors, such as inter-individual variability in gut microbiota composition or different clinical parameters at baseline, such as BMI, may have influenced the results. However, the associations between microbial composition and BP remained significant after adjustment for BMI among other variables, as reported in our previous work (Calderón-Pérez et al., 2020), indicating that the differences were intrinsically related to the hypertensive condition. Also PC bioavailability might be influenced by uncontrolled external factors, such as food processing and reciprocal interactions with the gut microbiota, affecting the intestinal biotransformation of PCs into their metabolites (Ozdal et al., 2016). It would be of great interest in the future to design in vitro culture models simulating lower-intestinal-tract conditions in order to test whether the reported associations indicate an effect or reflect a possible mechanism of action of PCs from plants.

5. Conclusion

In summary, plant-derived dietary PCs appear to be associated with discriminant gut bacterial taxa and faecal metabolites in the hypertensive and healthy states. The most remarkable findings are the multiple-way positive relationships found in HT subjects among coffee PCs, systolic and diastolic BP, faecal SCFAs and the most discriminant bacterial taxa, *B. plebeius* and *B. coprocola*, and the negative associations in NT subjects between coffee PCs and the beneficial bacteria *F. prausnitzii* and Christensenellaceae R-7. These results suggest a new pattern in which coffee PCs could precede the rise in BP. Additionally, olive fruit PCs were positively associated with Ruminococcaceae UCG-010, Christensenellaceae R-7 and plasma SCFAs in NT subjects, reinforcing the previously reported role of these PCs in the prevention of hypertension.

Finally, the overall results suggested the potential role of specific food phenolic sources as precursors for human gut microbiome modulation involved in hypertension pathogenesis or its prevention. However, further studies on the causal relationship between plant-derived PCs and the gut microbiome will lead to a better understanding of their mutual interaction effects on BP.

CRediT authorship contribution statement

Lorena Calderón-Pérez: Formal analysis, Writing - original draft, Visualization, Investigation, Writing - review & editing. **Elisabet Llauradó:** Investigation, Writing - review & editing. **Judit Companys:** Investigation, Writing - review & editing. **Laura Pla-Pagà:** Investigation, Writing - review & editing. **Anna Pedret:** Supervision, Investigation, Writing - review & editing. **Laura Rubió:** Project administration, Supervision, Investigation, Writing - review & editing, Data curation, Validation, Formal analysis. **Maria José Gosalbes:** Data curation, Validation, Formal analysis. **Silvia Yuste:** Investigation, Writing - review & editing. **Rosa Solà:** Project administration, Conceptualization, Investigation, Writing - review & editing. **Rosa M Valls:** Project administration, Conceptualization, Supervision, Investigation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to acknowledge the support of the Institut d'Investigació Sanitària Pere Virgili (IISPV) in the form of a *Sara Borrell postdoctoral grant* (CD14/00275) to L. Rubió, the EURECAT-Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut (Reus, Spain), the University of Lleida, and the Centre for Public Health Research (FISABIO-Salud Pública, Valencia, Spain). Additionally, we wish to acknowledge the volunteers of the study. A. Pedret is the recipient of a post-doctoral grant (PTQ-15-08068; Spain). The NFOC-Salut group is a consolidated research group of Generalitat de Catalunya, Spain (2017 SGR522).

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128567.

References

Al Khodor, S., Reichert, B., & Shatat, I. F. (2017). The microbiome and blood pressure: Can microbes regulate our blood pressure? *Frontiers in Pediatrics, 5*, 138. https://doi.org/10.3389/fped.2017.00138.

Calderón-Pérez, L., Gosalbes, M. J., Yuste, S., Valls, R. M., Pedret, A., Llauradó, E., … Solà, R. (2020). Gut metagenomic and short chain fatty acids signature in hypertension: A cross-sectional study. *Scientific Reports, 10*(1), 6436. https://doi.org/10.1038/s41598-020-63475-w.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581–583. https://doi.org/10.1038/nmeth.3869.

Chassard, C., & Bernalier-Donadille, A. (2006). H2 and acetate transfers during xylan fermentation between a butyrate-producing xylanolytic species and hydrogenotrophic microorganisms from the human gut. *FEMS Microbiology Letters, 254*(1), 116–122. https://doi.org/10.1111/j.1574-6968.2005.00016.x.

Chiva-Blanch, G., Magraner, E., Condines, X., Valderas-Martínez, P., Roth, I., Arranz, S., … Estruch, R. (2015). Effects of alcohol and polyphenols from beer on atherosclerotic biomarkers in high cardiovascular risk men: A randomized feeding trial. *Nutrition, Metabolism, and Cardiovascular Diseases: NMCD, 25*(1), 36–45. https://doi.org/10.1016/j.numecd.2014.07.008.

Costa, C., Tsatsakis, A., Mamoulakis, C., Teodoro, M., Briguglio, G., Caruso, E., … Fenga, C. (2017). Current evidence on the effect of dietary polyphenols intake on chronic diseases. *Food and Chemical Toxicology, 110*, 286–299. https://doi.org/10.1016/j.fct.2017.10.023.

Danneskiold-Samsøe, N. B., Dias de Freitas Queiroz Barros, H., Santos, R., Bicas, J. L., Cazarin, C. B. B., Madsen, L., … Maróstica Júnior, M. R. (2019). Interplay between food and gut microbiota in health and disease. Food Research International, 115 (April 2018), 23–31. https://doi.org/10.1016/j.foodres.2018.07.043.

de Brito Alves, J. L., de Sousa, V. P., Cavalcanti Neto, M. P., Magnani, M., de A. Braga, V., … da Costa-Silva, J. H. (2016). New insights on the use of dietary polyphenols or probiotics for the management of arterial hypertension. *Frontiers in Physiology, 7*, 448. https://doi.org/10.3389/fphys.2016.00448.

Duenas, M., Munoz-Gonzalez, I., Cueva, C., Jimenez-Giron, A., Sanchez-Patan, F., Santos-Buelga, C., … Bartolome, B. (2015). A survey of modulation of gut microbiota by dietary polyphenols. *BioMed Research International, 2015*, Article 850902. https://doi.org/10.1155/2015/850902.

Esh, H., Agabiti, E., France, M. A., Uk, A. D., Germany, F. M., Kerins, M., … France, I. D. (2018). 2018 ESC/ESH Guidelines for the management of arterial hypertension The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of. https://doi.org/10.1097/HJH.

Fernández-Ballart, J. D., Piñol, J. L., Zazpe, I., Corella, D., Carrasco, P., Toledo, E., … Martn-Moreno, J. M. (2010). Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *British Journal of Nutrition, 103*(12), 1808–1816. https://doi.org/10.1017/S0007114509993837.

Fernandez-Navarro, T., Salazar, N., Gutierrez-Diaz, I., Sanchez, B., Ruas-Madiedo, P., de Los Reyes-Gavilan, C. G., … Gonzalez, S. (2018). Bioactive compounds from regular diet and faecal microbial metabolites. *European Journal of Nutrition, 57*(2), 487–497. https://doi.org/10.1007/s00394-016-1332-8.

Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R., & White, B. A. (2008). Polysaccharide utilization by gut bacteria : potential for new insights from genomic analysis, 6 (february), 121–131. https://doi.org/10.1038/nrmicro1817.

Hidalgo, M., Prieto, I., Abriouel, H., Villarejo, A. B., Ramirez-Sanchez, M., Cobo, A., … Martinez-Canamero, M. (2018). Changes in gut microbiota linked to a reduction in systolic blood pressure in spontaneously hypertensive rats fed an extra virgin olive oil-enriched diet. *Plant Foods for Human Nutrition (Dordrecht, Netherlands), 73*(1), 1–6. https://doi.org/10.1007/s11130-017-0650-1.

Husain, K., Ansari, R. A., & Ferder, L. (2014). Alcohol-induced hypertension: Mechanism and prevention. *World Journal of Cardiology, 6*(5), 245–252. https://doi.org/10.4330/wjc.v6.i5.245.

Interamericana, M.. (2008). TABLAS DE COMPOSICIÓN DE ALIMENTOS DEL CESNID. *Revista Española de Salud Pública*. https://doi.org/10.1590/s1135-57272004000300011.

Jaquet, M., Rochat, I., Moulin, J., Cavin, C., & Bibiloni, R. (2009). Impact of coffee consumption on the gut microbiota: A human volunteer study. *International Journal of Food Microbiology, 130*(2), 117–121. https://doi.org/10.1016/j.ijfoodmicro.2009.01.011.

Kaakoush, N. O. (2015). Insights into the role of erysipelotrichaceae in the human host. *Frontiers in Cellular and Infection Microbiology, 5*, 84. https://doi.org/10.3389/fcimb.2015.00084.

Kiritsakis, A., & Shahidi, F. (2017). Olives and Olive Oil as Functional Foods: Bioactivity, Chemistry and Processing. Wiley. Retrieved from https://books.google.es/books?id=HNkoDwAAQBAJ.

Kumar Singh, A., Cabral, C., Kumar, R., Ganguly, R., Kumar Rana, H., Gupta, A., … Pandey, A. K. (2019). Beneficial effects of dietary polyphenols on gut microbiota and strategies to improve delivery efficiency. *Nutrients, 11*(9). https://doi.org/10.3390/nu11092216.

Marques, F. Z., Mackay, C. R., & Kaye, D. M. (2018). Beyond gut feelings: How the gut microbiota regulates blood pressure. *Nature Reviews Cardiology, 15*(1), 20–32. https://doi.org/10.1038/nrcardio.2017.120.

Martin-Pelaez, S., Mosele, J. I., Pizarro, N., Farras, M., de la Torre, R., Subirana, I., … Fito, M. (2017). Effect of virgin olive oil and thyme phenolic compounds on blood lipid profile: Implications of human gut microbiota. *European Journal of Nutrition, 56*(1), 119–131. https://doi.org/10.1007/s00394-015-1063-2.

Alexander Medina-Remon, Barrionuevo-Gonzalez, A., Zamora-Ros, R., Andres-Lacueva, C., Estruch, R., Martinez-Gonzalez, M.-A., … Lamuela-Raventos, R. M. (2009). Rapid Folin-Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake. *Analytica Chimica Acta, 634*(1), 54–60. https://doi.org/10.1016/j.aca.2008.12.012.

Medina-Remon, A., Zamora-Ros, R., Rotches-Ribalta, M., Andres-Lacueva, C., Martinez-Gonzalez, M. A., Covas, M. I., … Lamuela-Raventos, R. M. (2011). Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk. *Nutrition, Metabolism, and Cardiovascular Diseases: NMCD, 21*(5), 323–331. https://doi.org/10.1016/j.numecd.2009.10.019.

Moorthy, M., Chaiyakunapruk, N., Jacob, S. A., & Palanisamy, U. D. (2020). Prebiotic potential of polyphenols, its effect on gut microbiota and anthropometric/clinical markers: A systematic review of randomised controlled trials. *Trends in Food Science & Technology, 99*, 634–649. https://doi.org/10.1016/j.tifs.2020.03.036.

Moreno-Luna, R., Munoz-Hernandez, R., Miranda, M. L., Costa, A. F., Jimenez-Jimenez, L., Vallejo-Vaz, A. J., … Stiefel, P. (2012). Olive oil polyphenols decrease blood pressure and improve endothelial function in young women with mild hypertension. *American Journal of Hypertension, 25*(12), 1299–1304. https://doi.org/10.1038/ajh.2012.128.

Most, J., Penders, J., Lucchesi, M., Goossens, G. H., & Blaak, E. E. (2017). Gut microbiota composition in relation to the metabolic response to 12-week combined polyphenol supplementation in overweight men and women. *European Journal of Clinical Nutrition, 71*(9), 1040–1045. https://doi.org/10.1038/ejcn.2017.89.

Nash, V., Ranadheera, C. S., Georgousopoulou, E. N., Mellor, D. D., Panagiotakos, D. B., McKune, A. J., … Naumovski, N. (2018). The effects of grape and red wine polyphenols on gut microbiota – A systematic review. *Food Research International (Ottawa, Ont.), 113*, 277–287. https://doi.org/10.1016/j.foodres.2018.07.019.

Natarajan, N., Hori, D., Flavahan, S., Steppan, J., Flavahan, N. A., Berkowitz, D. E., & Pluznick, J. L. (2016). Microbial short chain fatty acid metabolites lower blood pressure via endothelial G protein-coupled receptor 41. *Physiological Genomics, 48*(11), 826–834. https://doi.org/10.1152/physiolgenomics.00089.2016.

Neveu, V., Perez-Jimenez, J., Vos, F., Crespy, V., du Chaffaut, L., Mennen, L., … Scalbert, A. (2010). Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. Database : The Journal of Biological Databases and Curation, 2010, bap024. https://doi.org/10.1093/database/bap024.

Nissensohn, M., Roman-Vinas, B., Sanchez-Villegas, A., Piscopo, S., & Serra-Majem, L. (2016). The effect of the mediterranean diet on hypertension: A systematic review and meta-analysis. *Journal of Nutrition Education and Behavior, 48*(1), 42–53.e1. https://doi.org/10.1016/j.jneb.2015.08.023.

Ozdal, T., Sela, D. A., Xiao, J., Boyacioglu, D., Chen, F., & Capanoglu, E. (2016). The reciprocal interactions between polyphenols and gut microbiota and effects on bioaccessibility. *Nutrients, 8*(2), 78. https://doi.org/10.3390/nu8020078.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research, 41*(Database issue), D590–D596. https://doi.org/10.1093/nar/gks1219.

Rinninella, E., Cintoni, M., Raoul, P., Lopetuso, L. R., Scaldaferri, F., Pulcini, G., … Mele, M. C. (2019). Food components and dietary habits: Keys for a healthy gut microbiota composition. *Nutrients, 11*(10). https://doi.org/10.3390/nu11102393.

Selma, M. V., Espín, J. C., & Tomás-Barberán, F. A. (2009). Interaction between phenolics and gut microbiota: Role in human health. *Journal of Agricultural and Food Chemistry, 57*(15), 6485–6501. https://doi.org/10.1021/jf902107d.

Tomas-Barberan, F. A., Selma, M. V., & Espin, J. C. (2016). Interactions of gut microbiota with dietary polyphenols and consequences to human health. *Current Opinion in Clinical Nutrition and Metabolic Care, 19*(6), 471–476. https://doi.org/10.1097/MCO.0000000000000314.

Vallbona Calbó, C., Roure Cuspinera, E., Violan Fors, M., & Alegre Martín, J. (2007). Guia de prescripció d'exercici físic per a la salut (PEFS). Barcelona.

Valls, R. M., Pedret, A., Calderón-Pérez, L., Llauradó, E., Pla-Pagà, L., Companys, J., … Solà, R. (2020). Effects of hesperidin in orange juice on blood and pulse pressures in mildly hypertensive individuals: A randomized controlled trial (Citrus study). *European Journal of Nutrition*. https://doi.org/10.1007/s00394-020-02279-0.

Wang, D., Özen, C., Abu-Reidah, I. M., Chigurupati, S., Patra, J. K., Horbanczuk, J. O., … Atanasov, A. G. (2018). Vasculoprotective effects of pomegranate (Punica granatum L.). *Frontiers in Pharmacology, 9*, 544. https://doi.org/10.3389/fphar.2018.00544.

Xie, L., Lee, S. G., Vance, T. M., Wang, Y., Kim, B., Lee, J.-Y., … Bolling, B. W. (2016). Bioavailability of anthocyanins and colonic polyphenol metabolites following consumption of aronia berry extract. *Food Chemistry, 211*, 860–868. https://doi.org/10.1016/j.foodchem.2016.05.122.

Yamamura, R., Nakamura, K., Kitada, N., Aizawa, T., Shimizu, Y., Nakamura, K., … Tamakoshi, A. (2020). Associations of gut microbiota, dietary intake, and serum short-chain fatty acids with fecal short-chain fatty acids. *Bioscience of Microbiota, Food and Health, 39*(1), 11–17. https://doi.org/10.12938/bmfh.19-010.

Yan, Q., Gu, Y., Li, X., Yang, W., Jia, L., Chen, C., … Ma, Y. (2017). Alterations of the gut microbiome in hypertension. *Frontiers in Cellular and Infection Microbiology, 7*, 381. https://doi.org/10.3389/fcimb.2017.00381.

Yang, T., Santisteban, M. M., Rodriguez, V., Li, E., Ahmari, N., Carvajal, J. M., … Mohamadzadeh, M. (2015). Gut dysbiosis is linked to hypertension. Hypertension (Dallas, Tex. : 1979), 65(6), 1331–1340. https://doi.org/10.1161/HYPERTENSIONAHA.115.05315.

Zhang, M., Zhou, L., Wang, Y., Dorfman, R. G., Tang, D., Xu, L., … Yu, C. (2019). Faecalibacterium prausnitzii produces butyrate to decrease c-Myc-related metabolism and Th17 differentiation by inhibiting histone deacetylase 3. *International Immunology, 31*(8), 499–514. https://doi.org/10.1093/intimm/dxz022.

Supplementary Table 1 | Mean daily intake according to food groups.

Food group intake *(g/day)*	**Hypertensive (n=27)**	**Normotensive (n=32)**	**P-value**
Dairy products	240.17 ± 150.38	329.26 ± 295.26	0.513
Whole dairy	65.62 ± 59.35	103.40 ± 154.12	0.610
Semi-skimmed dairy	86.28 ± 112.30	105.46 ± 151.72	0.766
Skimmed dairy	76.28 ± 115.24	85.78 ± 112.70	0.926
Eggs	25.25 ± 12.80	27.11 ± 28.71	0.537
Meats	193.07 ± 122.15	148.55 ± 106.27	0.140
Red meat	64.95 ± 44.70	53.30 ± 50.40	0.150
White meat	82.09 ± 81.11	65.71 ± 78.23	0.125
Processed meat	46.03 ± 28.52	29.54 ± 25.13	0.016
Fish and seafood	103.98 ± 57.60	107.83 ± 71.10	0.976
Lean fish	37.40 ± 26.50	36.46 ± 27.75	0.691
Fatty fish	42.86 ± 23.09	48.13 ± 43.72	0.517
Shellfish	23.72 ± 18.93	23.20 ± 16.14	0.770
Vegetables	467.05 ± 261.35	433.03 ± 217.74	0.533
Tubers	66.22 ± 38.16	40.37 ± 32.58	0.005
Fresh fruits	399.99 ± 239.93	403.71 ± 338.54	0.420
Dried fruit	10.81 ± 22.90	11.07 ± 24.17	0.713
Nuts	12.18 ± 17.51	6.61 ± 7.35	0.132
Olive fruit	12.57 ± 13.19	24.77 ± 27.83	0.107
Legumes	27.72 ± 8.78	25.45 ± 11.92	0.417
Cereals	213.50 ± 176.76	238.93 ± 188.35	0.553
Refined cereals	121.42 ± 86.69	104.10 ± 85.13	0.312
Whole-grain cereals	26.51 ± 42.88	50.63 ± 68.66	0.034
Oils and fats	23.13 ± 14.96	27.70 ± 21.99	0.503
Olive oil	20.50 ± 14.61	25.30 ± 22.18	0.374
Sunflower oil	0.36 ± 0.89	1.17 ± 2.64	0.511
Butter	2.03 ± 4.58	1.21 ± 1.73	0.407
Pastries	14.52 ± 12.60	20.39 ± 19.76	0.583

Chocolate	7.03 ± 7.95	6.36 ± 8.40	0.590
Pre-cooked food	53.70 ± 40.00	39.53 ± 23.82	0.191
Non-alcoholic beverages	235.82 ± 172.11	144.79 ± 136.20	0.004
Sugary carbonated drinks	28.85 ± 45.34	9.46 ± 16.31	0.190
Soft drinks	12.66 ± 34.15	22.70 ± 56.08	0.713
Commercial juices	30.63 ± 96.65	18.83 ± 50.27	0.574
Natural juices	85.32 ± 81.47	27.88 ± 45.24	0.008
Coffee	60.11 ± 45.30	44.70 ± 57.45	0.033
Tea	18.23 ± 33.01	21.18 ± 36.97	0.906
Alcoholic beverages	160.80 ± 145.21	131.70 ± 162.22	0.108
Wine	57.60 ± 81.08	41.35 ± 61.76	0.278
Beer	101.90 ± 102.93	88.83 ± 112.07	0.213
Distilled spirits	1.28 ± 2.56	1.52 ± 4.05	0.756

Data expressed as mean ± standard deviation. Daily mean intake by food groups was estimated from adapted Food Frequency Questionnaires performed in 59 subjects. P-value estimated by Student's t-test and Mann-Whitney U test. The level of statistical significance was set at $p<0.05$.

Supplementary Table 2 | Correlations of total phenolic intake, total polyphenol excretion in urine and phenolic compounds from food sources and phenolic classes with systolic and diastolic blood pressure in all subjects

	Systolic Blood Pressure (mmHg)		Diastolic Blood Pressure (mmHg)	
	r	**p**	**r**	**p**
TPI, *mg/day*	-0.046	0.365	-0.058	0.331
TPE, *GAE/g creatinine*	0.183	0.083	0.166	0.103
Age, *y*	0.567	**0.000**	0.528	**0.000**
Dietary fiber, *g/day*	-0.070	0.298	-0.110	0.202
Energy intake, *kcal/day*	-0.044	0.369	-0.094	0.237
Phenolic compound dietary source, m*g/day*				
Tubers	0.162	0.108	0.143	0.138
Cereals	-0.070	0.296	-0.028	0.417
Whole-grain cereals	-0.119	0.189	-0.138	0.153
Vegetables	-0.008	0.476	-0.148	0.130
Legumes	-0.066	0.308	-0.008	0.476
Fresh fruits	-0.099	0.227	-0.155	0.119
Berries	0.056	0.336	0.029	0.414
Dried fruit	-0.018	0.445	0.016	0.453
Nuts	-0.011	0.467	-0.030	0.411
Olive fruit	-0.104	0.216	-0.080	0.273
Oils	0.005	0.486	-0.148	0.129
Olive oil	0.004	0.487	-0.147	0.131
Chocolate	-0.198	0.064	-0.175	0.091
Non-alcoholic beverages	0.300	**0.010**	0.344	**0.004**
Natural fruit juices	0.227	**0.040**	0.125	0.171
Coffee	0.278	**0.016**	0.295	**0.011**
Tea	0.005	0.486	0.048	0.359
Alcoholic beverages	0.236	**0.035**	0.259	**0.023**
Wine	0.231	**0.038**	0.218	**0.047**
Beer	0.135	0.152	0.295	**0.011**
Seasonings	-0.044	0.369	0.005	0.484
Phenolic compound class and subclass, *mg/day*				
Flavonoids	0.144	0.136	0.123	0.174
Anthocyanins	0.203	0.060	0.198	0.065
Chalcones	0.202	0.061	0.343	**0.004**
Dihydrochalcones	-0.107	0.208	-0.218	**0.047**
Dihydroflavanols	0.231	**0.038**	0.218	**0.047**
Flavanols	0.040	0.380	0.031	0.407
Flavanones	0.226	**0.042**	0.208	0.055
Flavones	-0.119	0.182	-0.207	0.056
Flavonols	0.050	0.351	-0.020	0.441
Isoflavonoids	-0.056	0.335	0.029	0.413
Phenolic acids	0.284	**0.014**	0.258	**0.023**
Hydroxybenzoic acid	0.030	0.409	0.021	0.436
Hydroxycinnamic acids	0.310	**0.008**	0.283	**0.014**
Stilbenes	0.234	**0.036**	0.224	**0.043**
Lignans	0.148	0.129	-0.023	0.432
Other polyphenols	0.028	0.416	0.038	0.385
Alkymethoxyphenols	0.290	**0.012**	0.348	**0.003**
Alkylphenols	-0.019	0.443	0.006	0.483
Curcuminoids	-0.023	0.432	-0.029	0.413
Hydroxybenzaldehydes	0.226	**0.041**	0.217	**0.048**
Hydroxybenzoketones	-0.033	0.402	0.097	0.231
Hydroxycoumarins	0.080	0.272	0.225	**0.042**
Methoxyphenols	0.285	**0.014**	0.299	**0.010**
Tyrosols	0.056	0.336	0.018	0.447

SBP. systolic blood pressure; DBP. diastolic blood pressure; TPI. total polyphenol intake; TPE. total polyphenol excretion.
Significant correlations were set at $p<0.05$ (depicted in **bold**). Hypertensive n=28; Normotensive n=32; All subjects n=60.
r: Pearson correlation coefficient; p: 1-tailed test of significance for Pearson correlations.

Supplementary Table 3 | Correlations of phenolic compounds from food sources and phenolic classes with faecal short-chain fatty acid concentrations in hypertensive and normotensive subjects

	Propionate *(mg/g feces)*				**Butyrate** *(mg/g feces)*				**Acetate** *(mg/g feces)*				**Valerate** *(mg/g feces)*			
	Hypertensive		**Normotensive**		**Hypertensive**		**Normotensive**		**Hypertensive**		**Normotensive**		**Hypertensive**		**Normotensive**	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
TPI. *mg/day*	-0.256	0.188	0.292	0.105	-0.267	0.170	0.134	0.464	-0.196	0.319	0.223	0.221	-0.226	0.248	0.239	0.188
TPE. *GAE/g creatinine*	0.157	0.415	0.237	0.199	0.135	0.485	0.290	0.113	0.290	0.127	0.244	0.185	-0.193	0.316	0.274	0.136
Phenolic compound dietary source, *g/day*																
Tubers	-0.119	0.545	0.044	0.810	0.023	0.908	0.177	0.332	0.011	0.955	0.079	0.668	-0.182	0.353	0.078	0.673
Whole-grain cereals	0.192	0.174	0.262	0.077	0.201	0.162	0.189	0.154	0.321	**0.055**	0.119	0.261	-0.054	0.396	0.040	0.416
Vegetables	-0.115	0.561	0.009	0.962	-0.110	0.578	0.175	0.339	0.028	0.887	0.007	0.969	-0.152	0.439	0.048	0.794
Legumes	-0.315	0.103	0.143	0.433	-0.362	0.059	-0.027	0.882	-0.334	0.082	0.108	0.558	-0.103	0.603	0.032	0.862
Fresh-fruits	0.116	0.557	0.349	0.051	0.205	0.296	0.243	0.180	0.147	0.456	0.292	0.104	0.054	0.784	0.311	0.083
Berries	0.266	0.172	0.231	0.203	0.045	0.821	0.192	0.292	0.128	0.516	0.141	0.441	-0.092	0.643	0.102	0.579
Dried fruits	0.375	**0.049**	0.142	0.440	0.246	0.207	0.158	0.388	0.385	**0.043**	0.105	0.569	0.196	0.318	0.212	0.243
Nuts	-0.219	0.263	-0.060	0.745	-0.216	0.269	0.177	0.332	-0.216	0.270	-0.056	0.759	-0.158	0.422	-0.024	0.894
Olive fruits	-0.117	0.553	-0.001	0.997	-0.171	0.383	0.156	0.395	-0.025	0.899	-0.064	0.729	-0.291	0.133	0.102	0.580
Olive oil	-0.036	0.857	0.280	0.120	-0.016	0.935	0.103	0.575	0.056	0.779	0.202	0.267	-0.064	0.745	0.240	0.185
Chocolate	0.112	0.572	0.239	0.187	0.062	0.755	-0.124	0.499	0.240	0.219	0.129	0.483	-0.285	0.142	0.075	0.683
Natural fruit juices	0.045	0.821	0.166	0.365	-0.074	0.708	0.284	0.115	0.105	0.595	0.092	0.618	-0.170	0.388	0.201	0.270
Coffee	0.475	**0.006**	0.270	0.165	0.013	0.949	0.079	0.667	0.401	**0.023**	0.191	0.329	0.506	**0.003**	0.271	0.263
Tea	-0.023	0.908	-0.040	0.828	-0.118	0.551	0.017	0.928	0.039	0.844	-0.046	0.803	-0.140	0.476	0.052	0.779
Wine	-0.229	0.241	0.029	0.875	-0.173	0.379	-0.101	0.583	-0.309	0.109	0.143	0.436	-0.049	0.806	0.138	0.452
Beer	-0.040	0.841	0.248	0.170	0.165	0.402	0.147	0.422	0.057	0.774	0.264	0.144	-0.058	0.768	0.508	**0.003**
Seasonings	-0.017	0.931	0.031	0.867	-0.078	0.692	0.149	0.416	0.013	0.947	0.070	0.703	-0.204	0.297	-0.095	0.606
Phenolic compound class, *mg/day*																
Anthocyanins	0.048	0.807	0.152	0.405	-0.022	0.913	0.213	0.241	-0.111	0.573	0.095	0.606	-0.070	0.724	0.099	0.589
Chalcones	0.019	0.924	0.242	0.183	-0.033	0.870	0.139	0.446	0.024	0.902	0.261	0.148	-0.046	0.816	0.505	**0.003**
Dihydrochalcones	-0.031	0.875	0.316	0.078	-0.112	0.570	0.137	0.456	-0.060	0.761	0.368	**0.038**	-0.215	0.271	0.367	**0.039**
Dihydroflavanols	-0.228	0.242	0.028	0.877	-0.173	0.378	-0.101	0.584	-0.308	0.111	0.144	0.431	-0.049	0.803	0.137	0.456
Flavanols	-0.060	0.763	0.273	0.130	-0.085	0.666	0.034	0.853	-0.053	0.791	0.279	0.122	-0.153	0.437	0.246	0.174

Flavanones	0.121	0.539	0.201	0.271	0.212	0.278	-0.008	0.963	0.122	0.537	0.159	0.384	0.141	0.474	0.217	0.234
Flavones	0.180	0.360	-0.312	0.082	-0.042	0.831	-0.316	0.078	0.074	0.709	-0.246	0.174	-0.233	0.233	-0.173	0.343
Flavonols	-0.241	0.217	0.041	0.825	-0.282	0.146	0.003	0.986	-0.141	0.474	0.018	0.921	-0.231	0.237	0.060	0.743
Isoflavonoids	-0.298	0.124	-0.146	0.425	-0.218	0.265	-0.041	0.826	-0.161	0.413	-0.126	0.491	-0.149	0.448	-0.009	0.960
Hydroxybenzoic acid	0.130	0.510	0.132	0.473	0.167	0.395	0.284	0.115	0.119	0.547	0.081	0.658	0.120	0.541	0.150	0.411
Hydroxycinnamic acids	0.454	**0.009**	0.193	0.325	-0.096	0.625	0.129	0.481	0.376	**0.034**	0.122	0.537	0.583	**0.000**	0.060	0.762
Stilbenes	-0.232	0.235	0.034	0.855	-0.176	0.370	-0.100	0.585	-0.316	0.102	0.143	0.434	-0.050	0.799	0.143	0.435
Lignans	-0.240	0.218	-0.059	0.748	-0.261	0.180	-0.011	0.954	-0.149	0.450	-0.080	0.663	-0.236	0.226	-0.079	0.669
Alkymethoxyphenols	0.450	**0.010**	0.234	0.230	0.076	0.701	0.076	0.680	0.411	**0.019**	0.213	0.277	0.566	**0.001**	0.236	0.227
Alkylphenols	-0.138	0.483	0.037	0.839	0.031	0.876	0.009	0.962	-0.125	0.526	0.071	0.700	-0.071	0.719	-0.069	0.706
Curcuminoids	-0.038	0.846	-0.137	0.456	-0.192	0.328	-0.147	0.422	0.064	0.746	-0.052	0.779	-0.087	0.662	0.076	0.680
Hydroxybenzaldehydes	-0.222	0.257	0.042	0.820	-0.160	0.415	-0.092	0.617	-0.295	0.128	0.159	0.384	-0.049	0.806	0.156	0.395
Hydroxybenzoketones	-0.044	0.825	0.015	0.936	0.170	0.386	-0.021	0.908	0.050	0.799	0.084	0.649	-0.057	0.773	0.161	0.379
Hydroxycoumarins	-0.044	0.822	0.244	0.178	0.180	0.361	0.138	0.451	0.061	0.759	0.263	0.146	-0.039	0.844	0.506	**0.003**
Methoxyphenols	0.434	**0.013**	0.262	0.179	0.010	0.959	0.045	0.806	0.383	**0.031**	0.197	0.314	0.482	**0.005**	0.262	0.179
Tyrosols	-0.141	0.475	0.139	0.449	-0.214	0.275	0.191	0.295	-0.101	0.608	0.078	0.672	-0.245	0.208	0.250	0.168

SBP, systolic blood pressure; DBP, diastolic blood pressure. Significant correlations were set at $p<0.05$ (depicted in **bold**). Hypertensive n=28; Normotensive n=32. r: Pearson correlation coefficient; p: 2-tailed test of significance for Pearson correlations.

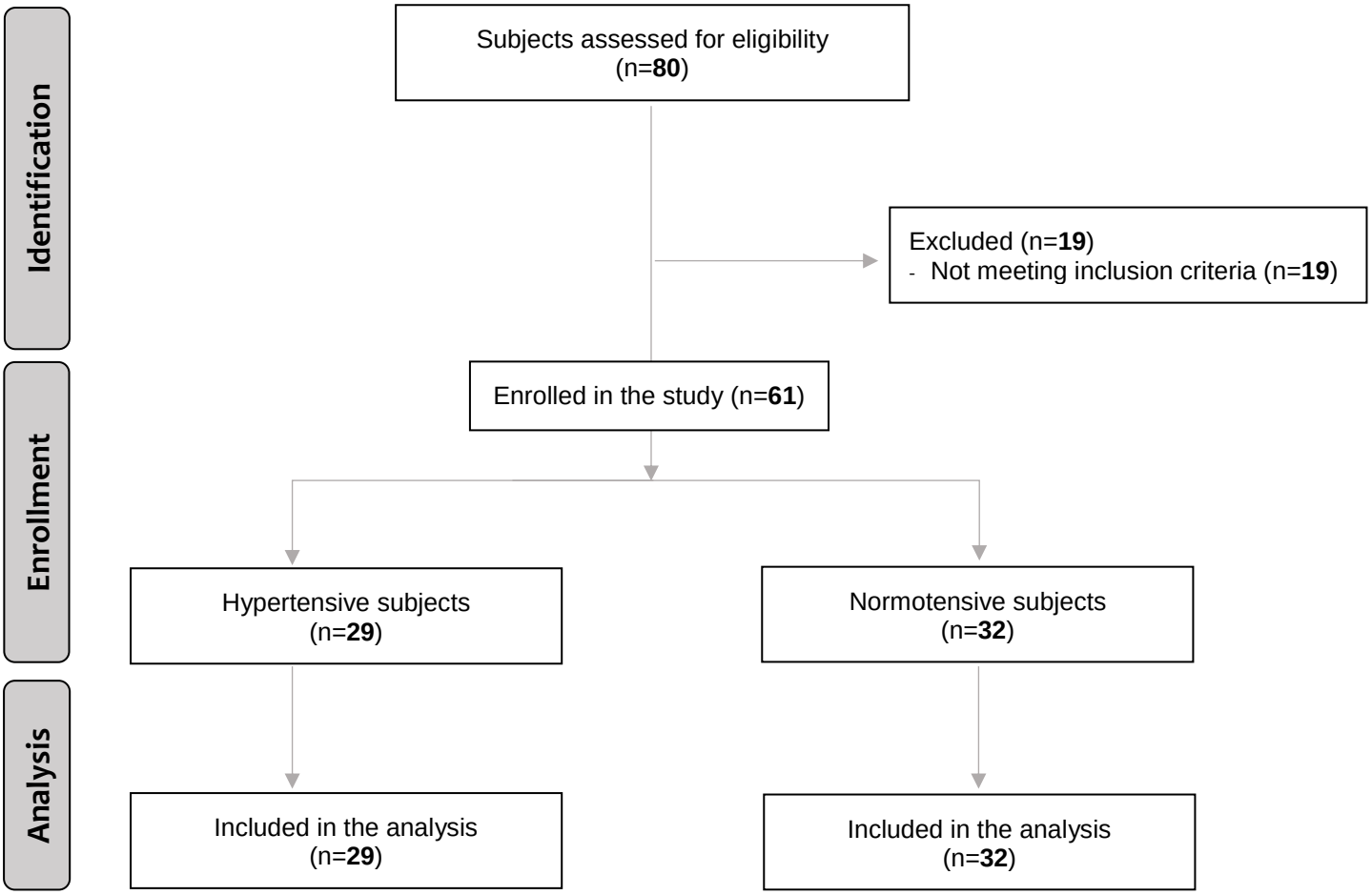
Supplementary Table 4| Correlations of phenolic compounds from food sources and phenolic classes with plasma short-chain fatty acid concentrations in hypertensive and normotensive subjects

	Propionate *(mg/mL plasma)*				**Butyrate** *(mg/mL plasma)*				**Acetate** *(mg/mL plasma)*				**Valerate** *(mg/mL plasma)*			
	Hypertensive		**Normotensive**		**Hypertensive**		**Normotensive**		**Hypertensive**		**Normotensive**		**Hypertensive**		**Normotensive**	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Phenolic compound dietary source, *g/day*																
Tubers	0.173	0.378	0.199	0.282	0.158	0.423	-0.106	0.569	0.159	0.418	0.060	0.750	0.119	0.546	0.054	0.774
Whole-grain cereals	0.036	0.857	-0.207	0.263	-0.108	0.583	-0.177	0.341	-0.188	0.338	-0.140	0.454	-0.123	0.533	-0.069	0.711
Vegetables	-0.026	0.894	0.109	0.559	0.052	0.793	-0.207	0.265	0.244	0.210	0.330	0.070	-0.102	0.606	-0.303	0.097
Legumes	0.186	0.344	-0.172	0.354	-0.167	0.395	0.080	0.671	0.185	0.345	-0.053	0.776	-0.119	0.547	0.168	0.367
Fresh-fruits	0.060	0.760	0.197	0.288	0.053	0.790	**0.368**	**0.042**	0.172	0.380	0.142	0.446	0.185	0.345	**0.576**	**0.001**
Berries	-0.028	0.889	-0.073	0.696	-0.136	0.489	0.250	0.175	-0.109	0.582	0.056	0.765	-0.009	0.963	0.326	0.073
Dried fruits	-0.066	0.737	-0.001	0.997	0.155	0.432	0.197	0.289	-0.164	0.405	0.054	0.771	0.144	0.465	0.291	0.112
Nuts	0.140	0.476	0.026	0.889	-0.179	0.363	0.287	0.117	0.204	0.298	0.148	0.426	-0.258	0.184	0.311	0.089
Olive fruits	-0.139	0.479	0.011	0.952	-0.267	0.170	**0.356**	**0.049**	-0.025	0.900	0.192	0.301	-0.264	0.174	**0.415**	**0.020**
Olive oil	0.078	0.693	-0.011	0.953	-0.098	0.619	-0.065	0.727	0.027	0.891	0.116	0.534	-0.121	0.539	0.087	0.642
Chocolate	0.026	0.894	-0.107	0.567	0.028	0.888	-0.058	0.755	-0.112	0.571	-0.172	0.356	0.054	0.785	0.074	0.691
Natural fruit juices	0.047	0.811	0.011	0.954	0.063	0.752	0.189	0.309	-0.041	0.834	0.272	0.139	0.284	0.143	0.002	0.992
Coffee	0.107	0.588	0.034	0.857	-0.152	0.439	-0.189	0.309	0.309	0.110	0.049	0.792	-0.238	0.222	-0.052	0.780
Tea	0.353	0.065	-0.028	0.880	0.138	0.483	-0.226	0.221	**0.403**	**0.034**	-0.176	0.344	0.011	0.955	-0.181	0.331
Wine	-0.191	0.330	0.060	0.748	-0.201	0.304	-0.152	0.415	0.104	0.597	0.204	0.271	-0.228	0.244	-0.243	0.188
Beer	0.251	0.198	0.037	0.842	-0.101	0.610	-0.168	0.367	0.026	0.895	0.269	0.144	-0.110	0.576	-0.149	0.423
Seasonings	0.209	0.286	0.046	0.805	0.141	0.473	-0.160	0.391	0.183	0.351	-0.066	0.725	0.169	0.391	-0.252	0.171
Phenolic compound class, *mg/day*																
Anthocyanins	-0.185	0.345	-0.048	0.796	-0.208	0.289	0.347	0.056	-0.201	0.304	0.146	0.435	-0.070	0.723	**0.360**	**0.047**
Chalcones	0.326	0.091	0.044	0.815	0.005	0.979	-0.165	0.375	**0.415**	**0.028**	0.275	0.134	0.037	0.852	-0.151	0.417
Dihydrochalcones	0.123	0.535	0.235	0.204	-0.037	0.852	0.047	0.802	-0.008	0.968	0.216	0.243	0.005	0.979	0.194	0.295
Dihydroflavanols	-0.194	0.323	0.060	0.747	-0.204	0.298	-0.152	0.415	0.104	0.598	0.201	0.277	-0.231	0.237	-0.240	0.194
Flavanols	0.168	0.394	0.086	0.645	0.026	0.896	-0.220	0.234	0.215	0.271	-0.014	0.940	-0.033	0.866	-0.038	0.838
Flavanones	-0.061	0.759	0.108	0.564	0.002	0.991	0.041	0.826	0.152	0.439	0.047	0.804	0.116	0.556	-0.030	0.872
Flavones	0.004	0.983	0.014	0.942	-0.138	0.484	-0.186	0.317	-0.037	0.851	-0.096	0.607	-0.002	0.991	-0.129	0.490

Flavonols	0.218	0.265	0.196	0.292	0.094	0.633	-0.110	0.555	**0.446**	**0.017**	0.230	0.213	-0.045	0.822	-0.071	0.704
Isoflavonoids	0.305	0.115	0.130	0.487	0.280	0.148	**0.485**	**0.006**	**0.477**	**0.010**	0.289	0.115	0.291	0.133	0.340	0.061
Hydroxybenzoic acid	0.156	0.427	-0.063	0.736	-0.129	0.512	0.207	0.263	0.259	0.184	0.133	0.475	-0.254	0.193	0.222	0.229
Hydroxycinnamic acids	0.065	0.744	0.226	0.221	-0.241	0.217	-0.140	0.452	0.229	0.241	0.188	0.311	-0.303	0.117	0.126	0.501
Stilbenes	-0.191	0.331	0.060	0.748	-0.208	0.287	-0.150	0.422	0.099	0.617	0.211	0.255	-0.227	0.245	-0.246	0.182
Lignans	0.020	0.919	0.033	0.862	-0.054	0.784	0.083	0.658	0.245	0.208	0.173	0.353	-0.127	0.518	0.052	0.782
Alkymethoxyphenols	0.154	0.434	0.046	0.806	-0.177	0.367	-0.236	0.202	0.256	0.189	0.104	0.576	-0.259	0.183	-0.127	0.496
Alkylphenols	0.125	0.527	-0.029	0.879	0.001	0.996	-0.042	0.822	-0.010	0.958	-0.041	0.829	0.078	0.692	-0.114	0.541
Curcuminoids	**0.422**	**0.025**	-0.177	0.341	0.121	0.539	-0.216	0.244	**0.520**	**0.005**	-0.014	0.940	0.023	0.906	-0.257	0.163
Hydroxybenzaldehydes	-0.182	0.355	0.059	0.754	-0.203	0.301	-0.157	0.400	0.106	0.592	0.202	0.276	-0.241	0.217	-0.230	0.212
Hydroxybenzoketones	0.187	0.341	0.075	0.689	-0.123	0.533	-0.036	0.849	-0.061	0.757	0.211	0.255	-0.139	0.480	-0.054	0.771
Hydroxycoumarins	0.223	0.255	0.043	0.820	-0.060	0.762	-0.167	0.369	-0.013	0.946	0.273	0.137	-0.073	0.714	-0.151	0.417
Methoxyphenols	0.075	0.704	0.037	0.842	-0.160	0.415	-0.210	0.256	0.272	0.162	0.032	0.863	-0.241	0.216	-0.092	0.624
Tyrosols	-0.301	0.120	0.196	0.291	-0.326	0.090	0.098	0.599	-0.103	0.604	0.254	0.167	-0.374	0.050	0.294	0.108

SBP, systolic blood pressure; DBP, diastolic blood pressure. Significant correlations were set at $p<0.05$ (depicted in **bold**). Hypertensive n=28; Normotensive n=32. r: Pearson correlation coefficient; p: 2-tailed test of significance for Pearson correlations.

Supplementary Figure 1 | Flowchart based on the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) statement



SUMMARY OF RESULTS: PART I

Summary of results – Article 1. Gut metagenomic and short-chain fatty acids signature in hypertension: A cross-sectional study

Characteristics of the study participants

A total of 61 participants were assigned to HT group (n = 29) and NT group (n = 32). Of the 61 candidates, 10 females and 19 males with a mean age of 53.7 years were in the HT group, and 16 females and 16 males with a mean age of 41.1 years were in the NT group. Significant differences between groups were found in different clinical variables including age, weight, BMI, waist circumference and fat mass; and in analytical parameters, including fasting blood glucose, total cholesterol and LDL cholesterol, being higher in HT group ($P>0.05$).

Microbiota composition analysis and identification of gut microbiota-based biomarkers associated with hypertension

A total of 59 faecal samples were filtered by length, quality and chimera giving a total of 7,171,741 filtered sequences with an average of 129,125 sequences per sample in HT and 114,237 sequences per sample in NT. 4491 ASVs were generated, and taxonomic assignment was performed at ASV and genus level. Firmicutes and Bacteroidetes were the two major phyla in both groups, with predominant genera such as *Faecalibacterium*, *Ruminococcus*, *Lachnospira*, *Phascolarctobacterium*, *Roseburia* and *Dialister* in Firmicutes, and *Bacteroides*, *Prevotella* and *Alistipes* taxa in Bacteroidetes genus.

Not significant differences were detected in bacterial richness and diversity indexes between groups at ASV or genus level. However, specific alterations were found in particular bacterial taxa. In this way, the LEfSe analysis

identified a total of 67 ASVs biomarkers that had significant different abundance between HT and NT groups (LDA score > 2). Of these, three ASVs classified as *Bacteroidetes coprocola* and *Bacteroidetes plebeius* genus (s41, s16 and s92) showed the highest LDA scores (3.08, 3.05 and 2.9, respectively), highlighting their role as potent bacterial biomarkers in HT. In contrast, in NT, different genera of Ruminococcaceae, Lachnospiraceae, Acidaminococcaceae and Christensenellaceae families, were the most frequent taxa. Particularly, in NT group, *Faecalibacterium prausnitzii* and *Roseburia hominis*, both described as the main SCFA-producers in the healthy status, presented the highest discriminant power (LDA score > 2.5). However, a depletion of SCFA-producers was observed in HT group.

After performing random forest analysis, the LEfSe results were confirmed. A discriminatory power of the area under the ROC curve of 0.84 was obtained with 21 ASVs as the most discriminatory to classify individuals into HT and NT.

Correlation of gut-microbiota based biomarkers with blood pressure

Correlation analysis showed several positive associations between ASVs biomarkers in HT group and BP values. Particularly, Lachnospiraceae and Ruminococcaceae taxa (ASV s394 and s718, respectively) were correlated positively with SBP ($r > 0.38$, $P < 0.05$). ASV s718 was assigned as *Pygmaiobacter* genus, and was associated with both SBP and DBP in HT. Thus, these results indicated that higher relative abundance of these ASVs could suggest higher values of SBP and DBP and a negative effect on CVDs status.

Also, was reported that *Faecalibacterium prausnitzii* (ASV s372) and Christensenellaceae R-7 group (ASV s524), discriminant biomarkers in NT group, were negatively correlated with SBP and DBP ($r > -0.40$, $P \leq 0.01$).

Differences in microbiota metabolic functions

From the metagenomic analysis the functional composition of the microbiota was elucidated. TIGRFAM database of prokaryotic protein family models allowed to assign main roles and subroles to the identified ASV biomarkers discriminant between groups. The results showed that NT group presented higher abundance of the subrole "Signal Transduction_Two Component Systems" ($P = 0.015$), however, HT-associated microbiota was enriched in genes involved in the energy metabolism (subroles: electron transport and anaerobic), cellular processes (subrole: DNA transformation), and DNA metabolism (subrole: DNA replication, recombination and repair) ($P < 0.05$).

Microbial metabolites analysis

Targeted metabolomics analysis revealed interesting results in microbiota-derived metabolites. SCFAs showed antagonistic results in faeces and plasma samples in HT group. Whereas the concentrations of acetate, propionate, butyrate and valerate were significantly higher in faeces ($P < 0.05$), plasma results showed significant lower circulating levels of acetate ($P < 0.001$), butyrate, isobutyrate and isovalerate ($P < 0.05$) compared to NT. Therefore, these results support the hypothesis that a lower efficiency in the absorption of SCFAs could occur in HT subjects and that an imbalanced host-microbiome crosstalk in SCFAs production may be an important cause of hypertension.

Additionally, the relation between SCFAs, BP and dietary intake was also tested. As a result, most of faecal SCFAs detected in faeces appeared to have a positive correlation with SBP and DBP, highlighting their role as metabolite-

based biomarkers for hypertension. In relation to diet, the intake of total dietary fiber was negatively correlated with faecal propionate, valerate and isobutyrate in NT group.

On the other hand, no significant differences in fasting plasma TMAO concentrations between HT and NT groups were found, although a great inter-individual variability was noted. There were also no significant associations between fasting TMAO and dietary intake.

Overall, these results reveal that individuals with hypertension in a first disease grade prior to drug treatment hold a particular bacterial signature. This study provides a new approach to distinguish HT individuals from healthy subjects according to a specific bacterial and SCFAs profile. In addition, higher faecal excretion of SCFAs together with lower plasmatic levels is associated with BP and could be indicative of hypertension in humans.

Summary of results – Article 2. Interplay between dietary phenolic compound intake and the human gut microbiome in hypertension: A cross-sectional study

Characteristics of the study participants

As described in ***Article 1***, a total of 61 participants, including 29 HT and 32 NT subjects, were also enrolled in this second study. In this second article, further assessment of dietary compounds intake was performed. Total dietary fiber intake was greater in NT group ($P < 0.05$). Moreover, data from the FFQ revealed significantly higher daily mean intakes of processed meat, tubers, natural juices and coffee, and significantly lower intake of whole-grain cereals in HT group than in NT.

PCs intake and excretion

PCs from vegetables, legumes, fresh fruits, chocolate, coffee and wine, together with flavonoids and phenolic acids, were the major contributors to total polyphenol intake (TPI) in both groups. Although no significant differences were found between groups in TPI and total polyphenol excretion (TPE), as objective marker of PCs intake, HT group had a significantly higher mean daily PC intake from non-alcoholic beverages, mainly coffee, and from alcoholic fermented beverages, such as wine and beer, and a lower PC intake from vegetables and whole-grain cereals than NT group ($P < 0.05$). Also, HT group consumed more hydroxycinnamic acids, alkylmethoxyphenols and methoxyphenols than NT ($P < 0.05$).

Associations between PCs and blood pressure

Multiple linear regression analysis showed positive associations between PCs from coffee and hydroxycinnamic acids with SBP ($\beta > 0.27$, $P < 0.05$).

Moreover, coffee and beer PCs, alkylmethoxyphenols and chalcones were positively related to DBP ($\beta > 0.25$, $P < 0.05$).

Associations between PCs and gut microbiota-based biomarkers

From the 67 ASVs biomarkers identified in the ***Article 1***, in this second study, a total of 14 ASVs in NT and 11 ASVs in HT were selected with the strongest Pearson correlations ($r \geq 0.30$) with PC dietary sources and classes. The multiple linear regression analysis revealed, in the NT group, interesting positive associations between PCs from olive fruits and Ruminococcaceae UCG-010 (ASVs s408 and s1019), Christensenellaceae R-7 (ASV s430) and *Bilophila wadsworthia* genus from the Desulfovibionaceae family (ASV s1147) ($p < 0.001$). This bacterial associations remained significant with anthocyanins, also derived from olive fruits ($p < 0.001$). Moreover, PCs from dried fruits (including dried apricots, plums, dates, figs and raisins) showed a positive correlation with the abundance of Ruminococcaceae NK4A214 (ASV s127) ($P = 0.002$) and Christensenellaceae R-7 (ASV s3173) ($P < 0.001$). Conversely, coffee PCs and hydroxycinnamic acids presented negative associations with the abundances of Faecalibacterium prausnitzii (ASV s372) and Christensenellaceae R-7 (ASV s3173) ($P < 0.05$).

On the other hand, in the HT group, coffee PCs, together with alkylmethoxyphenols and methoxyphenols, were identified as positive contributors to *Bacteroides plebeius* (ASV s92) and *Bacteroides coprocola* (ASVs s16 and s41) abundance ($P < 0.05$). Also, in HT, PCs from whole-grain cereals and flavanols were positively related to the abundance of *Bacteroides vulgatus* (ASV s651) ($P < 0.001$).

Therefore, all these associations provide new insights into the interplay of dietary PCs with human gut microbiota composition, and suggest that

specific dietary PCs could be precursors for the occurrence of particular bacterial taxa that differs in the guts of HT and NT subjects.

Associations between PCs and faecal and plasma SCFAs

In last instance, in order to assess the influence of dietary PCs on SCFAs levels, Pearson correlations were conducted between PCs and the faecal and plasma concentrations of SCFAs. As a result, in the HT group, coffee PCs, hydroxycinnamic acids, alkylmethoxyphenols and methoxyphenols were positively correlated with faecal levels of propionate, acetate and valerate ($P < 0.05$), which could explain a poor efficiency in intestinal absorption of these SCFAs. However, in the NT group, significant positive correlations were noted for fresh fruit and olive fruit PCs, and anthocyanins, with plasma butyrate and valerate levels ($P < 0.05$), suggesting a favoured SCFAs absorption. Thus, these associations, and particularly those observed between coffee and olive fruit PCs with SCFAs in both HT and NT, evidence a role of PCs in gut microbiota modulation.

Overall, the interplays found between BP, PCs, gut microbiota composition and metabolites represent a complex pathway by which PCs modulate gut microbiome and could be involved in either the pathogenesis or prevention of hypertension.

PART II. Discovering circulating bioactive lipids as promising early biomarkers of human hypercholesterolemia and other causal and modifiable CVD risk factors

PART II.I The *Bioclaims* study

Article 3. Serum lysophospholipidome of dietary origin as a suitable susceptibility/risk biomarker of human hypercholesterolemia: A cross-sectional study

Lorena Calderón-Pérez[+], Susana Suárez-García[+], Anna Pedret, Manuel Suárez, Elisabet Llauradó, Laura Rubió, Josep M del Bas, Antoni Caimari, Francesc Puiggrós, Lluís Arola, Rosa Solà, and Rosa M Valls.

Manuscript accepted in Clinical Nutrition on Nov 29th 2021

PART II.II Systematic review and meta-analysis

Article 4. The effects of fatty-acid based dietary interventions on circulating bioactive lipid levels as intermediate biomarkers of health, cardiovascular disease and cardiovascular disease risk factors: A systematic review and meta-analysis of randomized clinical trials

Lorena Calderón-Pérez, Judit Companys, Rosa Solà, Anna Pedret, and Rosa M Valls.

Manuscript submitted to Critical Reviews in Food Science and Nutrition

PART II.I The *Bioclaims* study

In this second part, the methods and results of the *Bioclaims* study are described. The *Bioclaims* study (BIOmarkers of Robustness of Metabolic Homeostasis for Nutrigenomics-derived Health CLAIMS Made on Food) emerged from a European project founded in 2009 by a consortium of researchers from different European institutions within the context of the Seventh Framework Programme. This large-scale project lasted up to 2015, and the main objective was the identification and characterization of nutrigenomic-based, early, robust biomarkers predictive of a healthy metabolic phenotype during ageing, and validating these novel biomarkers against traditional ones (Bioclaims Group, 2015). Moreover, the response of identified biomarkers to bioactive food components in animal and human models was aimed. Animals and human models observations were integrated and further validated. In this way, the biomarkers discovered in one model would be assessed in the other models and under other conditions. Different work packages (WPs) were planned and involved observational and intervention studies at three levels: in mice (WP1), in rats and hamsters (WP2), and in humans (WP3) (***Figure 24***). While WP1 and WP2 packages provided the possibility for highly controlled and wider systemic analysis, WP3 package concentrated all human studies which were crucial for biomarker validation (Bioclaims Group, 2015).

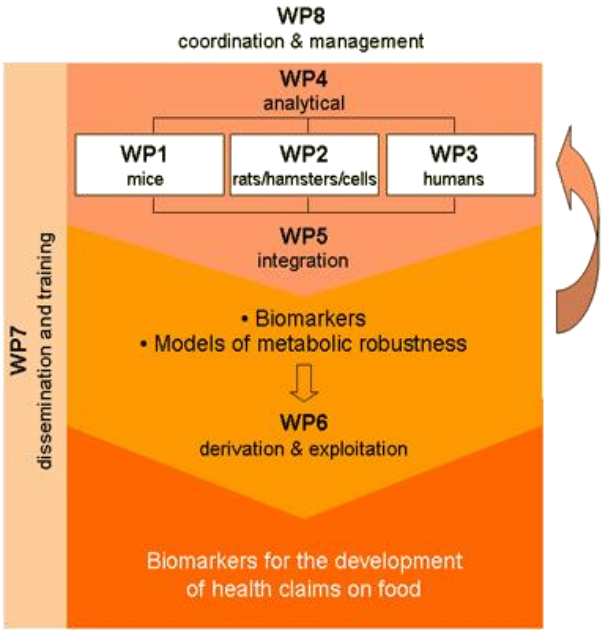


Figure 24 | PERT Diagram of the *BIOCLAIMS* Work Packages. Including scientific and administrative coordination, & management and exploitation of results. Source: (Bioclaims Group 2015).

The work conducted during the PhD period and presented in this thesis involve *WP3* analysis of human data and stored biological specimens derived from a cross-sectional study carried out in subjects with moderate to high hypercholesterolemia compared to healthy subjects. Through the application of targeted metabolomics and lipidomics, new circulating biomarkers of hypercholesterolemia risk were identified in human, and further validated with data from a second *in vivo* study in hamsters with hypercholesterolemia induced by diet, within the *WP2* work package (***Figure 25***). These targeted biomarkers could precede the increase of LDL cholesterol levels, and therefore represent promising early indicators of human hypercholesterolemia.

In addition, the relationships between targeted circulating metabolites and dietary animal source foods, particularly FAs, were tested. Also, in order to discern possible hepatic mechanisms, the relationships between targeted

metabolites and serum liver transaminases were further examined (***Figure 25***).

As a result of this work, one scientific article has been written (***Article 3***).

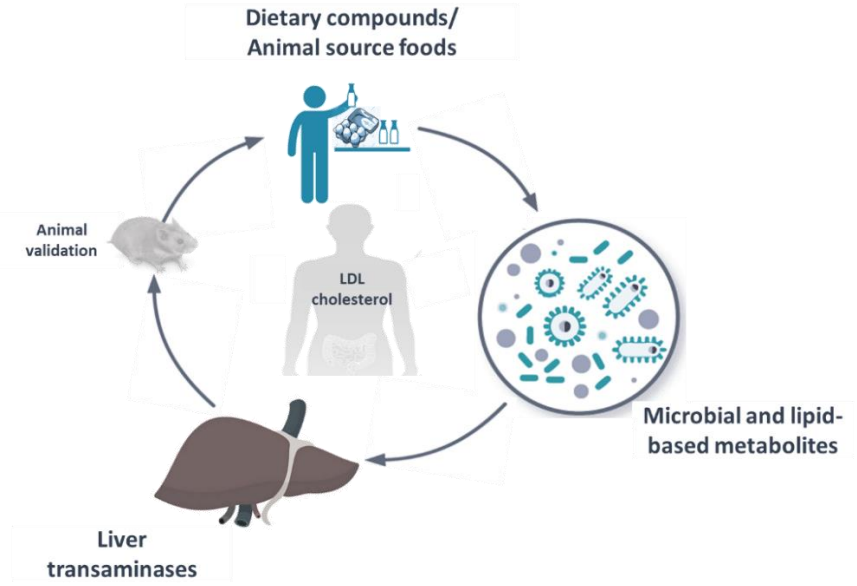


Figure 25 | Diagram of the different approaches in the discovery of early biomarkers of hypercholesterolemia assessed in the *Bioclaims* study. Own source.

METHODS

Study aims

As previously mentioned, the main purpose of the *Bioclaims* study, in context with the motive of the present thesis, was to identify novel early biomarkers of hypercholesterolemia in human. The following specific aims were set:

- To assess whether serum lyso-PLs and plasma TMAO may be suitable susceptibility/risk biomarkers of human hypercholesterolemia based on their observed increase prior to LDL cholesterol alteration.
- To evaluate the relationships between targeted circulating lyso-PLs and TMAO with diet composition, with special focus on FAs intake.
- To examine the relationships between targeted lyso-PLs and serum liver transaminases to speculate possible hepatic mechanisms involved in lyso-PL metabolism in humans.
- To verify the human results in a secondary *in vivo* study in hamsters to elucidate the possible mechanisms of action of lyso-PLs and their role in hypercholesterolemia risk.

Subjects and study design

A cross-sectional study involving 70 low (L-LDL-c) and 48 moderate to high (MH-LDL-c) LDL cholesterol male and female subjects was performed. The classification of LDL cholesterol levels was based on the *2019 ESC/EAS Guidelines for the Management of Dyslipidaemias* (Mach et al., 2020). The following criteria were accomplished:

Inclusion criteria:

- LDL cholesterol ≤ 115 mg/dL (L-LDL-c)
- LDL cholesterol among 116 - 190 mg/dL (MH-LDL-c)
- Age over 18 years old
- Willingness to provide informed consent before starting the study

Exclusion criteria:

- Family history of CVDs or chronic metabolic disorders
- Triglycerides > 150 mg/dL

- BMI ≥ 30 kg/m^2
- Using lipid-lowering treatment or other nutraceutical
- Altered renal function
- Suffering anemia (hemoglobin ≤13 mg/dL in men and ≤12 mg/dL in women)
- Suffering chronic gastrointestinal, respiratory or hepatic disorders
- Pregnant or breastfeeding women
- Smoking
- Participating in another study

Two separate visits were performed at the HUSJ and Eurecat, Reus. In a first screening visit (V0), a clinical interview to verify that participants met all the eligibility criteria was done, and a routine blood test was collected. In the second visit (V1), clinical and lifestyle information was registered and blood samples were collected. There were no subject withdrawals between first and second visit so data were available for all subjects.

Study outcomes and measurement

A summary of main study clinical, lifestyle and biochemical outcomes with the corresponding methodology applied for their analysis is provided in ***Table 12***.

Outcome	Methodology for determination and analysis
Clinical	
SBP DBP	Multiple automated sphygmomanometer *(OMRON HEM-907; Peroxfarma, Barcelona, Spain)* Two readings and calculation of the average value
Weight BMI	Body composition analyser *(Tanita SC 330-S; Tanita Corp., Barcelona, Spain)*
Waist circumference	150-cm anthropometric steel measuring tape At the umbilicus level
Lifestyle	

Diet composition Daily energy and nutrients intake	3-day dietary record Spanish Food Composition Tables *(Cesnid)*
Dietary choline and L-carnitine rich foods	3-day dietary record Spanish Food Composition Tables *(Cesnid)*
Amino acid intake	3-day dietary record Spanish Food Composition Tables *(Cesnid)* USDA Food and Nutrient database *(available online)*
Physical activity	Validated questionnaire *(Physical Activity Questionnaire Class AF)* (Vallbona Calbó et al., 2007)
Biochemical	
Lipid profile Blood glucose Liver transaminases	Serum sample Automated enzymatic methods in an autoanalyzer *(Beckman Coulter-Synchron, Galway, Ireland)*
Microbe-derived metabolites: TMAO	Plasma sample Ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)
Lipid metabolites: Lyso-PLs	Serum sample UPLC coupled to a triple quadrupole mass spectrometer (MS) and electrospray ionization (ESI) source MassHunter Quantitative software (v.7.0) Internal standards reference curves

Table 12 | Outcomes and their analytical measurement in the *Bioclaims* study. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; Lyso-PLs, lysophospholipids; SBP, systolic blood pressure; TMAO, trimethylamine N-oxide. Own source.

In addition to targeted metabolomics and lipidomics, a comprehensive statistical analysis was performed at two different levels: univariate and multivariate. The multivariate analysis allowed to analyze more complex sets of data, including predictive models such as Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), Receiver Operative Characteristic (ROC) curves, and heatmap. The application of multivariate predictive models permitted the identification of discriminant lyso-PLs between groups as promising biomarkers.

In vivo study in hamsters

In the secondary exploratory *in vivo* study a total of 16 male Golden Syrian hamsters feeding a moderate HFD (21% energy as fat) or a LFD (10% energy as fat), were enrolled.

Briefly, after 2 weeks of acclimatization with a LFD, the animals were randomly distributed into two groups with comparable body weights (n = 8 per group) and fed ad libitum either the LFD or a HFD for 30 days. On day 30, blood samples were collected and the liver was perfused, patted dry and then weighted. The white adipose tissue was also excised for determination of the relative weight. In addition, hepatic biopsies were performed for histology, in order to assess the degree of hepatic steatosis in both animal groups.

Targeted lipidomics was used to quantify lyso-PLs levels in hamster's liver and plasma as described in humans.

Article 3

Serum lysophospholipidome of dietary origin as a suitable susceptibility/risk biomarker of human hypercholesterolemia: A cross-sectional study

Lorena Calderón-Pérez[+], Susana Suárez-García[+], Anna Pedret, Manuel Suárez, Elisabet Llauradó, Laura Rubió, Josep M del Bas, Antoni Caimari, Francesc Puiggrós, Lluís Arola, Rosa Solà and Rosa M Valls.

[+] These authors contributed equally to the study.

Manuscript accepted in Clinical Nutrition on Nov 29th 2021

Clinical Nutrition 41 (2022) 489–499

Contents lists available at ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Original article

Serum lysophospholipidome of dietary origin as a suitable susceptibility/risk biomarker of human hypercholesterolemia: A cross-sectional study

Lorena Calderón-Pérez [a, b, 1], Susana Suárez-García [c, 1], Anna Pedret [a, b, *], Manuel Suárez [c, **], Elisabet Llauradó [b], Laura Rubió [d], Josep M. del Bas [a], Antoni Caimari [a], Francesc Puiggrós [a], Lluís Arola [c], Rosa Solà [b, e, 2], Rosa M. Valls [a, b, 2]

[a] Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain
[b] Universitat Rovira i Virgili, Facultat de Medicina i Ciències de la salut, Functional Nutrition, Oxidation, and Cardiovascular Diseases Group (NFOC-Salut), Reus, Spain
[c] Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Nutrigenomics Research Group, Spain
[d] Food Technology Department, XaRTA-TPV, Agrotecnio Center, Escola Tècnica Superior d'Enginyeria Agrària, University of Lleida, Lleida, Catalonia, Spain
[e] Hospital Universitari Sant Joan de Reus, Reus, Spain

ARTICLE INFO

Article history:
Received 14 September 2021
Accepted 29 November 2021

Keywords:
Lysophospholipids
Lysophosphatidylcholines
Lysophosphatidylethanolamines
Trimethylamine-N-Oxide
Hypercholesterolemia
Polyunsaturated fatty acids

SUMMARY

Background & aims: Whether bioactive lysophospholipids (lyso-PLs) and trimethylamine-N-oxide (TMAO) serve as non-invasive biomarkers in early human hypercholesterolemia (HC) is unknown. This study aimed to assess whether serum lyso-PLs and plasma TMAO may be suitable susceptibility/risk biomarkers of HC in humans. Secondarily, we aimed to evaluate the relationships between targeted metabolites, diet composition and circulating liver transaminases, and verify these results in hamsters.
Methods: A targeted metabolomics and lipidomics approach determined plasma TMAO and serum lysophosphatidylcholines (lyso-PCs) and lysophosphatidylethanolamines (lyso-PEs) in low (L-LDL-c) and moderate to high (MH-LDL-c) LDL-cholesterol subjects. Additionally, the relationships between targeted metabolites, liver transaminases and diet, particularly fatty acid intake, were tested. In parallel, plasma and liver lyso-PL profiles were studied in 16 hamsters fed a moderate high-fat (HFD) or low-fat (LFD) diet for 30 days.
Results: Predictive models identified lyso-PC15:0 and lyso-PE18:2 as the most discriminant lyso-PLs among groups. In MH-LDL-c (n = 48), LDL-cholesterol and saturated FAs were positively associated with lyso-PC15:0, whereas in L-LDL-c (n = 70), LDL-cholesterol and polyunsaturated fatty acids (PUFAs) were negatively and positively related to lyso-PE18:2, respectively. Interestingly, in MH-LDL-c, the lower lyso-PE 18:2 concentrations were indicative of higher LDL-cholesterol levels. Intrahepatic accumulation of lyso-PLs-containing essential n-6 PUFAs, including lyso-PE18:2, were higher in HFD-fed hamsters than LFD-fed hamsters.
Conclusions: Overall, results revealed a possible hepatic adaptive mechanism to counteract diet-induced steatosis in animal and hypercholesterolemia progression in humans. In particular, low serum lyso-PE18:2 suggests a suitable susceptibility/risk biomarker of HC in humans.

* Corresponding author. Facultat de Medicina, Universitat Rovira i Virgili, C/ Sant Llorenç, 21, 43201, Reus, Spain.
** Corresponding author. Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, C/ Marcel·lí Domingo, 1, 43007, Tarragona, Spain.
E-mail addresses: anna.pedret@urv.cat (A. Pedret), manuel.suarez@urv.cat (M. Suárez).
[1] LC-P and SS-G contributed equally to the study.
[2] Senior authors.

https://doi.org/10.1016/j.clnu.2021.11.033

Abbreviations list

ALA	Alpha-linolenic acid
ALT	Alanine transaminase
AST	Aspartate transaminase
DBP	Diastolic blood pressure
DEI	Daily energy intake
EL	Endothelial lipase
FDR	False discovery rates
GGT	Gamma-glutamyl transferase
HC	Hypercholesterolemia
HFD	High-fat diet
LA	Linoleic acid
L-LDL-c	Low LDL-cholesterol
LFD	Low-fat diet
Lyso-PCs	Lysophosphatidylcholines
Lyso-PEs	Lysophosphatidylethanolamines
Lyso-PLS	Lysophospholipids
MH-LDL-c	Moderate to high LDL-cholesterol
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
PUFAs	Polyunsaturated fatty acids
ROC	Receiver operating characteristic
SFAs	Saturated fatty acids
SBP	Systolic blood pressure
TC	Total cholesterol
TG	Triglycerides
TMAO	Trimethylamine N-oxide

1. Introduction

Hypercholesterolemia (HC), an imbalanced and pathologic state of cholesterol homeostasis, is a major risk factor for cardiovascular disease (CVD) [1]. Severe primary HC alone is characterized by an elevation of low density lipoprotein (LDL)-cholesterol > 190 mg/dL, with normal values of triglycerides (TG) [1]. Elevated plasma LDL-cholesterol levels have been a primary target of therapy for many years to reduce the risk of adverse cardiovascular events [2]. Thus, despite the great efforts and health plans that have been designed to improve the early detection and management of HC subjects, the population remains underdiagnosed in most cases, which translate into late detection and therefore prescription of lipid-lowering agents [3].

Cholesterol metabolism in humans is complex, and several pathways have been identified for the net flow of cholesterol particles through major tissue compartments [4]. Many exogenous and endogenous metabolites act as intermediates in cholesterol metabolism, which suggests the applicability of integrative omics approaches such as nuclear magnetic resonance (NMR)-based metabolomics and lipidomics for profiling novel early metabolic biomarkers [5,6].

Among various cellular metabolites, lysophospholipids (lyso-PLs), which are present in most cellular membranes, constitute a diverse group of bioactive molecules involved in a broad range of physiological and pathological processes in humans [7]. Lyso-PLs activities are primarily mediated by specific G protein-coupled receptors (GPCRs), implicating them in the aetiology of a growing number of disorders, such as inflammation, reproduction, angiogenesis, carcinogenesis, atherosclerosis and obesity [7]. Many lines of evidence have revealed a mechanistic link between both lyso-PLs and TMAO levels and CVD risk [8,9] and the incidence of major adverse cardiovascular events, such as myocardial infarction, stroke, and even death [10]. Lysophosphatidylcholines (lyso-PCs), the most abundant lyso-PLs in human blood have been suggested to promote atherosclerosis by altering the functions of a number of cells eliciting an inflammatory response [11]. However, controversy exists surrounding lyso-PC pro- or anti-atherogenic/inflammatory properties [11,12].

Additionally, lyso-PL simple structures are primarily composed of a hydrophobic tail of fatty acid residue and the hydrophilic head group, as well as the type of fatty acyl chain, that largely determine their health effects [12]. Group 1b phospholipase A_2 (PLA2g1b) is the major enzyme responsible for phospholipid hydrolysis in the intestinal lumen, yielding lyso-PLs and free fatty acids that are absorbed into enterocytes [13]. Concurrently, free omega 3 (n-3) PUFAs from diet may be able to bind to lyso-PLs, altering their fatty acyl composition, as previously suggested in C57BL/6 mice fed n-3 PUFA-enriched diets [14]. In the intestine, hydrolysed lyso-PLs become a substrate for trimethylamine (TMA) synthesis [13], a direct intestinal precursor of trimethylamine N-oxide (TMAO) [15]. Several gut bacteria belonging to the Firmicutes, Actinobacteria and Proteobacteria phyla, can form TMA through several enzyme complexes involved in anaerobic choline and L-carnitine metabolism [16]. Outside the intestinal tract, TMA is further oxidized to TMAO in the host liver by flavin monooxygenase enzymes, primarily FMO_3 [9].

Dietary factors are important determinants of circulating TMAO and lyso-PL levels. In this sense, Western-like diets, which are characterized by high intake of animal protein and saturated fat and low fibre, have been related with increased plasma TMAO levels in mice [17]. Similarly, a HFD enriched in phosphatidylcholine has demonstrated to increase TMAO levels in plasma and to induce dyslipidemia in rats [18]. On the other hand, in vivo studies have identified altered serum lyso-PLs levels in dyslipidemic hamsters after HFD administration, pointing out the role of lyso-PLs in the management of lipid disorders [19]. Nevertheless, the evidence on the potential relationships between dietary components and lysophospholipidome is scarce in humans. Thus, early identification of novel bioactive molecules is required to predicrt HC susceptibility and guide preventive strategies in clinical practice.

To address these issues, we performed a cross sectional study in which we carried out targeted metabolomics and lipidomics with special emphasis on identifying circulating metabolites and bioactive lyso-PLs of HC susceptibility. We hypothesize was that particular serum lyso-PLs and plasma TMAO concentrations could be precursors of HC progression because their rise occurs prior to LDL-cholesterol increase. Therefore, the overall goal of this study was to assess whether serum lyso-PLs and plasma TMAO may be suitable susceptibility/risk biomarkers of HC progression in humans. Furthermore, because these metabolites depend highly on diet and dietary animal-based foods, we also aimed to evaluate potential associations with diet composition, with special emphasis on fatty acid intake. Finally, considering that the liver is highly involved in extracellular lyso-PL level regulation by modifying the expression of several enzymes [19], to extend our knowledge on possible hepatic mechanisms involved in lyso-PL metabolism, we examined relationships with serum liver transaminases. In addition, the results were verified in a second *in vivo* study in hamsters with HC induced by chronic intake of a HFD.

2. Materials and methods

2.1. Subjects and study design

A cross sectional study involving male and female subjects undergoing blood testing was performed. All subjects were recruited from March to October 2014 at the *Hospital Universitari Sant Joan de Reus* (HUSJ, Spain) and Eurecat, Reus. Based on the 2019 ESC/EAS Guidelines for the Management of Dyslipidaemias [20], subjects were divided into two groups with low (≤115 mg/dL) and moderate to high (116–190 mg/dL) serum LDL-cholesterol, as shown in Supplemental Fig. 1. Subjects were eligible for enrolment in the study if they were over 18 and provided written informed consent, and were excluded if they met any of the following criteria: were diagnosed with any chronic metabolic disorder; had triglycerides levels >150 mg/dL; had a body mass index (BMI) > 30 kg/m^2; used prescribed lipid-lowering treatment or other nutraceutical; had altered renal function; suffering anaemia (hemoglobin ≤13 mg/dL in men and ≤12 mg/dL in women); suffering chronic gastrointestinal, respiratory or hepatic disorders; were pregnant or breastfeeding; were smoking; or were participating in another study.

The protocol was conducted in accordance to the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization (ICH GCP) and was approved by the Ethical Committee of Clinical Research of the HUSJ (with 09-12-17/proj1 reference).

2.2. Clinical and lifestyle data collection

All clinical information was collected according to standard procedures at two different visits performed at the HUSJ and Eurecat, Reus. In the first pre-screening visit, after indicating informed consent, a clinical interview was used to verify that participants met all the eligibility criteria, and a routine blood test was collected under fasting conditions. In addition, clinical information related to a history of disease and use of medications or supplements was obtained, and a physical examination was performed, including blood pressure and anthropometric parameters, such as weight, height, BMI, and waist circumference measures. Accordingly, the following procedures were applied:

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were monitored with participants in a seated position after resting for 2–5 min using a multiple automated sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain). Anthropometric parameters including weight and body composition were measured with a body composition analyser (Tanita SC 330-S; Tanita Corp., Barcelona, Spain). Waist circumference was measured at the umbilicus level using a 150 cm anthropometric steel measuring tape.

After the first pre-screening visit, participants who met all the eligibility criteria were included in the study and scheduled for a second visit in one week. At the second visit, blood pressure and anthropometrical parameters were measured again, and a fasting blood sample was collected for biochemical analysis. No subject was using antibiotics, probiotics or diuretics that could alter the levels of circulating metabolites at the time of inclusion. In addition, lifestyle information including dietary habits and physical activity, was recorded.

Data on diet composition and mean daily energy and nutrients intake were assessed through a 3-day dietary record (2 labour and 1 week-end consecutive days) and calculated by Spanish Food Composition Tables (CESNID) [21]. The percentage (%) of daily energy intake (% DEI) from primary macronutrients (proteins, carbohydrates and fatty acids) was calculated by dividing the calories derived from each by the total calories consumed. In addition, from the 3-day dietary record, the intake of well-known choline animal-based foods, according to the US Department of Agriculture (USDA) Database for the Choline Content of Common Foods [22], was grouped as follows: red meat, white meat, processed meat, fatty fish, lean fish, shellfish, whole milk, low-fat milk, skim milk, yogurt, cheese, butter and eggs. These foods provided between 250 and 14 mg total choline/100g, with eggs being the largest contributors and dairy products, particularly yogurt, the lowest.

Physical activity was evaluated by completion of the "Physical Activity Questionnaire Class AF" validated questionnaire [23]. There were no subject withdrawals between the first and second visits; thus, data were available for all subjects.

2.3. Determination of biochemical parameters

Blood samples were taken under over-night fasting conditions. Briefly, total cholesterol (TC), LDL-cholesterol, HDL-cholesterol, TG, and glucose and the hepatic enzymes aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyl transferase (GGT) concentrations were measured in serum by standardized enzymatic automated methods in an autoanalyzer (Beckman Coulter-Synchron, Galway, Ireland).

2.4. Targeted metabolomics and lipidomics analysis of plasma TMAO and serum lysophospholipids

Quantification of TMAO in plasma samples was performed as described previously [24]. Briefly, for sample extraction, 25 μL of plasma was mixed with 80 μL of methanol with labelled IS working solution (TMAO-d9; Cambridge Isotope Laboratories, Massachusetts, USA) and mixed 30 s to precipitate proteins. The samples were centrifuged at 9000 rpm for 5 min at room temperature, and the supernatants were diluted with 150 uL of Milli-Q water. Diluted samples were filtered with PVDF filters 0.22 μm and transferred into HPLC vials for analysis. The analysis was performed by liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) (Waters, Milford, MA, USA) using a column Acquity UPLC BEH HILIC (1.7 μm 2.1 × 100 mm).

Lyso-PLs content was determined in a subsample of 24 L-LDL-c and 26 MH-LDL-c subjects through targeted lipidomic analysis. A previously reported methodology [25] based on protein precipitation with cold methanol assisted by ultrasounds was used. The quantitative lyso-PLs evaluation and analysis is detailed in Supplemental Information 1.

2.5. In vivo study to validate circulating lysophospholipid suitability as susceptibility/risk biomarkers and to inquire into hepatic mechanisms

2.5.1. Animals and diets

A second exploratory in vivo study was performed using 16 male Golden Syrian hamsters (Charles River Laboratories, Barcelona, Spain) fed a moderate HFD (21% energy as fat) or LFD (10% energy as fat). Golden Syrian hamsters were used because they display the greatest similarity to humans with regard to lipoprotein metabolism [26]. The animal protocol was approved by the Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain), and all of procedures have been performed in accordance with the European Communities Council Directive (86/609/EEC). The detailed procedure for sample extraction is described in Supplemental Information 2. Briefly, after 2 weeks of adaptation to a LFD, the animals were randomly distributed into two groups with comparable body weights (n = 8 per group) and fed either the LFD or a HFD *ad libitum* for 30 days (the composition of the diets is shown in Supplemental Information 3). On day 30, blood samples

were withdrawn by cardiac puncture, and the liver was perfused, patted dry and then weighed for the determination of the relative weight. White adipose tissue was also excised for determination of the relative weight. All samples were snap frozen in liquid nitrogen and then stored at -80 °C until further analyses.

Targeted lipidomics was used to quantify lyso-PL levels in hamster liver and plasma following the same methodology described above.

2.5.2. Liver histology

The detailed procedure on histological examination is described in Supplemental Information 2. Briefly, frozen liver tissue samples were thawed and fixed by immersion in 4% paraformaldehyde for 24 h, dehydrated, and embedded in paraffin at 52 °C. Then, paraffin blocks were sectioned and prepared in slides for automated staining. Morphometric evaluation was conducted by expert pathologists to assess the degree of hepatic steatosis in the biopsies [27]. The steatosis grade was determined estimating the percentage of hepatocytes containing lipid droplets: absent – score 0, <5%; mild – score 1, 5–33%; moderate – score 2, >33%–66%; severe – score 3, >66% [27]. The diagnosis also included determination of the cytoplasmic configuration (microvesicular and macrovesicular steatotic patterns), tissue distribution and inflammation extent.

2.6. Statistical analysis

Data on clinical, biochemical and dietary parameters in humans are expressed as means ± SDs, and the normality of variables was assessed using the Kolmogorov–Smirnov test. The differences among groups were assessed using the Mann–Whitney test and the Student's t-test for comparison of non-normally and normally distributed variables, respectively. Fisher's exact test was used for categorical variables comparisons. Correlations between circulating lyso-PLs, TMAO, and clinical and dietary variables in humans were evaluated using Spearman's correlation coefficients (r) adjusting for the age, sex, BMI and energy intake confounders. To manage false-positive correlations, multiple testing using the Benjamini-Hochberg correction was used, and adjusted p values were set [28]. Stepwise multiple linear regression analysis was then used to predict the strength of the associations, setting clinical and dietary variables as predictors, and TMAO and discriminant lyso-PLs as response variables. In hamsters, the results of plasma lipid markers, plasma lyso-PL concentrations and liver histology are expressed as means ± SEM from the indicated number of hamsters (n = 8) in each group. Correlations between plasma and liver lyso-PL levels in hamsters were evaluated using Pearson's correlation coefficient (r). A two-tailed value of $p<0.05$ was considered statistically significant for all human and animal tests. Univariate statistical analysis was performed with Statistical Package for Social Sciences (IBM SPSS Statistics, version 25.0).

Lyso-PL content was also comprehensively examined in both the MH-LDL-c and L-LDL-c groups using principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The variable importance in projection (VIP) score from the PLS-DA model was calculated to estimate the importance of lyso-PLs and their contribution to the overall differentiation between groups. The predictive models were further validated by conducting receiver operating characteristic (ROC) curves. The optimal combination of the most important identified lyso-PLs predictive of HC was that associated with the highest predictive accuracy (maximum sensitivity and specificity). All multivariate analysis were performed after data normalization and autoscaling with the use of the software MetaboAnalyst (version 4.0) available online.

Sample size was calculated using the GRANMO freeware based on the number of participants required to detect significant differences in LDL-cholesterol levels at baseline in each of the groups. Based on means ± SDs for serum concentration of LDL-cholesterol from previous studies in the Spanish population [29,30], it was calculated that a minimum of 27 subjects/group would be required.

3. Results

3.1. Subject characteristics

From 138 subjects who were assessed for eligibility, 20 were excluded due to not fulfilling the inclusion criteria or because their informed consent was withdrawn. 118 subjects, 70 L-LDL-c and 48 MH-LDL-c, were enrolled and analysed. The flowchart of the study according to the STROBE statement is depicted in Supplemental Fig. 1.

Baseline characteristics of the study population are illustrated in Table 1. MH-LDL-c subjects were significantly older than L-LDL-c subjects ($p < 0.001$) and had higher SBP, DBP, BMI and waist circumference ($p < 0.05$). Concerning biochemical parameters, the MH-LDL-c group, had higher serum concentrations of TC and TG than the L-LDL-c group ($p < 0.001$). Despite these differences in lipid markers between groups, it is important to highlight that their baseline levels remained within normal values except for LDL-cholesterol (MH-LDL-c, 144.0 ± 19.9 mg/dL; L-LDL-c, 86.1 ± 18.2 mg/dL; $p < 0.001$).

Daily energy and nutrients intake was similar between groups except for complex carbohydrates, the most consumed macronutrient in both groups (MH-LDL-c, 102.57 ± 39.18 g/day; L-LDL-c, 114.51 ± 44.13 g/day). In the L-LDL-c group, complex carbohydrate intake, which is expressed as the percentage of daily energy intake, was significantly higher than that in the MH-LDL-c group ($p = 0.010$). In the MH-LDL-c group, the mean ethanol intake was significantly higher than that in the L-LDL-c group ($p = 0.006$) (Supplemental Table 1). Reported data from 3-day dietary records showed significantly greater daily mean intake of lean fish ($p = 0.039$), animal protein ($p = 0.023$) and several amino acids ($p < 0.05$) in MH-LDL-c subjects than in L-LDL-c subjects (Supplemental Table 1). No significant differences were found in other dietary parameters analysed or in physical activity.

3.2. Identification of circulating metabolites involved in hypercholesterolemia susceptibility

After targeted metabolomics analysis, a total of 27 serum lyso-PLs (19 lyso-PCs and 8 lyso-PEs) were identified and plasma TMAO concentrations were assessed (Table 2). There were no significant differences in TMAO concentrations between groups, while particular lyso-PLs differed significantly among L-LDL-c and MH-LDL-c groups. The most of the identified lyso-PCs and lyso-PEs were highly concentrated in the MH-LDL-c group compared to L-LDL-c group, primarily lyso-PC 15:0 (1.12 ± 1.40 vs. 1.05 ± 0.26 μM, $p < 0.001$). Exceptionally, lyso-PE 18:2 was significantly higher in L-LDL-c and lower in MH-LDL-c (6.08 ± 1.51 vs. 5.32 ± 1.16 μM, $p = 0.05$). Additionally, in L-LDL-c group, lyso-PC 14:0, 16:1, 18:1, 18:2, 18:3 and 20:4, and lyso-PE 18:1 and 20:4, were more concentrated (Table 2). The projection scores (VIPs) in the first component of the PLS-DA model indicated seven lyso-PLs, with VIPs>1.0 contributing to the overall differentiation between groups (Table 2). In particular, lyso-PC 15:0 and lyso-PE 18:2 showed the highest scores (2.74 and 2.24, respectively). Consistent with this, when the individual scores were hierarchically clustered in a heatmap, two distinct patterns in the serum lyso-PL profile, characterized by higher lyso-PC 15:0 and lower lyso-PE 18:2 in the MH-LDL-c group, and lower

Table 1
Baseline characteristics of study population.

Variables	Low LDL-cholesterol	Moderate to high LDL-cholesterol	P-value
N	*70*	*48*	
Age, *y*	35.4 ± 10.3	46.6 ± 11.9	<0.001
Gender, *(F/M)*	(36/34)	(25/23)	0.547
Anthropometry			
Weight, *kg*	65.0 ± 10.4	66.8 ± 11.3	0.380
BMI, kg/m^2	22.4 ± 2.5	24.0 ± 2.8	0.002
Waist circumference, *cm*	79.1 ± 8.7	83.4 ± 8.8	0.010
Blood chemistry *(mg/dL)*			
Total cholesterol	166.1 ± 24.1	227.3 ± 25.6	<0.001
LDL-cholesterol	86.1 ± 18.2	144.0 ± 19.9	<0.001
HDL-cholesterol	66.4 ± 15.0	64.8 ± 15.1	0.568
Triglycerides	67.5 ± 26.1	88.0 ± 27.7	<0.001
FBG	84.0 ± 8.9	86.7 ± 9.4	0.125
Blood pressure *(mm Hg)*			
Systolic BP	114.7 ± 14.3	120.4 ± 15.2	0.038
Diastolic BP	67.9 ± 9.6	72.4 ± 9.3	0.017

Data expressed as mean ± SD. P-value for gender was calculated by Fisher's exact test. P-value for the rest of the variables was calculated by Student's t-test and Mann–Whitney U test. Abbreviations: BMI, body mass index; BP, blood pressure; FBG, fasting blood glucose; HDL, high density lipoproteins; LDL, low density lipoproteins.

Table 2
Mean concentrations (μM) and variable importance in projection (VIP) score of serum lysophospholipid and plasma TMAO in low and moderate to high LDL-cholesterol groups.

Metabolite	Low LDL-cholesterol	Moderate to high LDL-cholesterol	Metabolite VIP score
Lyso-PC [a]			
14:0	2.19 ± 0.83	2.18 ± 0.68	0.78
15:0	1.05 ± 0.26	1.12 ± 0.22**	**2.74**
16:0	63.46 ± 4.87	64.10 ± 3.59	0.09
16:1	4.28 ± 1.44	4.14 ± 0.67	0.65
17:0	4.06 ± 1.03	4.51 ± 0.97	**1.40**
17:1	4.83 ± 0.31	4.74 ± 0.34	0.44
18:0	60.98 ± 10.44	67.02 ± 11.15*	0.12
18:1	66.06 ± 12.55	65.49 ± 12.70	**1.55**
18:2	94.84 ± 13.17	89.38 ± 11.67	**1.04**
18:3	1.84 ± 0.71	1.75 ± 0.69	0.51
20:0	1.12 ± 0.26	1.34 ± 0.34*	0.36
20:1	2.66 ± 0.66	2.79 ± 0.71	0.30
20:2	4.41 ± 0.92	4.58 ± 0.99	0.94
20:3	25.06 ± 9.04	25.27 ± 7.66	0.31
20:4	90.21 ± 19.24	88.27 ± 17.76	**1.15**
20:5	10.62 ± 7.67	12.67 ± 11.66	0.74
22:4	1.95 ± 0.67	1.79 ± 0.46	0.03
22:5	5.81 ± 1.80	5.90 ± 1.73	0.68
22:6	26.77 ± 6.50	30.56 ± 10.25	0.66
Lyso-PE [a]			
16:0	2.89 ± 0.75	3.00 ± 0.64	0.78
16:1	0.27 ± 0.07	0.25 ± 0.05	0.29
18:0	2.41 ± 0.63	2.67 ± 0.49	0.96
18:1	2.64 ± 0.84	2.41 ± 0.73	0.74
18:2	6.08 ± 1.51*	5.32 ± 1.16	**2.24**
20:4	4.20 ± 0.99	4.09 ± 0.92	0.24
20:5	0.39 ± 0.16	0.41 ± 0.20	0.08
22:6	2.53 ± 0.56	2.83 ± 0.77	**1.07**
TMAO [b]			
	5.83 ± 4.03	6.25 ± 5.12	0.77

Metabolite concentrations expressed as mean ± SD. VIP scores of the first component of the PLS-DA model, VIP score >1.0 depicted in **bold**.
Significance for differences in metabolite concentrations between groups was measured using independent Student's *t*-test: *$p \leq 0.05$; **$p < 0.001$. Abbreviations: lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; TMAO, trimethylamine N-oxide.
[a] Lysophospholipids available in a sub-group of n = 50 subjects.
[b] TMAO available in n = 107 subjects.

lyso-PC 15:0 and higher lyso-PE 18:2 in the L-LDL-c group, were evidenced (Fig. 1-A).

A PCA model was built including the seven most discriminant lyso-PLs to further test the independent predictive capability of the identified metabolites. As the three-dimensional score plot showed, it was possible to distinguish a wide separation between the MH-LDL-c and L-LDL-c groups (Fig. 1-B). Thus, 62.4% of the variance was explained when the scores of the first three principal components were represented. The PLS-DA model upgraded the differentiation between groups (Fig. 1-C), with lyso-PC 15:0 and lyso-PE 18:2 being the major contributors to the separation. The model reported a good fit of the data ($R^2Y = 0.68$) and an optimal quality assessment statistic ($Q^2 = 0.54$), over the threshold of 0.4 stipulated for biological models [31]. In addition, lyso-PC 15:0 and lyso-PE 18:2 explained 59.3% and 40.7% of the variance when the scores of the two principal components were computed (Fig. 1-D). ROC curves using lyso-PC 15:0 and lyso-PE 18:2 achieved optimal predictive power with an accuracy of 70.4%, and an AUC of 0.777 (95% CI 0.65-0.932) (Fig. 1-E). These models confirmed that two particular lyso-PLs, lyso-PC 15:0 and lyso-PE 18:2, indicated good clinical accuracy and could be suitable susceptibility/risk biomarkers for HC in preliminary stages of the disease.

3.3. Associations of circulating lysophospholipids with TMAO

A correlation study with the 27 serum lyso-PLs identified in the MH-LDL-c and L-LDL-c groups was performed to investigate the contribution of lyso-PLs to TMAO concentrations. As shown in Supplemental Fig. 2, after the multiple linear regression analysis lyso-PC 20:5 and lyso-PE 20:5 were positively associated with TMAO concentrations in MH-LDL-c subjects ($\beta = 0.216$ and $\beta = 0.562$, respectively) ($p < 0.05$), while in L-LDL-c subjects, lyso-PC 14:0 showed a positive contribution to TMAO concentrations ($\beta = 0.382$, $p < 0.05$). These positive associations indicated that particular polyunsaturated and saturated chain lyso-PLs could be precursors for TMA and TMAO synthesis in HC progression.

3.4. Associations of discriminant lysophospholipids and TMAO with classical cardiovascular risk factors and dietary components

3.4.1. Associations with lipid-related markers

No significant correlations were found in either group between serum lipid markers and TMAO concentrations. However, positive correlations were observed for lipid markers with the most discriminant serum lyso-PLs (Table 3). In MH-LDL-c subjects, TC, LDL-cholesterol and the atherogenic ratio LDL-cholesterol/HDL-cholesterol were positively correlated with lyso-PC 15:0 levels ($r > 0.46$, $p < 0.01$ and p.adj<0.05), supporting lyso-PC 15:0 suitability as susceptibility/risk biomarker of HC. In L-LDL-c subjects, an inverse correlation was observed between TC and LDL-cholesterol with lyso-PE 18:2 ($r > -0.30$, $p < 0.05$ and p.adj = 0.134).

3.4.2. Associations with dietary components

A correlation study was performed at three different levels: nutrient intake, amino acid intake, and with choline animal-based foods (Supplemental Table 2).

In the MH-LDL-c group, saturated fatty acids (SFAs) were positively associated with lyso-PC 15:0 ($r = 0.52$, $p < 0.001$ and p.adj = 0.002) and lyso-PC 17:0 ($r = 0.63$, $p < 0.001$ and p.adj = 0.002). In addition, dairy products including yogurt, cheese and whole milk were positively associated with lyso-PC 15:0 ($r > 0.35$, $p < 0.05$ and

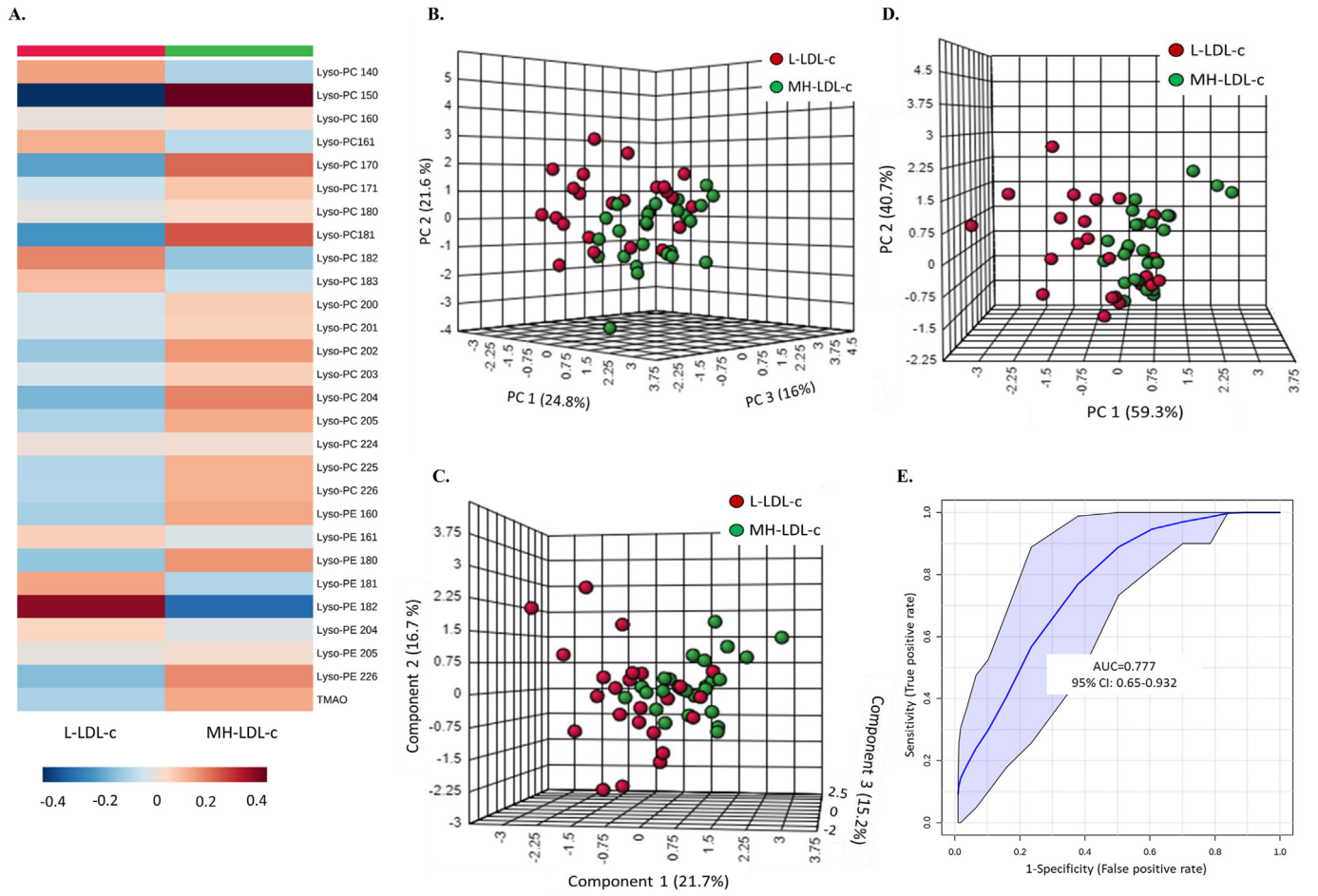


Fig. 1. Multivariate analysis of the circulating lysophospholipid and TMAO levels identified in 24 low LDL-cholesterol and 26 moderate to high LDL-cholesterol subjects. (**A**) Heatmap plot from hierarchical clustering analysis of lyso-PLs and TMAO levels in low and moderate to high LDL-cholesterol subjects. Each row represents a metabolite colored by its range-scaled abundance intensity. The scale from -0.4 (blue) to +0.4 (red) represents the normalized abundance in arbitrary units. (**B**) Three-dimensional score plot of the PCA model and (**C**) PLS-DA predictive model with the use of 7 serum lyso-PLs with the highest variable importance in projection (VIP) scores. (**D**) Two-dimensional score plot of the PCA model using of lyso-PC 15:0 and lyso-PE 18:2. (E) ROC curve analysis of the PLS-DA model using lyso-PC 15:0 and lyso-PE 18:2. The area under the curve (AUC) and corresponding 95% confidence interval (CI) are reported in the inset. PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; ROC, receiver operating characteristic. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Spearman correlations between serum lysophospholipid biomarkers, classical lipid-related markers and dietary components in low and moderate to high LDL-cholesterol groups.

Lysophospholipid, μM	Lipid-related marker Dietary component	Spearman correlation index	p.value	Adjusted p. value
Low LDL-cholesterol				
Lyso-PE 18:2	Total cholesterol	-0.309	0.028	0.134
	LDL-cholesterol	-0.318	0.024	0.134
	PUFAs	0.389	0.005	0.033[a]
Moderate to high LDL-cholesterol				
Lyso-PC 15:0	Total cholesterol	0.462	<0.001	0.003[a]
	LDL-cholesterol	0.482	<0.001	0.002[a]
	LDL-cholesterol/HDL-cholesterol ratio	0.543	0.006	0.042[a]
	SFAs	0.520	<0.001	0.002[a]
	Glutamic acid	0.432	0.001	0.016[a]
	Yoghurt 0.532		0.005	0.043[a]
	Cheese	0.467	0.021	0.181
	Whole milk	0.350	0.052	0.525

Lysophospholipid available in a sub-group of n = 50 subjects. Significant correlations were set at $p < 0.05$.
[a] Significant Bonferroni-Hochberg adjusted p value < 0.05. Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; Lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; PUFAs, polyunsaturated fatty acids; SFA, saturated fatty acid.

p.adj ≤ 0.05) and whole milk with lyso-PC 17:0 (r = 0.35, p = 0.058 and p.adj > 0.05) (Table 3). However, yogurt and whole milk were inversely associated with unsaturated lyso-PCs 20:4 and 20:5 (r > -0.39, p < 0.05 and p.adj>0.05) (Supplemental Table 2). Vitamin D and fatty fish were positively associated with lyso-PC 20:5 and lyso-PE 20:5 (r > 0.33, p ≤ 0.05). Concerning the L-LDL-c group, ethanol, total fatty acids, yogurt and cheese were positively correlated with Lyso-PC 14:0 (r > 0.40, p < 0.05 and p.adj < 0.05). In addition, in L-LDL-c, positive associations were stated between PUFAs and lyso-PE 18:2 (r = 0.38, p = 0.005 and p.adj = 0.033) (Table 3). Major associations of discriminant lyso-PLs with dietary components are shown in Supplemental Table 3.

A schematic of major correlations between TMAO and lyso-PL biomarkers and all clinical and dietary features in both groups is provided in Supplemental Fig. 3.

3.5. Linking lysophospholipid biomarkers and TMAO with liver transaminases and possible implication for hypercholesterolemia progression

Overall relationships for serum liver transaminases with discriminant lyso-PLs, TMAO and diet are shown in Supplemental Table 4, and significant correlations were included in a multiple linear regression analysis (Supplemental Fig. 4).

Table 4
| Lysophospholipid content in plasma and liver of low-fat diet (LFD) or high-fat diet (HFD)-fed hamsters after 30 days.

Metabolite	Plasma (nM)		Liver (μM/g)	
	LFD	HFD	LFD	HFD
Lyso-PC				
14:0	35.9 ± 1.4	30.7 ± 1.1*	79.8 ± 9.9	48.1 ± 1.9*
15:0	38.2 ± 3.3	29.3 ± 1.4*	78.4 ± 7.0	44.4 ± 2.4**
16:0	5263 ± 303	5006 ± 172	9458 ± 371	8533 ± 275
16:1	92.2 ± 5.5	62.3 ± 2.8***	218.1 ± 24.1	118.7 ± 7.6**
17:0	48.8 ± 5.6	39.1 ± 2.1*	227.8 ± 26.3	158.3 ± 7.3*
17:1	19.2 ± 1.6	13.6 ± 0.4*	18.5 ± 2.5	9.3 ± 0.9**
18:0	1327 ± 117	1498 ± 58	3723 ± 202	3511 ± 105
18:1	1470 ± 120	1365 ± 46	821.5 ± 57.4	794.3 ± 50.2
18:2	1532 ± 107	1616 ± 66	364.1 ± 18.7	533.5 ± 47.6**
18:3	18.7 ± 1.0	16.0 ± 0.4*	65.1 ± 9.8	75.5 ± 9.3
20:0	8.7 ± 0.2	8.9 ± 0.2	13.9 ± 0.8	14.4 ± 0.4
20:1	11.1 ± 0.2	14.0 ± 0.5***	13.9 ± 0.8	30.2 ± 1.2
20:2	17.1 ± 0.8	25.5 ± 1.3	24.3 ± 1.4	36.6 ± 1.9***
20:3	44.4 ± 5.1	33.7 ± 2.1	56.9 ± 3.4	139.1 ± 16.9**
20:4	170.5 ± 16.1	157.8 ± 4.9**	78.1 ± 5.6	161.4 ± 20.2**
20:5	16.1 ± 1.3	9.8 ± 0.3**	5.9 ± 1.0	6.6 ± 1.1
22:5	24.4 ± 2.0	15.6 ± 0.5**	10.4 ± 0.8	12.7 ± 1.4
22:6	66.9 ± 6.5	41.4 ± 2.6**	57.7 ± 4.2	96.2 ± 11.4**
Lyso-PE				
16:0	73.3 ± 4.2	61.6 ± 2.8*	1080 ± 101	728.8 ± 44.6**
16:1	7.0 ± 0.2	6.0 ± 0.1**	24.3 ± 2.7	18.4 ± 3.4
18:0	65.5 ± 1.6	70.3 ± 4.2	1671 ± 104	1347 ± 57.2*
18:1	34.8 ± 2.4	35.1 ± 1.7	666.7 ± 63.5	646.7 ± 82.6
18:2	40.4 ± 2.9	42.0 ± 2.0	56.5 ± 5.2	122.6 ± 14.1***
18:3	4.3 ± 0.1	4.1 ± 0.0*	7.0 ± 0.9	9.9 ± 1.3
20:1	4.2 ± 0.1	4.4 ± 0.1	7.2 ± 0.4	11.2 ± 0.5***
20:2	3.7 ± 0.1	3.9 ± 0.0	2.2 ± 0.2	4.1 ± 0.3***
20:3	5.3 ± 0.3	4.7 ± 0.1	3.3 ± 0.4	9.8 ± 1.6***
20:4	19.9 ± 1.4	20.3 ± 0.6	33.0 ± 3.6	95.6 ± 14.2***
22:4	3.7 ± 0.1	3.7 ± 0.0	0.4 ± 0.1	1.6 ± 0.3**
22:5	6.1 ± 0.3	4.9 ± 0.1**	7.5 ± 1.0	13.0 ± 1.7*
22:6	14.0 ± 1.8	9.1 ± 0.4*	24.4 ± 3.4	37.3 ± 4.2*

Hamsters were fed ad libitum either LFD or HFD during 30 days. Samples were obtained on the last day of dietary intervention after a 6 h fast. For each animal group, the data are presented as the mean ± SEM (n = 8). Significance regarding the LFD-fed group (control animals) was measured using independent Student's t-test: *p < 0.05; **p < 0.01; ***p < 0.001. Abbreviations: Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine.

MH-LDL-c group showed significantly higher concentrations of AST, ALT and GGT than the L-LDL-c group (p = 0.013, p < 0.001 and p = 0.009, respectively) (Supplemental Fig. 4-A). When correlations were investigated regardless of the group (n = 118), TMAO and ethanol intake were positively correlated with GGT levels (Supplemental Table 4). In the L-LDL-c group, Lyso-PC 14:0 was positively associated with GGT levels in L-LDL-c group (β = 0.574, p = 0.003) (Supplemental Fig. 4-B). In addition, a significant positive association was noted between TMAO and ethanol intake with GGT (Supplemental Table 4). In the MH-LDL-c group, lyso-PE 18:2 correlated positively with GGT levels (β = 0.495, p = 0.010) (Supplemental Fig. 4-C). Additionally, a positive association was observed between PUFA intake and GGT (β = 0.630, p < 0.001) (Supplemental Fig. 4-D).

3.6. Validation of the suitability of lysophospholipids as biomarkers involved in hypercholesterolemia progression in HFD-fed hamsters

3.6.1. Changes in circulating lipid markers and identification of plasma and liver lysophospholipids altered after the chronic intake of HFD

After 30 days of chronic treatment with HFD, lipid parameters and traditional indicators of liver impairment were determined in the animal's plasma (Supplemental Table 5). Hamsters fed the HFD exhibited significantly higher amount of total cholesterol than LFD-fed animals (p = 0.011), which resulted in a marginal increase (of 13%) of the atherogenic index TC/HDL-cholesterol (p = 0.046). An increasing trend of 30% LDL-cholesterol levels was observed in the HFD-fed group compared to the LFD-fed group (p = 0.008). No effects were observed on the transaminase levels.

Lyso-PC and lyso-PE levels were markedly modified in liver and plasma after chronic HFD intake (Table 4). A generalized drop (nearly 50%) in the circulating levels of lyso-PLs was noted in HFD-fed animals, which was accompanied by an intrahepatic accumulation of lyso forms, particularly polyunsaturated acyl chain lyso-PEs 18:2 (p < 0.001), 22:5 and 22:6 (p < 0.05). Conversely, saturated acyl chain lyso-PC 14:0, 15:0 and 17:0, and lyso-PE 16:0, were significantly reduced in both plasma and liver (p < 0.05).

An additional correlation analysis was performed between the 31 lyso-PLs identified in plasma and liver of hypercholesterolemic hamsters (Fig. 2-A). The lyso-PL content in both tissues was highly correlated predominantly in a negative sense. Lyso-PC 14:0, 15:0, 16:1, 17:1, 18:3, 20:5, 22:5 and 22:6, as well as Lyso-PE 16:1 and 22:6, were associated with a greater number of negative correlations (r > -0.5) and therefore were decreased in circulation.

3.6.2. Effect of the chronic administration of HFD on the hamster liver tissue

Histological analysis of the liver samples showed an increase in the steatotic profile in the liver of hamsters fed the HFD (Fig. 2). As shown in Fig. 2-B, diet-induced steatosis was defined by hepatocytes distended by multiple microvesicles filled of with lipids that did not displace the nucleus of the cell, but no evidences was found of macrovesicular fat accumulation. The relative liver weights of both groups were comparable (Fig. 2-C), and the score of steatosis was measured according to the percentage of hepatic parenchyma compromised, revealing that HFD-fed hamsters had significantly higher scores than LFD-fed animals (Fig. 2-D). Importantly, many of the HFD-fed hamsters that developed steatosis also presented inflammation signs with prevalent moderate intensity and lobular arrangement (Fig. 2-E). None of the biopsies exhibited signs of ballooning degeneration or fibrosis of the hepatocytes.

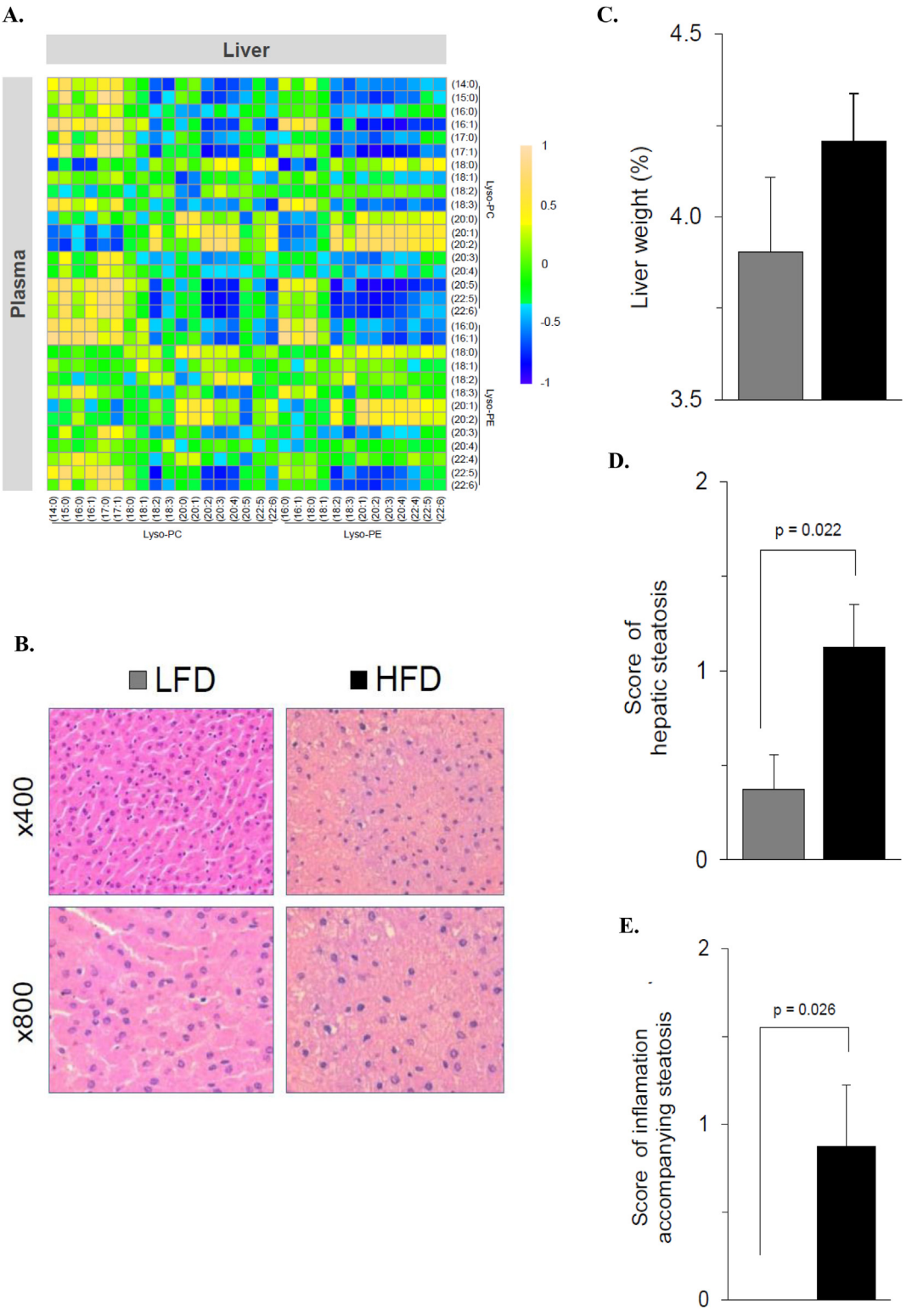


Fig. 2. Metabolic correlations across plasma and liver lysophospholipid contents and the effect of the thirty-day administration of low-fat diet (LFD) and a high-fat diet (HFD) on the hamster liver. (**A**) The normalized abundance of Lyso-PLs was correlated across both biological compartments using Pearson's correlation testing. The outgoing correlation coefficients (r) are displayed on a color-coded matrix ranging from -1 (blue) to +1 (orange). The bar chart shows the circulating metabolites with greater number of negative ($r < -0.5$) and positive ($r > 0.5$) correlations. (**B**) Histological analysis of liver tissue showing representative images of the study. Liver sections were stained with hematoxylin-eosin and were examined under a light microscope ($n = 8$ per group). The liver of HFD-fed animals shows hepatocellular vacuolization with microvesicular fat accumulation. (**C**) Relative liver weight in both animal groups, which was calculated by the formula (liver weight/body weight*100) and expressed as % of the total body weight. Data are expressed as mean ± SEM. (**D**) Score of hepatic steatosis. The score ranged from 0 to 3 and was determined by estimating the % of hepatocytes containing lipid droplets. (**E**) Score of inflammation (0–3) associated with steatosis. The significance was measured by conducting independent Student's *t*-test ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The circulating serum lysophospholipidome consists of a niche of bioactive lipids with promising applicability as risk biomarkers in preliminary stages of major lipid disorders. In tthis study, we confirm the hypothesis that particular lyso-PLs could be precursors of HC progression because their rise occurs prior to serum LDL-cholesterol increase. After targeted lipidomics analysis, a discrete number of lyso-PL species is shown to be suitable for use as susceptibility/risk biomarkers for HC in MH-LDL-c compared to L-LDL-c subjects. Lyso-PC 15:0 and lyso-PE 18:2 were identified as maximal contributors to the differentiation between both groups, showing optimal accuracy. In particular, lyso-PE 18:2 confers protective capabilities against the advancement of diet-induced hepatic steatosis in hamsters, and their low serum levels are suggestive of HC progression in humans. In addition, the strong relationships stated between lyso-PL biomarkers and dietary components, primarily fatty acids, add insights into the dietary origin of serum lyso-PLs and their role in diet-induced HC.

Lyso-PLs act as signalling molecules and are involved in a broad range of physiological and pathological processes [7]. However, whether they exert pro- or anti-atherogenic actions remains controversial. The influence of lyso-PCs in atherosclerosis appears to be due to the chain length and the degree of saturation of the fatty acyl moiety [12]. We found lyso-PC 15:0 to be the most discriminating lyso form in the MH-LDL-c group. This finding was accompanied by strong positive associations with classical lipid-related markers such as TC, LDL-cholesterol and the atherogenic index LDL-cholesterol/HDL-cholesterol, which is similar to previous human studies reporting a link for lyso-PC 15:0 to the incidence of type 2 diabetes [32] and increased diastolic BP [33]. Nonetheless, these results provides an added link between lyso-PC 15:0 to lipid disorders, highlighting its possible role as susceptibility/risk biomarker for HC. Also, the observed moderate positive correlations among lyso-PC 15:0 and dairy foods, such as yogurt, cheese and whole milk, indicated that lyso-PC 15:0 mainly originates from SFAs present in dairy. Conversely, in the L-LDL-c group, lyso-PE 18:2 showed the highest contribution to the differentiation and exhibited inverse associations with TC and LDL-cholesterol, which suggests a possible protective role for human HC in the healthy state. In contrast, in MH-LDL-c, the observed significantly lower concentrations of lyso-PE 18:2 in circulation would be indicative of LDL-cholesterol increase in a preliminary stage of the disease, making it a suitable susceptibility/risk biomarker. Supporting this theory, in the second *in vivo* study, we showed that after chronic treatment with HFD, hamsters experience an overall intrahepatic accumulation of PUFA-containing lyso-PLs, including lyso-PE 18:2, which could explain the existence of a hepatic response mechanism to the excess fat of the diet. This statement agrees with a previous animal study [19], in which lyso-PE 18:2 was among the lyso-PLs that exhibited the earliest alterations in response to induced dyslipidemia in hamsters. Thus, we speculate the existence of a hepatic response mechanism to excess fat in the diet, which is mediated by an accumulation of PUFA-containing lyso-PLs to counteract the increase in LDL-cholesterol. As recently reported in rat models [34], isolated HC induced by diet led to marked alterations in the hepatic lipid profile. These alterations were characterized by significant increases in fatty acid content, predominantly palmitoleic acid (C16:1n-7), oleic acid (C18:1n-9) and ALA (C18:3n-3) in hypercholesterolaemic rats, while higher linoleic acid (LA) (C18:2n-6) was present in normocholesterolaemic rats [34]. Thus, this previous study in animals [34] reinforces the proposed theory that in preliminary stages of HC, the hepatic accumulation of dietary omega (n)-PUFAs, primarily LA, could promote the synthesis of lyso-PE 18:2, which is a mechanism by which the liver protects itself from HC progression. Nonetheless, further human studies are required.

Also, we have reported that HFD promotes the development of microsteatosis in hamster liver, which is in accordance with other studies conducted in rodents in which the chronic administration of diets rich in fats led to intrahepatic accumulation of lipids [35,36]. However, in the proposed model, histological examination did not reveal hepatocyte balloon degeneration or fibrosis, and circulating transaminases were unaltered, suggesting that the steatosis degree induced by diet was still incipient and that the liver had not yet experienced substantial injuries. In contrast, when hypercholesterolemia is induced by strategies other than diet, such as chemical agents, steatotic signs are pronounced earlier, as previously reported in hamsters with dyslipidaemia induced by Poloxamer 407 compared to HFD-fed animals [19]. Therefore, the intrahepatic accumulation of polyunsaturated lyso forms could also protect against the benign progression of diet-induced hepatic steatosis in hamsters. In this sense, previous studies have described the ability of biologically active lyso-PLs to restrict fatty acid β-oxidation in rodent liver and thereby reduce energy expenditure in response to the chronic exposure to dietary fats [37].

Interestingly, the results of this study revealed that the polyunsaturated acyl chain of lyso-PLs can be highly influenced by dietary fatty acids. As pointed out the strong association between dietary PUFA intake and lyso-PE 18:2 in both groups, the intake of omega (n)-PUFAs such as LA could modulate the circulating lysophospholipidome, conferring lyso-PE 18:2 a protective role for the onset of human hypercholesterolemia and hepatic steatosis in hamsters. In addition to the known hydrolytic action that several secreted enzymes (e.g. LCAT, sPLA2 and lipases) exert on circulating phospholipid to produce lyso-PLs, direct hepatic secretion is an important source of lyso forms, particularly of those that contain PUFAs [38], which reinforces the importance of their dietary origin. Dietary intake of essential PUFAs, such as LA (C18:2n-6) and alpha-linolenic acid (ALA) 18:3 (n-3), in a balanced manner is necessary to maintain proper circulating LDL-cholesterol because they cannot be synthesized by humans [39]; however, whether the changes in circulating LDL-cholesterol are mediated by lyso-PL action deserves further investigation. The protective role of lyso-PE 18:2 in the liver could also be explained by a decrease in endothelial lipase (EL) expression. EL is highly involved in cholesterol removal from peripheral tissues to the liver [40], and lower expression levels have been previously reported in the liver of hamsters after HFD treatment [19].

In parallel to the liver accumulation of polyunsaturated lyso-PEs, from the *in vivo* study, we also revealed an intrahepatic replacement of saturated and monounsaturated acyl lyso forms in HFD-fed hamsters. Although the HFD was primarily enriched in SFAs and monounsaturated fatty acids, it could be explained by sequential processes of desaturation and elongation of saturated and monounsaturated lyso forms in the hamster liver to form polyunsaturated lyso-PL structures, as reported in several studies on free fatty acids [14,41,42]. Therefore, through this process dietary SFAs, such as myristic acid (14:0) or pentadecanoic acid (15:0), would regulate the bioavailability of LA and ALA in hepatocytes, as previously described in rats [42] and human [43]. The improved bioavailability of essential fatty acids in the liver would contribute to the synthesis of omega (n)-PUFA-enriched lyso-PLs as a protective mechanism against the progression of lipoapoptotic incidents. In this context, the strong positive associations found in MH-LDL-c subjects between SFA intake, including whole dairy products, and lyso-PCs 15:0 and 17:0 supports SFA intermediate involvement on the configuration of omega (n)-PUFA-containing lyso-PLs.

Liver transaminases, specifically GGT, has been suggested as a superior marker for predicting CVD risk in adults with mild

dyslipidemia [44]. For the first time, this study provides an integrated view of the interrelationships between liver transaminases, discriminant lyso-PLs and diet in humans. In this manner, the multiple-way positive interactions stated in the MH-LDL-c group for GGT with lyso-PE 18:2 and PUFAs add further insights into the previously speculated hepatic mechanism to mitigate HC progression. Conversely, in the L-LDL-c group, the observed increases in serum lyso-PC 14:0 levels together with the positive association with GGT also indicate this lyso form could be altered in response to higher GGT enzymatic activity. However, the positive association for lyso-PC 14:0 with TMAO indicated that it could be a precursor for TMAO synthesis in the healthy state, and therefore act better as a susceptibility/risk biomarker for HC. Indeed, lyso-PC 14:0 has been previously considered to be a good predictor for other cardiometabolic risk factors, such as obesity [45]. However, given the limited evidence, further studies to clarify lyso-PC 14:0 predictive capabilities are required.

In this study, TMAO was left out the primary susceptibility/risk biomarkers for HC. Despite their consolidated pro-atherogenic properties and the reported close relationships with cardiovascular events [8,10], the results of this study support the notion that in the preliminary stages of disease, certain lyso-PLs are better predictors for HC than TMAO. The multiple positive relationships stated in both groups with dietary components, particularly with lean fish in the MH-LDL-c group, indicate that fasting TMAO levels are strongly regulated by diet [46]. However, we cannot dismiss whether different gut microbial compositions among groups may have influenced the response of TMAO to animal-based foods; therefore, it is important to study this topic in more detail in the future.

Overall, the present results provide novel insights, particularly from a lipidomic angle, into the suitability of particular lyso-PLs as susceptibility/risk biomarkers for human HC progression, specifically in preliminary stages of the pathophysiological process. Based on the relationships of lyso-PL biomarkers with diet and hepatic transaminases in humans, we hypothesize the existence of a liver adaptive mechanism dependent of lyso-PLs to counteract HC progression, which is strengthened by our second *in vivo* study, where a contradictory response of the hamster liver to the long-term administration of HFD is evidenced. However, it cannot be excluded that lyso-PL levels depend largely on the hydrolysis of oxidized phospholipids in LDL and that the pro-hypercholesterolemic state in the MH-LDL-c group could be caused by a direct action of oxidized LDL rather than through the lyso-PL action. Despite this, the comprehensive analysis of dietary components allowed us to demonstrate interesting associations among particular lyso forms and diet, and revealed a more complex clinical presentation of HC than that represented by classical lipid markers. However, these associations should be interpreted with caution due to the cross-sectional design of this study that could limit the establishment of causality. Given this limitation, the results of this study support the idea of resuming this study in the future to obtain a prospective vision of the changes in the lysophospholipidome and better understand their role as prognostic biomarkers for HC.

5. Conclusion

Two distinct patterns in the serum lyso-PL profile, characterized by higher lyso-PC 15:0 and lower lyso-PE 18:2 in the MH-LDL-c group, and lower lyso-PC 15:0 and higher lyso-PE 18:2 in the L-LDL-c group, were evidenced. Therefore, among all the species of lyso-PLs, serum lyso-PC 15:0 and lyso-PE 18:2 constitute promising susceptibility/risk biomarkers for HC. Dietary essential PUFAs, such as LA (C18:2n-6), promote the intrahepatic synthesis of lyso-PE 18:2, being this an adaptive mechanism of liver to protect itself from diet-induced hepatic steatosis in animal and to counteract HC progression in humans. These biomarkers, particularly low serum levels of Lyso-PE 18:2 in humans, could be used in clinical practice to guide novel preventive strategies to tackle HC.

Funding sources and grants

The research leading to these results was supported by the European Commission through its Seventh Framework Programme "BIOmarkers of Robustness of Metabolic Homeostasis for Nutrigenomics-derived Health CLAIMS Made on Food" Project (FP7-KBBE-2009-3 BIOCLAIMS) under grant agreement number 244995. Additionally, it was financially supported by the Centre for the Development of Industrial Technology (CDTI) of the Spanish Ministry of Science and Innovation under grant agreement: TECNOMIFOOD project (CER-20191010).

Credit author contributions

The authors' responsibilities were as follows – RS, LA, JMdB, AC and FP: Project administration and Conceptualization. RMV, RS, AP, LC-P and EL: Investigation, Methodology and Validation of the human study. SS-G: Investigation, Methodology and Formal analysis of the animal study. LC-P and LR: Data curation. LC-P: Formal analysis, Writing - original draft and Visualization. RS, MS, RMV and AP: Writing – Reviewing and Editing, Supervision. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgments

We would like to thank Miguel Querol, Lorena Torrado and Lluïsa Iniesta, who were nurses ascribed to the Technological Unit of Nutrition and Health (Eurecat, Reus), for their enthusiastic support administering this study. Additionally, we gratefully acknowledge Niurka Llópiz (URV, Tarragona, Spain) for the valuable technical assistance provided in the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2021.11.033.

References

[1] Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, et al. ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS). Eur Heart J 2019;41:111–88. https://doi.org/10.1093/eurheartj/ehz455. 2020.
[2] Grundy SM, Cleeman JI, Merz CNB, Brewer HBJ, Clark LT, Hunninghake DB, et al. Implications of recent clinical trials for the national cholesterol education program adult treatment panel III guidelines. J Am Coll Cardiol 2004;44:720–32. https://doi.org/10.1016/j.jacc.2004.07.001.
[3] Nordestgaard BG, Chapman MJ, Humphries SE, Ginsberg HN, Masana L, Descamps OS, et al. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. Eur Heart J 2013;34:3478–3490a. https://doi.org/10.1093/eurheartj/eht273.
[4] Wang HH, Garruti G, Liu M, Portincasa P, Wang DQ-H. Cholesterol and lipoprotein metabolism and atherosclerosis: recent advances in reverse

cholesterol transport. Ann Hepatol 2017;16(Suppl 1):S27–42. https://doi.org/10.5604/01.3001.0010.5495.
[5] Song Z, Wang H, Yin X, Deng P, Jiang W. Application of NMR metabolomics to search for human disease biomarkers in blood. Clin Chem Lab Med 2019;57:417–41. https://doi.org/10.1515/cclm-2018-0380.
[6] Wang R, Li B, Lam SM, Shui G. Integration of lipidomics and metabolomics for in-depth understanding of cellular mechanism and disease progression. J Genet Genomics 2020;47:69–83. https://doi.org/10.1016/j.jgg.2019.11.009.
[7] Grzelczyk A, Gendaszewska-Darmach E. Novel bioactive glycerol-based lysophospholipids: new data – new insight into their function. Biochimie 2013;95:667–79. https://doi.org/10.1016/j.biochi.2012.10.009.
[8] Schiattarella GG, Sannino A, Toscano E, Giugliano G, Gargiulo G, Franzone A, et al. Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker : a systematic review and dose-response meta-analysis. 2018. p. 2948–56. https://doi.org/10.1093/eurheartj/ehx342.
[9] Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57–63. https://doi.org/10.1038/nature09922.
[10] Haghikia A, Li XS, Liman TG, Bledau N, Schmidt D, Zimmermann F, et al. Gut microbiota-dependent trimethylamine N-oxide predicts risk of cardiovascular events in patients with stroke and is related to proinflammatory monocytes. Arterioscler Thromb Vasc Biol 2018;38:2225–35. https://doi.org/10.1161/ATVBAHA.118.311023.
[11] Knuplez E, Marsche G. An updated review of pro- and anti-inflammatory properties of plasma lysophosphatidylcholines in the vascular system. Int J Mol Sci 2020;21:4501. https://doi.org/10.3390/ijms21124501.
[12] Akerele OA, Cheema SK. Fatty acyl composition of lysophosphatidylcholine is important in atherosclerosis. Med Hypotheses 2015;85:754–60. https://doi.org/10.1016/j.mehy.2015.10.013.
[13] Hui DY. Intestinal phospholipid and lysophospholipid metabolism in cardiometabolic disease. Curr Opin Lipidol 2016;27:507–12. https://doi.org/10.1097/MOL.0000000000000334.
[14] Balogun KA, Albert CJ, Ford DA, Brown RJ, Cheema SK. Dietary omega-3 polyunsaturated fatty acids alter the fatty acid composition of hepatic and plasma bioactive lipids in C57BL/6 mice: a lipidomic approach. PLoS One 2013;8:e82399. https://doi.org/10.1371/journal.pone.0082399.
[15] Wang Z, Zhao Y. Gut microbiota derived metabolites in cardiovascular health and disease. Protein Cell 2018;9:416–31. https://doi.org/10.1007/s13238-018-0549-0.
[16] Rath S, Heidrich B, Pieper DH, Vital M. Uncovering the trimethylamine-producing bacteria of the human gut microbiota. Microbiome 2017;5:54. https://doi.org/10.1186/s40168-017-0271-9.
[17] Chen K, Zheng X, Feng M, Li D, Zhang H. Gut microbiota-dependent metabolite trimethylamine N-oxide contributes to cardiac dysfunction in western diet-induced obese mice. Front Physiol 2017;8:139. https://doi.org/10.3389/fphys.2017.00139.
[18] Wang M, Wang F, Wang Y, Ma X, Zhao M, Zhao C. Metabonomics study of the therapeutic mechanism of Gynostemma pentaphyllum and atorvastatin for hyperlipidemia in rats. PLoS One 2013;8:e78731. https://doi.org/10.1371/journal.pone.0078731.
[19] Suárez-García S, Caimari A, Del Bas JM, Suárez M, Arola L. Serum lysophospholipid levels are altered in dyslipidemic hamsters. Sci Rep 2017;7:10431. https://doi.org/10.1038/s41598-017-10651-0.
[20] Guideline E. 2019 ESC/EAS guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. Atherosclerosis 2019;290:140–205. https://doi.org/10.1016/j.atherosclerosis.2019.08.014.
[21] CESNID. Tablas de composición de alimentos del Cesnid. Rev Esp Salud Publica 2004. https://doi.org/10.1590/s1135-57272004000300011.
[22] Patterson KY, Bhagwat S, Williams JR, Howe JC, Holden JM, Zeisel SH, et al. USDA database for the choline content of common foods. Nutr Data Lab 2008:1–37. https://doi.org/10.15482/USDA.ADC/1178141.
[23] Vallbona Calbó C, Roure Cuspinera E, Violan Fors M, Alegre Martín J. Guia de prescripció d'exercici físic per a la salut (PEFS). 2007. Barcelona.
[24] Zhao X, Zeisel SH, Zhang S. Rapid LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine N-oxide, and creatinine in human plasma and urine. Electrophoresis 2015;36:2207–14. https://doi.org/10.1002/elps.201500055.
[25] Suárez-García S, Arola L, Pascual-Serrano A, Arola-Arnal A, Aragonès G, Bladé C, et al. Development and validation of a UHPLC-ESI-MS/MS method for the simultaneous quantification of mammal lysophosphatidylcholines and lysophosphatidylethanolamines in serum. J Chromatogr B, Anal Technol Biomed Life Sci 2017;1055–1056:86–97. https://doi.org/10.1016/j.jchromb.2017.04.028.
[26] Russell JC, Proctor SD. Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis. Cardiovasc Pathol Off J Soc Cardiovasc Pathol 2006;15:318–30. https://doi.org/10.1016/j.carpath.2006.09.001.
[27] Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313–21. https://doi.org/10.1002/hep.20701.
[28] Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 1995;57:289–300.
[29] Guallar-Castillón P, Gil-Montero M, León-Muñoz LM, Graciani A, Bayán-Bravo A, Taboada JM, et al. Magnitude and management of hypercholesterolemia in the adult population of Spain, 2008-2010: the ENRICA Study. Rev Esp Cardiol (Engl Ed) 2012;65:551–8. https://doi.org/10.1016/j.recesp.2012.02.005.
[30] Ibarrola-Jurado N, Bulló M, Guasch-Ferré M, Ros E, Martínez-González MA, Corella D, et al. Cross-sectional assessment of nut consumption and obesity, metabolic syndrome and other cardiometabolic risk factors: the PREDIMED study. PLoS One 2013;8:e57367. https://doi.org/10.1371/journal.pone.0057367.
[31] Worley B, Powers R. Multivariate analysis in metabolomics. Curr Metabolomics 2013;1:92–107. https://doi.org/10.2174/2213235X11301010092.
[32] Kröger J, Zietemann V, Enzenbach C, Weikert C, Jansen EH, Döring F, et al. Erythrocyte membrane phospholipid fatty acids, desaturase activity, and dietary fatty acids in relation to risk of type 2 diabetes in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Am J Clin Nutr 2011;93:127–42. https://doi.org/10.3945/ajcn.110.005447.
[33] Petersen KS, Keogh JB, Lister N, Weir JM, Meikle PJ, Clifton PM. Association between dairy intake, lipids and vascular structure and function in diabetes. World J Diabetes 2017;8:202–12. https://doi.org/10.4239/wjd.v8.i5.202.
[34] Csonka C, Baranyai T, Tiszlavicz L, Fébel H, Szűcs G, Varga ZV, et al. Isolated hypercholesterolemia leads to steatosis in the liver without affecting the pancreas. Lipids Health Dis 2017;16:144. https://doi.org/10.1186/s12944-017-0537-z.
[35] Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, et al. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. Obesity (Silver Spring) 2011;19:1109–17. https://doi.org/10.1038/oby.2011.18.
[36] Nam M, Choi M-S, Jung S, Jung Y, Choi J-Y, Ryu DH, et al. Lipidomic profiling of liver tissue from obesity-prone and obesity-resistant mice fed a high fat diet. Sci Rep 2015;5:16984. https://doi.org/10.1038/srep16984.
[37] Labonté ED, Pfluger PT, Cash JG, Kuhel DG, Roja JC, Magness DP, et al. Postprandial lysophospholipid suppresses hepatic fatty acid oxidation: the molecular link between group 1B phospholipase A2 and diet-induced obesity. FASEB J Off Publ Fed Am Soc Exp Biol 2010;24:2516–24. https://doi.org/10.1096/fj.09-144436.
[38] Brindley DN. Hepatic secretion of lysophosphatidylcholine: a novel transport system for polyunsaturated fatty acids and choline. J Nutr Biochem 1993;4:442–9. https://doi.org/10.1016/0955-2863(93)90061-Z.
[39] Saini RK, Keum Y-S. Omega-3 and omega-6 polyunsaturated fatty acids: dietary sources, metabolism, and significance - a review. Life Sci 2018;203:255–67. https://doi.org/10.1016/j.lfs.2018.04.049.
[40] Annema W, Tietge UJF. Role of hepatic lipase and endothelial lipase in high-density lipoprotein-mediated reverse cholesterol transport. Curr Atheroscler Rep 2011;13:257–65. https://doi.org/10.1007/s11883-011-0175-2.
[41] Legrand P, Rioux V. The complex and important cellular and metabolic functions of saturated fatty acids. Lipids 2010;45:941–6. https://doi.org/10.1007/s11745-010-3444-x.
[42] Rioux V, Catheline D, Bouriel M, Legrand P. Dietary myristic acid at physiologically relevant levels increases the tissue content of C20:5 n-3 and C20:3 n-6 in the rat. Reprod Nutr Dev 2005;45:599–612. https://doi.org/10.1051/rnd:2005048.
[43] Dabadie H, Peuchant E, Bernard M, LeRuyet P, Mendy F. Moderate intake of myristic acid in sn-2 position has beneficial lipidic effects and enhances DHA of cholesteryl esters in an interventional study. J Nutr Biochem 2005;16:375–82. https://doi.org/10.1016/j.jnutbio.2005.01.010.
[44] Park E-O, Bae EJ, Park B-H, Chae S-W. The associations between liver enzymes and cardiovascular risk factors in adults with mild dyslipidemia. J Clin Med 2020;9. https://doi.org/10.3390/jcm9041147.
[45] Kim JY, Park JY, Kim OY, Ham BM, Kim H-J, Kwon DY, et al. Metabolic profiling of plasma in overweight/obese and lean men using ultra performance liquid chromatography and Q-TOF mass spectrometry (UPLC-Q-TOF MS). J Proteome Res 2010;9:4368–75. https://doi.org/10.1021/pr100101p.
[46] Canyelles M, Tondo M, Cedó L, Farràs M, Escolà-Gil JC, Blanco-Vaca F. Trimethylamine N-oxide: a link among diet, gut microbiota, gene regulation of liver and intestine cholesterol homeostasis and HDL function. Int J Mol Sci 2018;19:3228. https://doi.org/10.3390/ijms19103228.

Supplemental Information 1 | Lipidomics analysis of serum lysophospholipid content

The quantitative evaluation of lyso-PLs was performed using a UHPLC 1290 coupled to a QqQ 6490 mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA). The samples were randomized prior to metabolite extraction, and again prior to mass spectrometer analysis. The temperature in the autosampler was held at 4°C.

The mobile phase consisted of water / isopropanol / acetonitrile / 500 mM ammonium acetate (89:5:5:1 v/v/v/v) as solvent A, and isopropanol / acetonitrile / 500 mM ammonium acetate (50:49:1 v/v/v) as solvent B. A sample injection volume of 7.5 µL was loaded onto a 0.3 mL/min flow of 30% B passing through a reverse-phase column (Acquity UPLC BEH C8; 1.7 µm, 2.1 x 150 mm) (Waters Corporation, Milford, MA, USA) held at 50°C. The chromatographic separation was conducted using linear gradient elution to 75% B over 20 min, followed by a further increase to 100% B over 1 min, a 4-min solvent B hold and 2 min for return to the initial conditions. Thereafter, a post-time of 2 min was applied. The mass spectrometer was operated in positive ion mode (+ESI) with the following settings: nebulizer gas (nitrogen) pressure, 25 psi; gas flow rate, 12 L/min at 240°C; sheath gas flow rate, 12 L/min at 350°C; capillary and nozzle voltages, 4,500 and 500 V, respectively; cell accelerator voltage was set to 5 V and fragmentor to 380 V. The scan mode was dynamic multiple reaction monitoring using a retention time (RT) window of 1 min. The RTs of Lyso-PLs, monitored transitions and optimal collision energies were described in a previous study [30]. [M+H]+ ions were used as precursor ions. For each analyte, the most abundant transition was chosen for the quantification, whereas two transitions were used for qualitative purposes. The UHPLC and QqQ systems were operated using MassHunter Data Acquisition, whereas peak detection and integration were performed using MassHunter Quantitative software, both versions 7.0 from Agilent Technologies.

The quantitative analysis of endogenous Lyso-PLs was performed as described previously [30,31]. Briefly, calibration curves were obtained by mixing different concentrations (0.05 – 2000 µg/L) of the standard Lyso-PLs with constant levels (100 µg/L) of the internal standards Lyso-PC (13:0) and

Lyso-PE (17:1) and performing UHPLC-+ESI-MS/MS analysis. The peak abundance ratios (analyte / IS) were plotted versus the concentration ratios (analyte / IS) and fitted to a linear regression. Concentration levels of the Lyso-PLs identified in samples were calculated by using the closest related standard curve and expressed in molarity (μM).

Supplemental Information 2 | Detailed procedure for sample extraction and histological examination in the *in vivo* study

A total of 16 male Golden Syrian hamsters (Charles River Laboratories, Barcelona, Spain) weighting 143 ± 2 g were housed singly at 22°C with a light/dark period of 12 h and free access to water and food. After 2 weeks of acclimatization feeding a low-fat diet (LFD), they were randomly distributed into two groups with comparable body weights (n = 8 per group). The hamsters were fed ad libitum either the LFD or a HFD and feed, conserved at 4°C, were renewed daily. The LFD (3.9 kcal/g) and HFD (4.1 kcal/g) (Research Diets, New Brunswick, NJ, USA) provided 10% and 21% energy as fat, respectively. Throughout the study, food intake was monitored weekly.

On day 30, the animals were fasted for 6 h and anesthetized with i.p. pentobarbital sodium (80 mg per kg body weight). Blood was withdrawn by cardiac puncture and collected in BD Vacutainer® tubes containing K2EDTA as anticoagulant (Franklin Lakes, NJ, USA). Blood was conserved at 4°C for 1 h and plasma was separated by centrifugation at 2,000 g for 15 min. The liver relative weight was calculated following the formula (tissue weight / body weight * 100) and expressed as percentage of the total body weight. The white adipose tissue including mesenteric (MWAT), epididymal (EWAT) and retroperitoneal (RWAT) depots were also excised for determination of the relative weight. All the samples were snap frozen in liquid nitrogen and then stored at - 80°C until further analyses.

For the histological examination frozen liver tissue was thawed and fixed by immersion in 4% paraformaldehyde for 24 h, dehydrated successively in graded concentrations of ethanol (70%, 96 % and 100 %; plus xylol), and embedded in paraffin at 52°C (Citadel 2000). The paraffin blocks were sectioned at 2 μm thickness with a microtome (Microm HM 355S), and mounted in slides for automated staining with hematoxylin-eosin (Varistain Gemini ES).

Supplemental Information 3 | Composition of the animal diets. LFD, low-fat diet; HFD, moderate high-fat diet

	LFD	HFD
Macronutrients		
Protein (g/kg)	22	24
Carbohydrate (g/kg)	65	57
Fat		
g/kg	4	10
%	10	21
Energy (kcal/g)	4	4
Ingredients (g/kg)		
Casein, 80 mesh	220	220
L-Cysteine	3	3
Wheat starch	386.5	281
Maltodextrin 10	100	100
Dextrose	50	50
Sucrose	100	100
Cellulose, BW200	50	50
Coconut oil, 76	7.74	5
Flaxseed oil	5.16	4
Sunflower oil	30.1	10
Lard	0	71
Mineral mix S10022G	35	35
Vitamin mix V10037	10	10
Choline bitartrate	2.5	2.5
Cholesterol	0.03	0.92
Fatty acids		
SFA		
g/kg	10.9	28.6
%	26.5	33.7
MUFA		
g/kg	8.2	28.0
%	19.9	33.1
PUFA		
g/kg	22.1	28.2
%	53.6	33.2

Supplemental Table 1 | Lifestyle baseline characteristics of the study population

Variables	Low LDL-cholesterol	Moderate to high LDL-cholesterol	*P*-value
Diet, Macronutrients			
Energy intake, *Kcal/day*	2117.54 ± 502.28	2206.87 ± 600.15	0.383
Total protein, *g/day*	84.55 ± 22.52	91.82 ± 25.46	0.105
(% DEI)	*(16.0)*	*(16.9)*	0.101
Total carbohydrates, *g/day*	211.87 ± 65.09	206.17 ± 67.08	0.461
(% DEI)	*(40.0)*	*(37.7)*	0.061
Complex carbohydrates, *g/day*	114.51 ± 44.13	102.57 ± 39.18	0.180
(% DEI)	*(21.6)*	*(19.0)*	0.010
Simple carbohydrates, *g/day*	96.72 ± 37.42	101.22 ± 42.08	0.720
(% DEI)	*(18.4)*	*(18.7)*	0.769
Total fatty acids, *g/day*	100.02 ± 24.68	103.57 ± 32.55	0.502
(% DEI)	*(42.0)*	*(41.4)*	0.445
SFA, *g/day*	29.42 ± 9.30	28.74 ± 9.66	0.702
(% DEI)	*(12.3)*	*(11.5)*	0.116
MUFA, *g/day*	46.47 ± 11.76	50.79 ± 17.01	0.131
(% DEI)	*(19.9)*	*(20.2)*	0.721
PUFA, *g/day*	15.71 ± 6.63	15.35 ± 6.94	0.620
(% DEI)	*(6.6)*	*(6.2)*	0.158
Total cholesterol, *mg/day*	306.05 ± 124.87	348.73 ± 149.06	0.095
Dietary fibre, *g/day*	23.11 ± 11.74	22.62 ± 7.64	0.628
Ethanol, *g/day*	5.09 ± 7.08	11.26 ± 12.93	0.006
Diet, Micronutrients			
Sodium, mg/day	2495.47 ± 758.70	2588.97 ± 925.83	0.549
Potassium, *mg/day*	3365.09 ± 993.22	3748.33 ± 1050.71	0.047
Magnesium, *mg/day*	342.39 ± 155.01	340.90 ± 99.76	0.632
Iron, *mg/day*	13.82 ± 4.94	14.41 ± 4.88	0.595
Vitamin A, *µg/day*	873.73 ± 415.72	939.50 ± 540.60	0.641
Vitamin D, *µg/day*	2.55 ± 2.07	3.02 ± 3.23	0.861
Vitamin E, *µg/day*	13.65 ± 6.61	13.95 ± 4.84	0.461
Vitamin B6, *mg/day*	363.07 ± 147.69	371.38 ± 136.94	0.758
Vitamin B9, *µg/day*	2.26 ± 0.79	2.38 ± 0.65	0.187
Vitamin B12, *µg/day*	5.60 ± 3.13	7.10 ± 3.98	0.018
Diet composition, protein intake *g/day*			
Mean animal protein	54.10 ± 19.45	62.55 ± 19.90	0.023
Tryptophan	0.98 ± 1.19	1.00 ± 1.00	0.390
Threonine	2.21 ± 0.84	2.58 ± 0.88	0.021
Isoleucine	2.49 ± 0.93	2.86 ± 0.97	0.040
Leucine	4.29 ± 1.60	4.91 ± 1.70	0.048

Lysine	4.36 ± 1.72	5.08 ± 1.81	0.031
Methionine	1.37 ± 0.52	1.59 ± 0.55	0.032
Cysteine	0.55 ± 0.22	0.64 ± 0.22	0.043
Phenylalanine	2.25 ± 0.82	2.55 ± 0.87	0.060
Tyrosine	2.00 ± 0.72	2.26 ± 0.78	0.069
Valine	2.89 ± 1.04	3.27 ± 1.12	0.060
Arginine	2.83 ± 1.21	3.38 ± 1.22	0.017
Histidine	1.55 ± 0.61	1.79 ± 0.66	0.047
Alanine	2.68 ± 1.11	3.19 ± 1.13	0.017
Aspartic acid	4.65 ± 1.83	5.46 ± 1.89	0.021
Glutamic acid	8.60 ± 3.14	9.72 ± 3.33	0.068
Glycine	2.17 ± 0.98	2.64 ± 0.98	0.011
Proline	3.05 ± 1.08	3.30 ± 1.19	0.346
Serine	2.45 ± 0.87	2.77 ± 0.92	0.062
Diet composition, choline rich food sources *g/day*			
Red meat	24.37 ± 35.40	29.53 ± 41.44	0.666
White meat	42.66 ± 55.33	52.28 ± 47.05	0.093
Processed meat	48.30 ± 40.60	49.48 ± 39.14	0.807
Fatty fish	22.48 ± 30.37	28.58 ± 36.67	0.284
Lean fish	14.67 ± 24.78	32.24 ± 39.67	0.039
Shellfish	17.26 ± 30.79	24.58 ± 31.25	0.134
Total dairy	264.43 ± 149.02	243.83 ± 156.92	0.472
Total milk	168.34 ± 127.79	152.17 ± 122.73	0.435
Whole milk	39.19 ± 59.46	31.68 ± 41.02	0.878
Low-fat milk	87.07 ± 127.11	68.91 ± 108.14	0.454
Skim milk	42.06 ± 84.42	51.57 ± 94.92	0.917
Fermented dairy products	85.49 ± 56.70	80.10 ± 56.31	0.612
Yoghurt	59.58 ± 52.02	56.62 ± 53.50	0.674
Cheese	25.90 ± 24.18	23.48 ± 23.53	0.504
Butter	10.59 ± 12.32	11.55 ± 11.66	0.667
Eggs	31.44 ± 23.27	29.92 ± 17.15	0.904
Physical activity *%*			
Inactive	12.8	8.3	
Very low activity	4.3	8.3	
Low activity	8.6	8.3	0.578
Moderate activity	21.4	31.3	
High activity	52.9	43.8	

Data expressed as mean ± standard deviation or percentage. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; TPE, total polyphenol excretion. % DEI, mean percentage of daily energy intake. P-value for physical activity was calculated by Fisher's exact test. P-value for the rest of the variables was calculated by Student's t-test and Mann-Whitney U test.

Supplemental Table 2 | Spearman correlations between serum discriminant lysophospholipids and dietary components in low and moderate to high LDL-cholesterol subjects

Lysophospholipid, *μM*	Dietary component	Spearman correlation index	p.value	Adjusted p.value
Low LDL-cholesterol				
Lyso-PC 14:0	Ethanol	0.531	0.005	0.033*
	Complex carbohydrates	0.613	0.001	0.009 *
	Total fatty acids	0.533	0.007	0.031*
	Yoghurt	0.560	0.004	0.037*
	Cheese	0.408	0.003	0.019*
Lyso-PE 18:2	Total fatty acids	0.289	0.041	0.109
	PUFAs	0.389	0.005	0.033*
	Fatty fish	-0.426	0.037	0.319
Moderate to high LDL-cholesterol				
Lyso-PC 15:0	Complex carbohydrates	0.470	0.015	0.099
	Vitamin A	-0.430	0.027	0.153
	SFAs	0.520	<0.001	0.002*
	Glutamic acid	0.432	0.001	0.016*
	Yoghurt	0.532	0.005	0.043*
	Cheese	0.467	0.021	0.181
	Whole milk	0.350	0.052	0.525
Lyso-PC 17:0	Energy intake	0.434	0.027	0.071
	Total fatty acids	0.407	0.039	0.085
	SFAs	0.639	<0.001	0.002*
	MUFAs	0.500	0.009	0.043*
	PUFAs	0.459	0.018	0.059
	Vitamin A	-0.549	0.003	0.019*
	Glutamic acid	0.300	0.034	0.131
	Glycine	0.432	0.036	0.278
	Tryptophan	0.416	0.044	0.278
	Lean fish	0.376	0.058	0.262
	Shellfish	0.430	0.028	0.254
	Whole milk	0.354	0.058	0.503
	Butter	0.376	0.058	0.262
Lyso-PC 18:1	Fatty fish	0.426	0.029	0.253
Lyso-PC 20:4	Leucine	0.419	0.033	0.128
	Methionine	0.427	0.030	0.128
	Histidine	0.463	0.018	0.114
	Valine	0.474	0.015	0.114

	Yoghurt	-0.394	0.046	0.241
	Fermented dairy	-0.396	0.044	0.241
Lyso-PC 20:5	Vitamin D	0.666	1.2896^{e-07}	7.0926^{e-07*}
	Whole milk	-0.475	0.014	0.120
	Fatty fish	0.429	0.028	0.121
Lyso-PE 18:2	Total fatty acids	0.529	0.006	0.023*
	PUFAs	0.518	0.006	0.023*
	Total protein	0.542	0.004	0.022*
	Mean animal protein	0.396	0.044	0.455
	Histidine	0.403	0.042	0.369
	Total milk	0.539	0.005	0.042*
	Cheese	0.395	0.045	0.252
Lyso-PE 20:5	Ethanol	0.424	0.038	0.252
	Vitamin E	0.403	0.041	0.225
	Vitamin D	0.332	0.018	0.067
	Fatty fish	0.582	0.001	0.015*
	Low-fat milk	-0.512	0.007	0.041*
	Butter	0.390	0.048	0.205
Lyso-PE 22:6	Ethanol	0.466	0.016	0.105
	Simple carbohydrates	0.435	0.026	0.113
	Iron	0.415	0.034	0.150
	Vitamin D	0.380	0.055	0.150
	Fatty fish	0.402	0.041	0.234
	Processed meat	-0.418	0.033	0.234

Abbreviations: MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine. Lysophospholipid available in a sub-group of n=50 subjects. Significant correlations were set at $p<0.05$. *Significant adjusted p value <0.05 after Benjamini-Hochberg correction.

Supplemental Table 3 | Multiple linear regression results for associations of discriminant lysophospholipids with dietary components in low and moderate to high LDL-cholesterol groups

Lysophospholipid, *μM*	**Dietary component**	**R^2**	***β***	***p* value**
Low LDL-cholesterol				
Lyso-PC 14:0	Ethanol	0.255	0.505	0.012*
	Cheese	0.168	0.409	0.047*
Lyso-PE 18:2	PUFAs	0.153	0.391	0.048*
Moderate to high LDL-cholesterol				
Lyso-PC 15:0	SFAs	0.162	0.460	0.025*
	Yoghurt	0.222	0.471	0.015*
Lyso-PC 17:0	SFAs	0.244	0.493	0.010*
	Vitamin A	0.189	0.434	0.027*
Lyso-PC 20:4	Yoghurt	0.313	-0.559	0.003*
Lyso-PC 20:5	Vitamin D	0.773	0.879	<0.001*
	Whole milk	0.259	-0.430	0.028*
Lyso-PE 20:5	Vitamin D	0.623	0.790	<0.001*
	Fatty fish	0.193	0.439	0.025*

R^2, coefficient of multiple determination; *β*, standardized regression coefficient; *p* value, two-sided test of significance. Abbreviations: PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine. Dietary components expressed in g/day except for vitamin, expressed in μg/day.

*p value ≤ 0.05

Values from multiple stepwise regression analysis.

Only statistically significant results are shown.

Supplemental Table 4 | Spearman correlations between liver transaminase (U/L), TMAO (µM), lysophospholipid biomarkers (µM), and mean animal protein, fatty acid and ethanol intake (g/day) in all, low LDL-cholesterol and moderate to high LDL-cholesterol subjects

	AST			ALT			GGT		
	Spearman correlation index	**p.value**	**Adjusted p.value**	**Spearman correlation index**	**p.value**	**Adjusted p.value**	**Spearman correlation index**	**p.value**	**Adjusted p.value**
All subjects (n=118)									
TMAO	0.072	0.433	0.433	0.127	0.167	0.223	0.175	**0.056**	0.113
Ethanol	0.066	0.471	0.628	0.031	0.738	0.738	0.259	**0.004**	**0.009***
Mean animal protein	0.403	**<0.0001**	**<0.0001***	0.283	**0.001**	**0.001***	0.307	**0.0007**	**0.0009***
Fatty acids	0.130	0.157	0.157	0.224	**0.014**	**0.016***	0.257	**0.004**	**0.008***
PUFAs	0.267	**0.003**	**0.003***	0.275	**0.002**	**0.003***	0.248	**0.006**	**0.006***
MUFAs	0.096	0.299	0.299	0.154	0.094	0.110	0.223	**0.014**	**0.020***
SFAs	0.157	0.087	0.120	0.128	0.164	0.164	0.150	0.103	0.120
Lyso-PC14:0	0.148	0.302	0.302	0.156	0.277	0.302	0.318	**0.024**	**0.048***
Lyso-PC15:0	-0.191	0.182	0.364	-0.021	0.879	0.879	0.134	0.351	0.468
Lyso-PE18:2	0.040	0.844	0.946	-0.013	0.946	0.946	0.065	0.750	0.946
Low LDL-cholesterol (n=70)									
TMAO	0.215	0.073	0.097	0.177	0.142	0.142	0.277	**0.020**	**0.040***
Ethanol	0.035	0.769	0.769	0.037	0.755	0.769	0.136	0.259	0.518
Mean animal protein	0.405	**<0.0001**	**0.0001***	0.277	**0.020**	**0.026***	0.235	**0.049**	**0.049***
Fatty acids	0.165	0.170	0.239	0.126	0.296	0.346	0.065	0.592	0.592
PUFAs	0.174	0.147	0.172	0.189	0.116	0.163	-0.127	0.291	0.291
MUFAs	0.169	0.160	0.224	0.084	0.487	0.568	-0.033	0.781	0.781
SFAs	0.093	0.441	0.617	0.005	0.963	0.963	0.015	0.895	0.963
Lyso-PC14:0	0.318	0.129	0.172	0.277	0.189	0.189	0.552	**0.005**	**0.010***
Lyso-PC15:0	0.096	0.652	0.652	0.284	0.178	0.356	0.183	0.390	0.520
Lyso-PE18:2	-0.195	0.360	0.572	-0.121	0.572	0.572	-0.138	0.518	0.572
Moderate to high LDL-cholesterol (n=48)									
TMAO	0.010	0.944	0.944	0.047	0.747	0.944	0.081	0.580	0.944
Ethanol	0.085	0.563	0.563	0.091	0.535	0.563	0.204	0.162	0.325
Mean animal protein	0.169	0.250	0.501	0.102	0.487	0.649	0.060	0.684	0.684
Fatty acids	0.071	0.629	0.629	0.323	**0.025**	**0.035***	0.410	**0.003**	**0.008***
PUFAs	0.434	**0.002**	**0.004***	0.416	**0.003**	**0.005***	0.576	**<0.0001**	**<0.0001***
MUFAs	0.042	0.775	0.775	0.174	0.235	0.275	0.379	**0.007**	**0.013***
SFAs	0.256	0.079	0.082	0.259	0.074	0.082	0.267	0.066	0.082
Lyso-PC14:0	0.040	0.844	0.946	-0.013	0.946	0.946	0.065	0.750	0.946
Lyso-PC15:0	-0.474	**0.014**	**0.028***	-0.197	0.334	0.445	0.122	0.552	0.552
Lyso-PE18:2	0.232	0.252	0.252	0.333	0.095	0.127	0.523	**0.006**	**0.012***

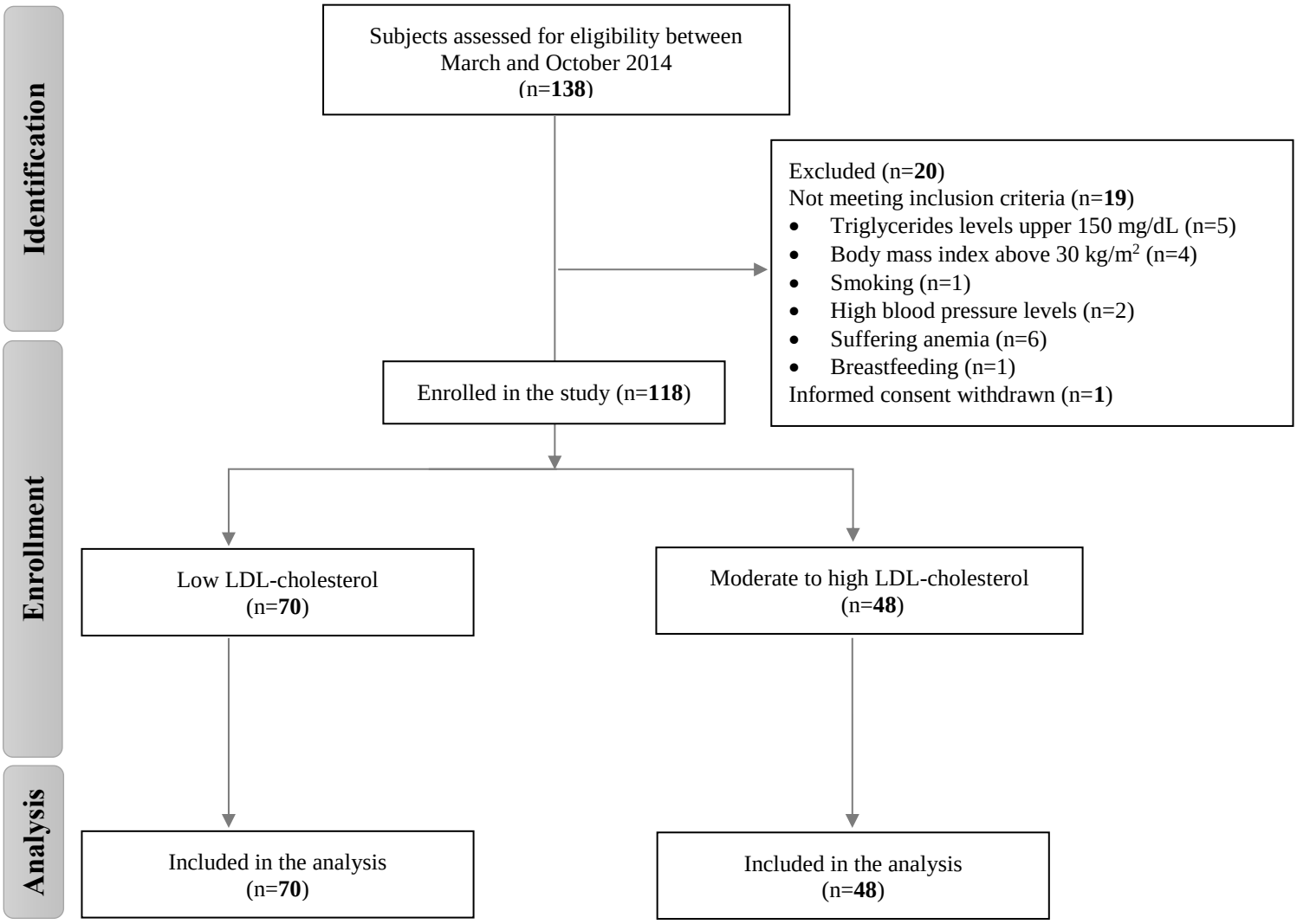
Abbreviations: Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine. Lysophospholipid available in a sub-group of n=50 subjects; TMAO available in n=107 subjects. Significant differences depicted in **bold**. Significant correlations were set at $p<0.05$.* Significant adjusted p value <0.05. Analysis adjusted for ethanol intake.

Supplemental Table 5 | Circulating parameters in hamsters fed a low- (LFD) or high-fat diet (HFD) for 30 days

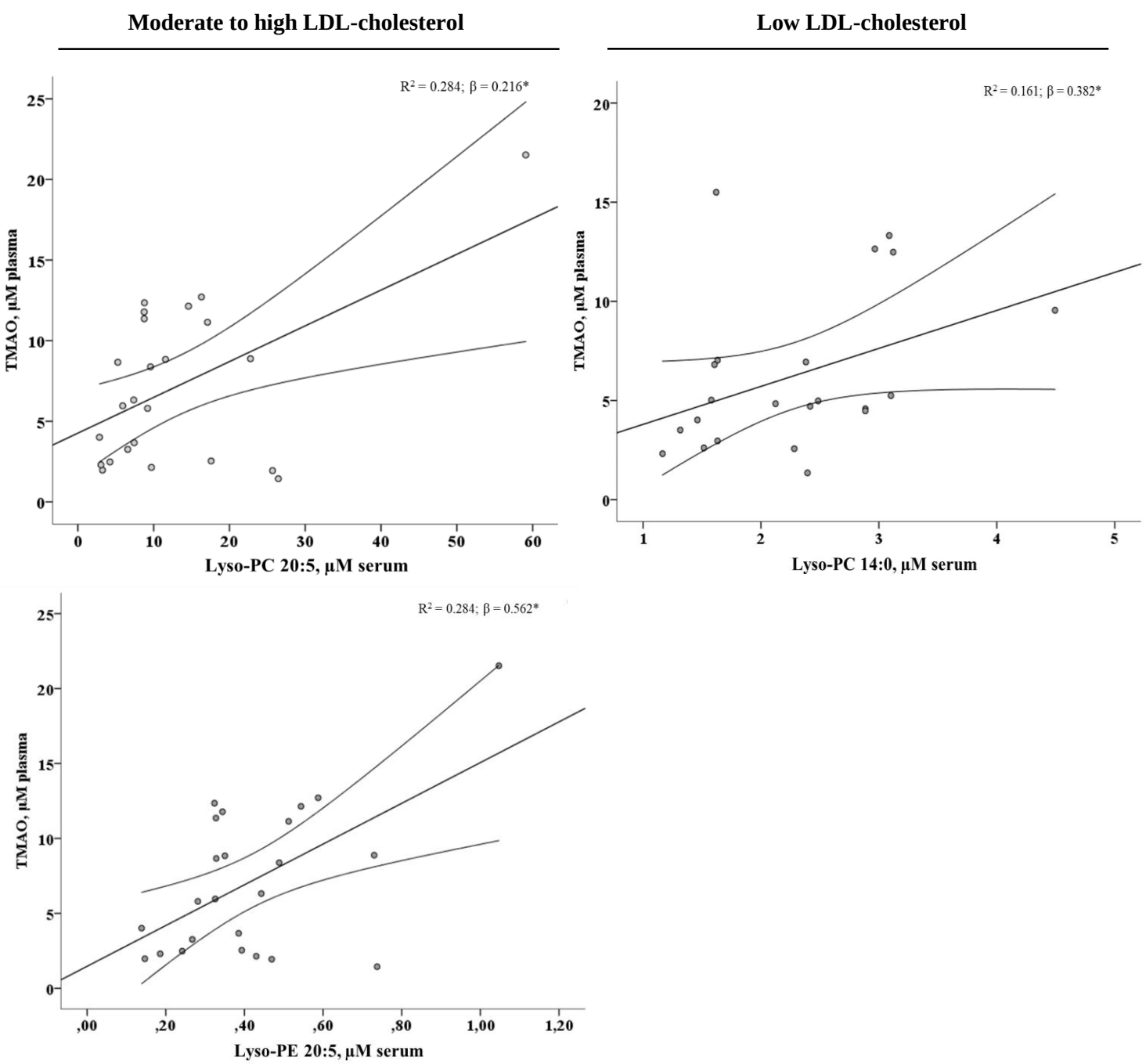
	Dietary group		
	LFD (n=8)	**HFD (n=8)**	***P*-value**
Triglycerides, *mmol/L*	1.82 ± 0.19	2.11 ± 0.23	0.370
Total cholesterol, *mmol/L*	5.43 ± 0.30	6.60 ± 0.25	0.011
LDL-cholesterol, *mmol/L*	1.64 ± 0.12	2.13 ± 0.25	0.088
HDL-cholesterol, *mmol/L*	2.03 ± 0.17	2.12 ± 0.09	0.662
Ratio TC/HDL-cholesterol	2.71 ± 0.10	3.07 ± 0.13	0.046
ALT	49.81 ± 9.65	46.89 ± 5.43	0.422
AST	29.39 ± 5.79	19.48 ± 3.50	0.168

Data expressed as mean ± SEM for each animal group (n=8). Two-tailed p-values were calculated using independent Student's t-test. Abbreviations: TC, total cholesterol; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; ALT, alanine transaminase; AST, aspartate transaminase; sPLA2, secreted phospholipase A2.

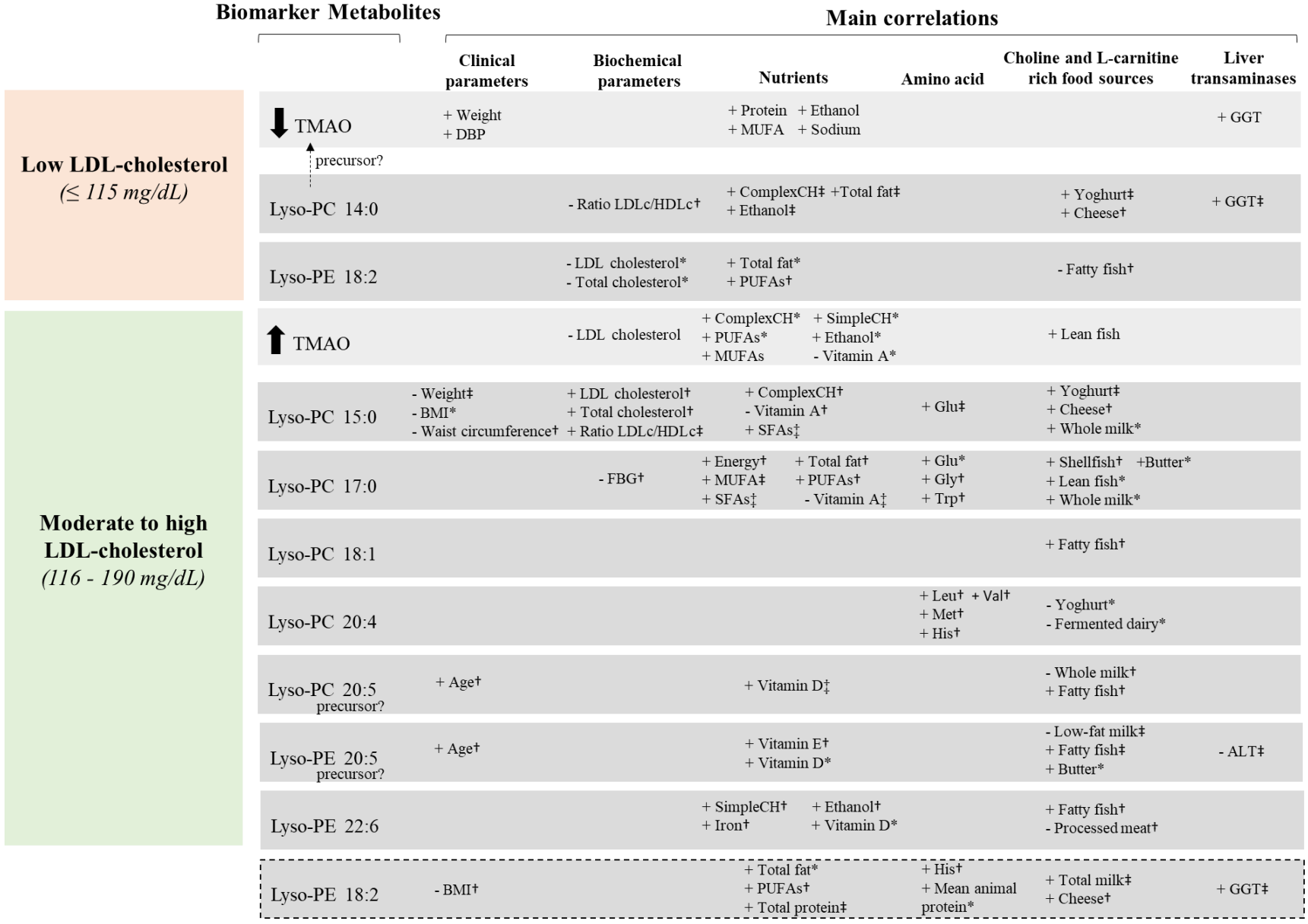
Supplemental Figure 1 | Data-collection flowchart based on the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) statement



Supplemental Figure 2 | Linear regression plots between lysophospholipids and TMAO in 24 low LDL-cholesterol and 26 moderate to high LDL-cholesterol subjects. The 95% CI is represented as curved lines on both sides of the linear regression line. Only significant associations are represented. *p<0.05

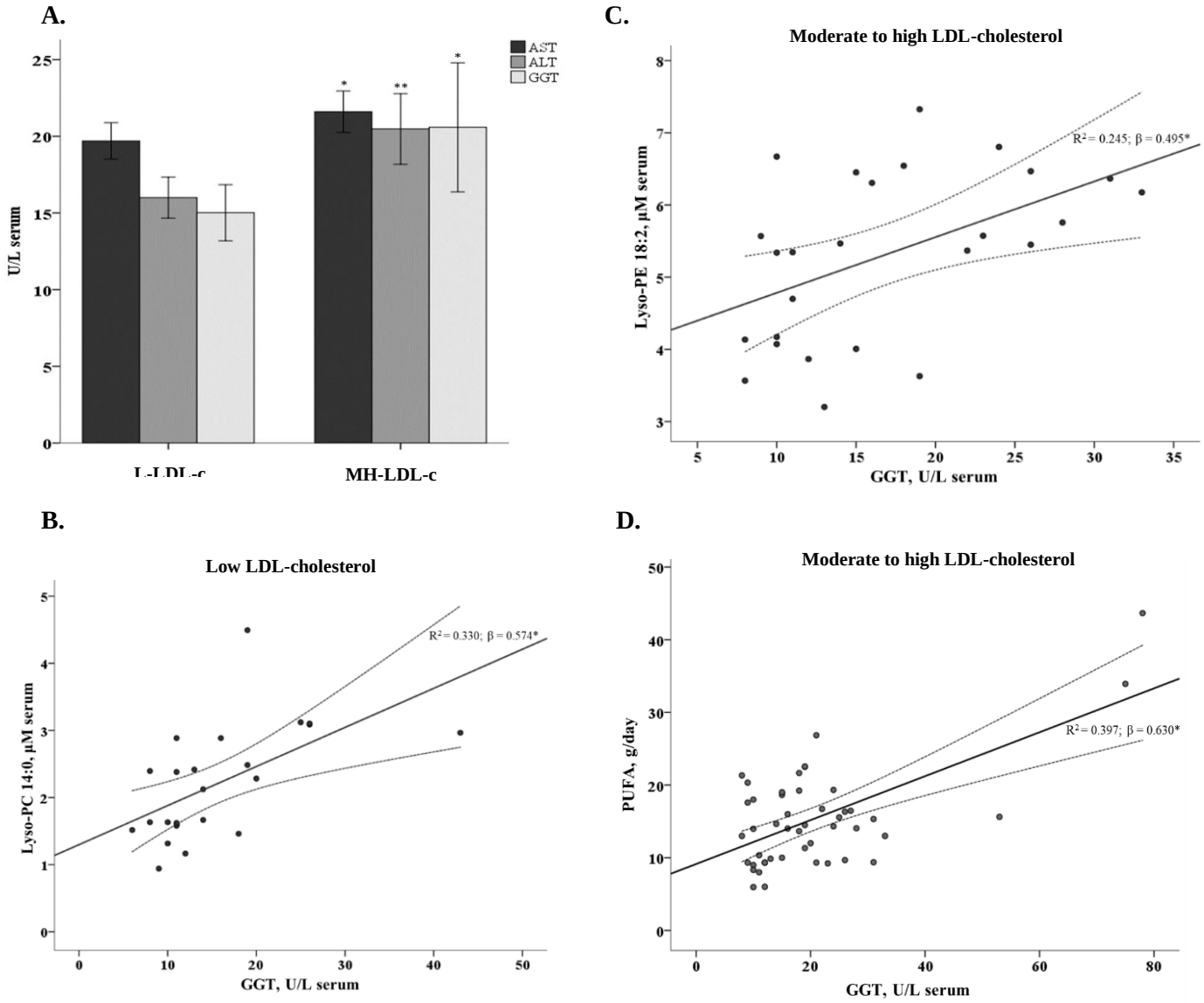


Supplemental Figure 3 | Scheme for major interrelationships between TMAO and discriminant lysophospholipids with all clinical and dietary features in low (L-LDL-c) and moderate to high (MH-LDL-c) LDL-cholesterol subjects. Positive and negative Spearman correlations are symbolized by (+) and (-), respectively. Only significant correlations are shown. P value set at <0.05



+: positive Spearman correlation; -: negative Spearman correlation
* r > 0.3; † r > 0.4; ‡ r > 0.5

Supplemental Figure 4 | Linear regression plots for significant relationships between liver transaminases and targeted metabolites. **(A)** Mean serum concentration (U/L) of liver transaminases in low (L-LDL-c) and moderate to high (MH-LDL-c) LDL-cholesterol groups. **(B)** Regression plot of Gamma-glutamyl transferase (GGT) with Lyso-PC 14:0 and **(C)** with Lyso-PE 18:2. **(D)** Regression plot of polyunsaturated fatty-acids (PUFA) (g/day) with GGT. The 95% CI is represented as curved lines on both sides of the linear regression line. $^{*}p<0.05$, $^{**}p<0.001$



SUMMARY OF RESULTS: PART II.I

Summary of results – Article 3. Serum lysophospholipidome of dietary origin as a suitable susceptibility/risk biomarker of human hypercholesterolemia: A cross-sectional study

Characteristics of the study participants

A total of 118 subjects, 70 L-LDL-c and 48 MH-LDL-c, were enrolled in the study and analyzed. MH-LDL-c subjects were significantly older than L-LDL-c subjects and had higher SBP, DBP, BMI and waist circumference ($P < 0.05$). The serum concentrations of total cholesterol and triglycerides were higher in MH-LDL-c subjects compared to L-LDL-c, although their baseline levels remained within normal values. In relation to dietary intake, MH-LDL-c group reported significantly greater daily mean intake of lean fish, animal protein and several amino acids, including threonine, lysine, arginine, alanine and glycine, among others ($P < 0.05$).

Identification of circulating microbial- and lipid-based metabolites involved in hypercholesterolemia susceptibility

After targeted metabolomics and lipidomics analysis, a total of 27 serum lyso-PLs (19 lyso-PC and 8 lyso-PE) were identified and plasma TMAO concentrations were assessed. Particular lyso-PLs, including several lyso-PC and lyso-PE species, differed significantly among the L-LDL-c and MH-LDL-c groups. In particular, in the MH-LDL-c group, lyso-PC 15:0 was highly concentrated ($P<0.001$) and contrary, lyso-PE 18:2 was significantly lower ($P<0.05$). In the L-LDL-c group, different monounsaturated and polyunsaturated chain lyso-PC and lyso-PE presented increased serum concentrations, mainly lyso-PE 18:2 ($P=0.05$). Moreover, lyso-PC 15:0 and lyso-PE 18:2 revealed the highest variable importance in projection (VIP)

score (VIPs > 2) in the differentiation between groups, and were the major contributors to the separation after applying PCA and PLS-DA models, explaining 59.3% and 40.7% of the variance, respectively, when the scores of the two principal components were computed. Finally, ROC curves using both lyso-PC 15:0 and lyso-PE 18:2 achieved optimal predictive power with an accuracy of 70.4% and an AUC of 0.777 (95% CI 0.65-0.932). Therefore, these predictive models highlighted lyso-PC 15:0 and lyso-PE 18:2 as suitable susceptibility/risk biomarkers for hypercholesterolemia in preliminary stages.

Associations of lysophospholipid biomarkers with classical lipid-related markers and dietary components

Spearman correlations showed a positive relationship between classical lipid-related markers, such as total cholesterol, LDL cholesterol and the atherogenic ratio LDL cholesterol/HDL cholesterol, with lyso-PC 15:0 levels in MH-LDL-c group ($r > 0.46$, $P < 0.01$). In contrast, in L-LDL-c group, an inverse correlation was observed between total cholesterol and LDL cholesterol with lyso-PE 18:2 ($r > -0.30$, $P < 0.05$), suggesting protective capabilities towards hypercholesterolemia.

Interesting associations were established between discriminant lyso-PLs and dietary components, particularly at FAs level. In L-LDL-c, a positive association was observed between omega-(n) PUFAs, such as linoleic acid (LA) (C18:2n-6), and lyso-PE 18:2 ($\beta = 0.39$, $P < 0.05$). In MH-LDL-c, different trends in relationships were found depending on the fatty acyl chain of lyso forms. While SFAs were positively associated with saturated lyso-PC 17:0 and 15:0 ($\beta > 0.47$, $P < 0.05$), inverse correlations were established between SFAs-

rich foods, including yogurt and whole milk, and unsaturated lyso-PC 20:4 and 20:5 ($\beta > -0.43$, $P < 0.05$).

Associations of lysophospholipid biomarkers with liver transaminases

MH-LDL-c group showed significantly higher concentrations of aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyl transferase (GGT) than L-LDL-c group ($P \leq 0.01$). Notably, a strong positive association was found between lyso-PC 14:0 and GGT levels in L-LDL-c group ($\beta = 0.57$, $P = 0.003$). However, in the MH-LDL-c group, a positive association was noted between lyso-PE 18:2 and GGT ($\beta = 0.49$, $P = 0.010$). Moreover, in MH-LDL-c, PUFAs intake was strongly related with GGT ($\beta = 0.63$, $P < 0.001$).

Validation of the suitability of lysophospholipid as biomarkers involved in hypercholesterolemia progression in animal models

After 30 days of chronic treatment with HFD or LFD, hamsters fed the HFD exhibited significantly higher levels of total cholesterol than LFD-fed animals. Also, an increasing trend of 30% LDL cholesterol levels was noted in the HFD-fed compared to the LFD-fed hamsters ($P=0.008$).

Remarkably, lyso-PC and lyso-PE levels were markedly modified in both liver and plasma after chronic HFD intake. A generalized drop (nearly 50%) in the circulating levels of lyso-PLs was noted in HFD-fed animals, which was accompanied by an intrahepatic accumulation (higher concentrations in liver tissue) of polyunsaturated lyso forms, particularly lyso-PEs 18:2 ($p<0.001$), 22:5 and 22:6 ($p<0.05$). Conversely, saturated acyl chain lyso-PC and lyso-PE were significantly reduced in both plasma and liver ($P<0.05$).

On the other hand, the histological analysis of the liver samples showed an increase in the steatotic profile in the liver of hamsters fed the HFD.

Overall, these results confirm the suitability of particular lyso-PL species, particularly lyso-PC 15:0 and lyso-PE 18:2, as susceptibility/risk biomarkers of hypercholesterolemia progression in MH-LDL-c compared to L-LDL-c subjects. Moreover, the strong relationships stated with dietary compounds, primarily with FAs intake, add insights into the dietary origin of lyso-PLs and their role in diet-induced hypercholesterolemia. The secondary *in vivo* study in hamsters strengthen the results in human and provide a possible mechanism by means of which the dietary intake of PUFAs, such as LA (C18:2n-6), could promote the intrahepatic synthesis of lyso-PE 18:2 being this an adaptive mechanism against diet-induced hepatic steatosis in animal, and to counteract hypercholesterolemia progression in human.

PART II.II Systematic review and meta-analysis

In this last part, the methods and results of a systematic review and meta-analysis of human randomized clinical trials, including RCTs and non-controlled randomized trials (RTs), are presented. Taking into account the results derived from ***Article 3***, we evidenced a paucity of human studies with conclusive results in relation to bioactive lipids dietary origin, and on how FAs intake could impact the lipidomic profile. In ***Article 3***, we observed strong relationships between dietary intake of SFAs and PUFAs from animal-based foods with particular lyso-PC and lyso-PE species. From these relationships, we speculated about the dietary origin of lyso-PLs and their role in diet-induced hypercholesterolemia. However, in translational research, reliable dietary assessments and interventional studies are essential beyond observational studies when attempting to understand the complex links between diet and health through novel biomarkers. Therefore, we proposed to expand our knowledge on this research topic by conducting a systematic review and meta-analysis compiling the evidence of human randomized clinical trials in last ten years.

METHODS

Main aim

- To evaluate the effect of SFAs, MUFAs, PUFAs and TFAs, provided as supplements, enriched food-components or diets, on the circulating bioactive lipid profile of healthy subjects, with CVD or CVD risk factors.

Study selection and eligibility criteria

A comprehensive study search was conducted in *PubMed*, *SCOPUS*, and *Cochrane Library* databases. It was filtered for English-language literature, published in last 10 years in peer-reviewed journals, and indexed up until October 2020. Search terms included the specific bioactive lipid classes, different FA categories, and CVD or CVD risk factors. All titles and abstracts found by the search strategy were screened for relevance, and then retrieved in full and evaluated for inclusion eligibility.

The Population, Intervention, Comparison, Outcomes and Study design (PICOS) criteria were used to define the inclusion and exclusion criteria. A brief summary of the main inclusion criteria is shown below:

Inclusion criteria:

Population

- RCTs and RTs involving adult humans (age 18 years or older)
- All sexes and races
- Healthy subjects or subjects with associated CV or cardiometabolic risk factors (type 2 diabetes, obesity, overweight, dyslipidemia, metabolic syndrome, hypertension), or with established CVD (stable CAD)

Intervention

- RCTs and RTs involving FA-based dietary interventions (SFAs, PUFAs, MUFAs and TFAs)
- FAs administered either as supplements, enriched food-components (EF-C), or enriched diets (ED)
- Sustained, postprandial or short-term interventions

Comparison

- Using placebo or control arms with dietary components, meals or diets as comparator

Outcomes

- Showing significant differences in main outcome (bioactive lipid levels) in terms of lipid class, lipid subclass, or enzymatic precursor, after exposure to dietary FAs

Study design

- Both RCTs and RTs
- Parallel or crossover design

Studies involving pregnant women and less common CV or autosomal disorders, such as non-alcoholic fatty liver disease, diabetic nephropathy, obstructive sleep apnea syndrome, or Fabry's disease, among other, were excluded. In addition, studies not involving FA-based dietary interventions, or assessing only the effects of pharmacological treatment or medicinal plant, were also excluded.

Summary measures

The data extracted from the included RCTs and RTs were summarized in two pre-specified tables for each CVD or CVD risk factor, and the healthy state. These tables collected key information such as the study design, characteristics of the population studied, details of the FA-based interventions and dosage, comparator used, duration of exposure, number of participants, and bioactive lipid classes and subclasses, among others.

Statistical analyses

The analysis was structured in two parts: a qualitative synthesis and the meta-analysis. In the qualitative synthesis, the net difference in the bioactive lipid mean values from the endpoint to baseline –effect before vs after

intervention–, and the corresponding standard deviations (SDs) or standard errors (SEs), were evaluated for each included study. In the meta-analysis, only RCTs assessing the effects of PUFA-supplemented dietary interventions (including n-3 and n-6 PUFAs) on circulating bioactive lipids or enzymatic precursors were included.

Moreover, all randomized clinical trials were assessed for study quality and risk of bias by using the *RevMan* software (v 5.4).

Article 4

The effects of fatty acid-based dietary interventions on circulating bioactive lipid levels as intermediate biomarkers of health, cardiovascular disease and cardiovascular disease risk factors: A systematic review and meta-analysis of randomized clinical trials

Lorena Calderón-Pérez, Judit Companys, Rosa Solà, Anna Pedret, and Rosa M Valls.

Manuscript submitted to Critical Reviews in Food Science and Nutrition

The effects of fatty acid-based dietary interventions on circulating bioactive lipid levels as intermediate biomarkers of health, cardiovascular disease and cardiovascular disease risk factors: A systematic review and meta-analysis of randomized clinical trials

Lorena Calderón-Pérez[1], Judit Companys[1], Rosa Solà[2,3,*], Anna Pedret[2,*], Rosa M.Valls[2]

[1] Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain.

[2] Universitat Rovira i Virgili, Facultat de Medicina i Ciències de la Salut, Functional Nutrition, Oxidation, and Cardiovascular Diseases Group (NFOC-Salut), Reus, Spain.

[3] Hospital Universitari Sant Joan de Reus, Reus, Spain.

[*]Correspondence:

Anna Pedret
Facultat de Medicina, Universitat Rovira Virgili.
C/ Sant Llorenç, 21, 43201, Reus, Spain.
Tel: (+34) 977 75 93 75
E-mail: anna.pedret@urv.cat

Rosa Solà
Facultat de Medicina, Universitat Rovira Virgili.
C/ Sant Llorenç, 21, 43201, Reus, Spain.
Tel: (+34) 977 75 93 69
E-mail: rosa.sola@urv.cat

ABSTRACT

Dietary fatty acids (FAs), primarily n-3 polyunsaturated fatty acids (PUFAs), have been associated with a FA-enrichment effect of circulating bioactive lipidome and with the enzymatic precursor lipoprotein-associated phospholipase A2 (Lp-PLA2) mass, however, the magnitude of this effect remains unclear. The effects of dietary FAs on the bioactive lipids profile of healthy subjects, with cardiovascular disease (CVD) and with CVD risk factors were evaluated in 21 randomized controlled trials (RCTs) and 6 non-controlled randomized trials (RTs) published in last ten years, and ten RCTs were selected for meta-analysis. In the meta-analysis, marine n-3 supplements (0.37-1.9 g/day) significantly increased pro-inflammatory lysophosphatidylcholines (lyso-PCs), mean (95%CI) +0.52(0.02,1.01) µM of lyso-PC(16:0) and +0.58(0.09,1.08) µM of lyso-PC(18:0), in obese subjects. Additionally, in meta-analysis, n-3 supplements (1-5.56 g/day) decreased plasma Lp-PLA2 mass, a well-known inflammatory marker, in healthy (-0.35(-0.59,-0.10) ng/mL), dyslipidemic (-0.36(-0.47,-0.25) ng/mL) and stable coronary artery disease subjects (-0.52 (-0.91,-0.12 ng/mL). In conclusion, daily n-3 PUFAs provided as EPA+DHA supplements, consumed from 1 to 6 months, exhibit positive effects reducing plasma Lp-PLA2 mass in healthy subjects, with CVD and CVD risk factors, suggesting an anti-inflammatory effect. However, an impaired saturated lyso-PCs response to n-3 is manifested in obese subjects.

Keywords: glycerophospholipids; lysoglycerophospholipids; sphingolipids; lipoprotein-associated phospholipase A2; polyunsaturated fatty acids; cardiovascular disease

Total word count: 9577

1. INTRODUCTION

Cardiovascular diseases (CVDs) involve a group of clinical disorders of the heart and blood vessels, being ischemic heart disease (IHD) and stroke the most common cardiovascular events and the leading cause of mortality and disability worldwide [1]. CVDs can be prevented if potential CVD risk factors, such as hypertension, elevated LDL-cholesterol, and a cluster of interrelated metabolic factors, are early detected and addressed [2]. In recent years, advances in lipidomic methodologies have opened a new frontier in the identification of specific lipid-based molecules able to mediate physiological and metabolic functions in the host, and with prominent roles as biomarkers in CVDs development [3,4]. Among various lipid molecules, glycerophospholipids (PLs), such as phosphatidylcholines (PCs), phosphatidylethanolamines (PEs) and phosphatidylinositols (PIs); lysoglycerophospholipids (lyso-PLs), including those containing a choline group, namely lysophosphatidylcholines (lyso-PCs), and lysophosphatidylethanolamines (lyso-PEs); and sphingolipids (SPs), including ceramides (Cer), dihydroceramides (diCer), glucosylceramides (gluCer), lactosylceramides (lacCer) or sphingomyelin (SM) classes; represent a large and structurally diverse group of circulating bioactive lipids generated by hydrolysis from membrane lipids [5]. Also, the plasma lipoprotein-associated phospholipase A2 (Lp-PLA2) enzyme plays an intermediate role in the hydrolysis of oxidized PLs in LDL-cholesterol particles, leading to the generation of plasma lyso-PLs and oxidized free fatty acids (FAs) [6].

Bioactive lipids can regulate key cell biological functions and signaling pathways, including growth regulation, cell differentiation, angiogenesis, autophagy, cell migration, and inflammatory response [7,8]. In this way, lyso-PCs, the most abundant lyso-PLs in human blood, have been widely regarded as pro-inflammatory mediators in atherosclerosis [9]. However, as recently reviewed, additional anti-inflammatory effects of lyso-PCs are evidenced in the vascular system, and these effects may be highly dependent on the lyso-PC acyl chains length and the degree of saturation [11,12]. Moreover, particular lyso-PLs have been postulated as non-invasive biomarkers of dyslipidemia in animal models [13], but the evidence in human is scarce. Other circulating bioactive lipids, such as Cer, have been linked with a wide range of pleiotropic actions in metabolism, and are measured clinically as prognostic indicators of major adverse cardiovascular events [14]. Also, in a 2-weeks prospective study, elevated Lp-PLA2 serum concentrations, has been proposed as a possible risk biomarker in IHD, especially in hospitalized patients with LDL-cholesterol lower than 130 mg/dL [15].

Overall, bioactive lipids and their mediators reflect different aspects of the development of CVDs and could provide useful tool for successful predictions. The simplicity of their chemical structures, mainly composed of a hydrophobic tail of FAs residue and a hydrophilic head group, could largely determine their biological functions. However, little is known about the effect of FAs from diet on bioactive lipids profile in healthy subjects, with CVD and CVD risk factors.

FAs conform a wide group of lipid molecules derived from the dietary fats breakdown and are divided into four categories: saturated (SFAs), mono-unsaturated (MUFAs), polyunsaturated (PUFAs), and trans (TFAs). Notably, essential FAs, including the very long-chain omega-3 (n-3) PUFAs eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, and omega-6 (n-6) PUFAs, have aroused great interest based on their beneficial properties. It has been reported that consumption of n-3 PUFAs ameliorates several CVD risk factors [17]. The primary sources of n-3 PUFAs in the diet of humans are marine fish, especially oily fish, and, if consumed, fish oil supplements. However, vegetable oils, such as soybean, nuts and canola oils, provide alternative plant forms of n-3 known as alpha-linolenic acid (ALA).

The n-3 PUFA levels in plasma PLs have been used as potential biomarkers of disease [19,20], showing dose-dependent responses to fish oil supplementation. Recently, a limited number of human intervention studies have reported a selective enrichment of long-chain n-3 PUFAs in plasma PLs and lyso-PLs following intake of both n-3-rich oily fish [21] and supplements [22]. This effect could be explained by an alteration of the fatty acyl moiety of bioactive lipids; however, the extent to which the enrichment effect of dietary n-3 PUFAs, or other common dietary FAs, modifies the plasma bioactive lipidome in healthy subjects, with cardiovascular disease (CVD) and CVD risk factors has not been widely evaluated.

In that context, the main goal of the present systematic review and meta-analysis of human randomized clinical trials was to evaluate the effect of SFAs, MUFAs, PUFAs and TFAs, provided as supplements, enriched food-components or diets, on the bioactive lipids profile of healthy subjects, with CVD and with CVD risk factors. The understanding of the pivotal role of dietary FAs on the human lipidome will allow the development of appropriate nutritional therapies for the management and prevention of CVDs or cardiometabolic risk factors.

2. METHODS

This systematic review and meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [23]. The protocol was prospectively registered at the PROSPERO database: registration number CRD42021218335.

2.1. Search strategy and eligibility criteria

We searched in PubMed (https://pubmed.ncbi.nlm.nih.gov/), SCOPUS (www.scopus.com), and Cochrane Library (https://www.cochranelibrary.com/) databases for English-language literature, published in last 10 years in peer-reviewed journals, and indexed up until October 2020. Search terms included the specific bioactive lipid classes, different FA categories, and CVD or cardiometabolic disease or their risk factors. We also reviewed

additional publications found through the references of the retrieved articles. The search strategy is detailed in ***Supplemental File 1***.

We included clinical trials involving adult humans (age 18 years or older), that reported effects of FA-based interventions, as either supplements or enriched food-components (EF-C) and enriched diets (ED), on circulating bioactive lipid levels. The selected studies included subjects who were either healthy, with ideal levels of blood glucose, triglycerides (TG), HDL cholesterol, blood pressure, waist circumference, and without using medications; or had any cardiovascular or cardiometabolic risk factor, including type 2 diabetes (T2D), obesity, overweight, dyslipidemia, metabolic syndrome (MetS) and hypertension; or with established CVD, primarily coronary artery disease (CAD). Excluded studies involved pregnant women and less common cardiovascular or autosomal disorders, such as non-alcoholic fatty liver disease, diabetic nephropathy, obstructive sleep apnea syndrome, or Fabry's disease, among other. Also, we excluded studies assessing the effects of medicinal plant or pharmacological therapy, and studies that did not specify bioactive lipid classes. The Population, Intervention, Comparison, Outcomes and Study design (PICOS) criteria were used to define the inclusion and exclusion criteria as listed in ***Table 1***.

2.2. Study selection and data extraction

All titles and abstracts found by the search strategy were screened for relevance, and then retrieved in full and evaluated for inclusion eligibility by LC-P and JC. Eligible results were compared and discussed between AP and RMV. The screening process was conducted through the *Covidence* web-based software (www.covidence.org), which allowed the asynchronous collaboration of every member. Data were extracted into pre-specified structured tables. Missing data was detailed as "not reported".

2.2.1. Summary measures

The data extracted from the selected trials were summarized in two pre-specified tables for each CVD risk factor, and the healthy state. In the first pre-specified table, we detailed the first author, the year of publication, the country, the study design, the population (including age range, sex, and main baseline features), the details of the FAs-based intervention and dosage, the comparator used, the duration of exposure, the number of participants in the study, and the intention-to-treat analysis. In the second pre-specified table, we reported the bioactive lipids or enzymatic precursor data. As to bioactive lipids, the reported data included the assessment method used for the lipidomic characterization [24], the identification reference (software or internal procedure used), the bioactive lipid class, in detail, the lipid subclass and molecular formula [25], and significant and non-significant changes in the particular bioactive lipid concentrations.

On the other hand, the enzymatic precursor data comprised the assessment method used for the measurement of enzymatic mass or activity, the enzyme class and subclass, and significant and non-significant changes in the enzymatic precursor.
We also reported the direction of change for each bioactive lipid and enzymatic precursor as "increased" or "decreased".

2.3. Statistical analyses

The systematic review and meta-analysis were performed using the Review Manager (RevMan) Cochrane Collaboration's software (v 5.4). For the qualitative synthesis, we evaluated the net difference in the bioactive lipids mean values from the endpoint to baseline –effect before vs after intervention–, and the corresponding standard deviations (SDs) or standard errors (SEs). When necessary, we estimated the SDs or SEs of the net change from reported variance data. If the SD or SE values were missing in the original article, we contacted the corresponding author for additional data (n = 4). The studies were clustered according to the CVD, CVD risk factor or the healthy state according to the population receiving the intervention.

For the meta-analysis, we selected randomized controlled trials (RCTs) assessing the effects of PUFA-supplemented dietary interventions (including n-3 and n-6) on bioactive lipids or Lp-PLA2 enzymatic precursor. This restriction of studies was done given the greater effects of the PUFA-supplemented interventions than EF-C or ED, as well as the inter-study similarity in terms of the administration form, the dose, and the expression of results. A set of meta-analyses was performed by using a fixed-effects model to assign a weight to each study included (n = 10) according to the within-study variability and the between-study heterogeneity. In parallel, a sensitivity analysis was performed to explore the degree of heterogeneity between studies, expressed by the heterogeneity statistic (I^2). An I^2 among 0% and 40% represented low heterogeneity [26], and therefore poor variability in the results.

Overall, the results of the meta-analyses are expressed as standard mean differences (SMDs) that are defined as the differences between the end and baseline values, and 95% confidence interval (CI). Exceptionally, in the meta-analysis for the effects of n-3 PUFAs on Lp-PLA2 mass in dyslipidemia, the effect was estimated from the net difference of the outcome compared to placebo.

Bioactive lipid levels are reported using the different metrics that can be used: molar concentration, relative abundance, relative amount, or molar percentage (%) of LDL or HDL surface lipids. The studies reporting only the peak intensities of lipid subclasses were excluded from the analysis (n = 2).

Given the limited number of studies included in the individual meta-analyses and their consistence in the supplementation method, dose, duration, and the way the effect was measured, we didn't consider performing additional meta-regression.

2.4. Quality assessment

All randomized trials were assessed for study quality and risk of bias. The quality assessment was conducted using the RevMan software (v 5.4). The following quality items were assessed: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other biases, such as publication bias. Each quality item was rated as Low risk/High risk/Unclear risk according to the Cochrane Collaboration's tool for assessing risk of bias in randomized trials [27]. After considering each quality item, an overall rating was assigned to each included study being "Low risk" the least bias and valid results; "Moderate risk", susceptibility to some bias but not enough to invalidate the results; and "High risk", significant bias that may invalidate the results. The risk of bias judgement was done by the first author (LC-P), and any inconsistency was resolved by a second author (JC). The overall quality classification, along with the target bioactive lipids assessed, are summarized in ***Table 2***.

3. RESULTS

3.1. Study selection

The literature search through PubMed, SCOPUS, and Cochrane databases yielded 362 articles to retrieve and review. One additional article was manually added. After duplicates removal (n = 72), we retrieved and reviewed 291 articles of which 229 were excluded for not meeting the eligibly criteria. Finally, 62 full-text studies were assessed for eligibility of which 27 were included in the overall systematic review, with 10 studies selected for meta-analysis, as detailed in ***Figure 1***.

3.2. Study characteristics

Of the 27 included studies, 21 were RCTs of which 19 involved sustained interventions with parallel design [22,28–45], except for one RCT that used a crossover design [46]. The one remaining RCT conducted a postprandial and crossover intervention [47]. The other 6 included studies were non-controlled RTs of which 2 were sustained interventions with a parallel [48] and crossover [21] design, 3 were postprandial test meals using parallel [49] and crossover [50,51] design, and 1 was a 4-day short-term study with a crossover design [52] (***Tables 3-9***). These studies represented about 2560 subjects (men and women) aged between 18 and 75 years. In one RCT, participants were restricted to postmenopausal women [38]. Over 78% of the enrolled

subjects presented at least one associated CVD or cardiometabolic disease risk factor, including obesity and overweight, dyslipidemia, MetS, T2D and hypertension, or established CVD, primarily stable CAD, whereas less than 22% were healthy (***Table 2***). The intervention period ranged from 1 to 6 months in sustained studies, and from 4- to 6-hour in postprandial test meals. In the crossover studies the washout periods varied from 4 days to 8 weeks after the first intervention. The populations studied were originated from upper to middle-income countries, including studies from Northern America (n = 11), Northern Europe (n = 6), Southern Europe (n = 4), Eastern Europe (n = 1), Eastern Asia (n = 2), Western Asia (n = 2), and Oceania (n = 1).

3.2.1. Characteristics of the studied bioactive lipid classes

All the studies reported the effects of FA-based dietary interventions on circulating bioactive lipid levels, among other biochemical compounds, after lipidomic profiling. PL classes, including PCs, PEs and PIs, were assessed by 11 studies, lyso-PL classes, including lyso-PCs, lyso-PEs and lyso-PIs, by 14 studies, and SP classes, including Cer and SM, by 11 studies (***Table 2***). The PLs, lyso-PLs and SPs molecular structures were diverse according to the configuration and the degree of saturation of their acyl chain (***Table 2***). Among studied PL subclasses, those containing very long chain PUFAs (VLC-PUFAs) in the sn-2 position of the glycerol backbone were predominant. In the lyso-PLs, the subclasses containing long chain PUFAs (LC-PUFAs) followed by MUFAs and SFAs in their acyl chains were most abundant. However, among SPs, predominated the subclasses containing SFAs and MUFAs (***Table 2***). In most of the cases, the circulating bioactive lipids were extracted with added internal standards of common lipid subclasses, and quantified using both targeted and untargeted mass spectrometry (MS) approaches, primarily liquid-phase separations coupled to MS (typically LC-MS). Then, they were characterized with data processing software, such as LipidView (v. 1.1), MATLAB (v. 7.2) or MZmine (v. 2.7). In addition, lipid databases, such as LipidBlast, Lipid MAPS, the Human Metabolome Database, METLIN or MassBank, were used for lipid identification.

Other intermediates, such as acylcarnitine (AC) and the enzyme Lp-PLA2, were assessed by immunoassay methods in 1 and 10 studies, respectively.

3.2.2. Characteristics of the studied fatty acid-based dietary interventions

The dietary interventions were variable between the studies, and FAs were administered in three different forms: as supplements (n = 12), EF-C (n = 9), or ED (n = 6) (***Table 2***).

The supplemented interventions mainly consisted of marine fish oil capsules providing n-3 PUFAs in the form of EPA+DHA with doses ranging from 0.6 to 5.56 g/day in the intervention groups (n = 9). In the control groups, corn oil, soybean oil and olive oil were used as placebo. In 2 studies [37,40] the n-3 EPA+DHA capsules were complexed with olive oil. In addition, in 2

studies the n-3 PUFAs supplement was combined with usual statin [40] and aspirin [41] therapy. One study assessed n-3 PUFAs from flaxseed oil, providing ALA at doses of 0.57 g/day [37]. Alternatively, two studies administered high fat enteral supplements in form of medium-chain fatty acids (MCFAs) [29] or long chain triglyceride (LCT) fat emulsion [49].

The studies including EF-C consisted of dietary fish (n = 2) or enriched plant oils (n = 3), spreads (n = 1), dairy products (n = 1) and cereal-based matrices (n = 2). Two studies assessed n-3 PUFAs-rich oily fish, such as salmon, provided in diets ranging from 160 to 400 g per week (1.0 to 1.6 g/day of EPA+DHA) [21,39]. One cross-over study compared an n-3 PUFAs enriched milk, providing 0.37 g/day EPA+DHA, with a phytosterol enriched milk [46]. In 6 studies n-6 PUFAs from four different plant oils, including soybean oil [36,38,51], sunflower oil [48], rapeseed oil [33] and canola oil [34], were provided as enriched oils or added into food matrices differing in their FAs content, such as soy-based dairy, soy- and sunflower-based cereals, and rapeseed oil-based spread (***Table 2***).

The studies using ED included diets with controlled FAs content (n = 6). In 5 studies the dietary interventions were based on the Mediterranean diet, which is rich in fruits and vegetables (sources of fiber and polyphenol), MUFAs and PUFAs [28,31,44,45,52]. Particularly, in 2 studies [28,44], the RESMENA (Reduction of Metabolic Syndrome in Navarra) diet, a new dietary strategy for reducing the MetS characterized by an increased meal frequency, high total antioxidant capacity and reinforced n-3 PUFAs intake [53], was used. Overall, the daily doses of PUFAs ranged from 0.4 to 6.8 % of total energy intake (TEI) (***Table 2***). The control diets usually provided lower % TEI as PUFAs. The remaining study, instead of the Mediterranean diet, involved two postprandial test meals differing in their SFAs content, including a high-saturated fat (24 g SFAs) vs. a high-carbohydrate meal (0 g SFAs).

3.3. Studies quality and risk of bias

The risk-of-bias assessment of the 27 included studies is shown in ***Supplemental Figure 1***. All included studies were randomized although 14 studies did not reported the methods used to generate the allocation sequence in sufficient detail to allow an assessment of whether it should produce comparable groups. In 15 studies the methods used to conceal the allocation sequence were not properly described, and 3 studies did not ensure the adequate concealment of allocations before assignment, supporting a selection bias. Blinding of both participants and personnel was performed correctly in 17 studies, however, 3 studies were susceptible and 7 studies were at high risk of performance bias due to the open-label or single-blind design. Only 13 studies correctly blinded the outcome assessment. Incomplete outcome data was adequately addressed in 18 studies, considering overall losses to follow-up, drop-outs, and whether an “intention-to-treat” analysis was performed. No systemic differences were found in any of the included studies

between planned and reported findings. However, we detected 10 studies that would be susceptible to measurement bias due to poor reliability of the diet monitoring during intervention. Overall, 12 studies were judged as "low risk", 2 studies as "high risk", and 13 studies as "unclear risk".

3.4. Results of individual studies and meta-analyses

3.4.1. Effects of dietary fatty acid-based interventions on bioactive lipids profile in subjects with CVD and CVD risk factors

We systematically reviewed a total of 18 RCTs and 1 RT that contained data on dietary FAs intake, including SFAs, MUFAs, PUFAs and TFAs, and circulating bioactive lipids, including PL, Lyso-PL or SP classes and subclasses, and Lp-PLA2 enzymatic precursor, in subjects with CVD or CVD risk factors. The complete information for each individual study is summarized in ***Tables 3-8***. Additionally, a set of fixed-effects model meta-analyses were performed in 7 RCTs, which assessed the effects of PUFA-supplemented dietary interventions, to investigate at what point the published RCT data were sufficient to yield the same results as were found in the qualitative synthesis (***Figures 2-4***).

3.4.1.1. Overweight and obesity

Five studies, involving 248 subjects, reported the effect of FA-based dietary interventions on bioactive lipids profile in overweight and obesity [22,28,38,46,48] (***Table 3***). The baseline body mass index (BMI) of included individuals ranged from 25 to 40 kg/m^2. In 2 studies [38,48], the effects of TFAs, SFAs and n-6 PUFAs from plant oils (partially hydrogenated soybean oil, palm oil and sunflower oil provided as EF-C into cereal-based matrices) on PCs, lyso-PCs, SM and Cer concentrations were reported after an overfeeding period. In detail, TFAs administered at daily doses of 15.7 g (26 g soybean oil) revealed significant increases in serum PC containing VLC-PUFAs, mainly PC(40:7), and SM(36:3) [38], whereas the administration of SFAs, mainly palmitate, at daily doses of 21.4 g (40 g palm oil) showed significant increases in saturated- and monounsaturated-chain Cer and SM subclasses, including diCer(16:0), gluCer(16:0), and SM(16:0, 18:0, 16:1, 18:1) [48]. In contrast, n-6 PUFA-rich oils at daily doses of 26.1g (40 g sunflower oil) mediated a marked decrease in several saturated-chain serum Cer subclasses, including Cer, diCer, gluCer and lacCer(18:0, 20:0 and 24:1) [48]. Both studies reported weight gain around 4 % after the overfeeding period, and it was preceded by a dietary weight loss program.

In one study [28], an overall decrease in serum concentrations of lyso-PC, lyso-PE and lyso-PI subclasses, significantly lyso-PCs(14:0, 15:0, 16:1, 18:4, 20:4) (-0.10 to -5.5 μM, $P<0.05$) and lyso-PE(22:6) (-0.24 μM, $P=0.05$), was reported after an ED intervention using the RESMENA

diet. In addition, positive relationships were established between decreased lyso-PCs and weight loss.

In two studies [22,46], the effects of n-3 PUFAs supplemented dietary interventions were analyzed. The n-3 PUFAs were administered in marine fish oil capsules or added into a milk matrix. The fixed-effects model meta-analysis found a significant increase in saturated lyso-PCs(16:0 and 18:0) [SMD (95% CI); 0.52 (0.02, 1.01) and 0.58 (0.09, 1.08) µM, respectively] with n-3 PUFAs supplemented at daily doses ranging from 0.37 to 1.9 g/day EPA+DHA (***Figure 2***). The heterogeneity between both studies was low (I^2=0%, P<0.05). Given the lack of similarity between the lipid subclasses studied in overweight and obesity, it was not possible to include the rest of the subclasses in the meta-analysis, therefore, they were systematically reviewed (***Table 3***).

3.4.1.2. Dyslipidemia

Three RCTs, involving 248 subjects and 6 experimental arms, evaluated the effect of n-3 PUFA EF-C, provided into a milk matrix, a plant oil-based spread or as oily fish, on the molar concentrations or percentage (%) of EPA and DHA-containing lyso-PLs of the serum LDL surface or plasma lyso-PLs, in dyslipidemia [33,39,46] (***Table 4***). Dyslipidemia was characterized by moderate to high serum LDL-cholesterol (among 135 and 196 mg/dL) or TG (among 150 and 2000 mg/dL), and scarce levels of HDL cholesterol (among 33 and 65 mg/dL) at baseline. None of the subjects were under lipid-lowering therapy. The results showed a significant increase in the LDL molar concentrations of saturated-chain lyso-PC(18:0) (+11 µg/100 mL, P=0.04) after 0.375 g/day EPA+DHA from a enriched milk [46]. In addition, the molar % of LC- and VLC-PUFAs-containing PC and lyso-PC subclasses, including lyso-PCs(18:2, 20:3, 20:4 and 20:5), were significantly increased (+0.01 to +0.17 %, P<0.05 or less) after the intake of 20 g/day of a plant stanol ester-enriched rapeseed oil spread (STAEST), providing 3.3 g n-6 LA and 1.3 g n-3 ALA [33]. In addition, LC- and VLC-PUFAs-containing lyso-PCs were negatively associated with LDL aggregation susceptibility, especially in lean individuals [33]. Remarkably, the plasma % of VLC lyso-PCs(20:5 and 22:6), containing n-3 EPA and DHA, was significantly increased after oily fish interventions, including salmon, herring and pompano, at doses of 80 g 5 days a week (1 to 1.6 g/day EPA+DHA) (+0.9 to +1.5%, P≤0.05 or less) [39]. However, the plasma % of lyso-PCs(18:2 and 20:4), both containing n-6, was significantly decreased after oily fish interventions (-1.2 and -2.9 %; P≤0.05, respectively). In two of the studies [39,46], a variation in lipid profile, mainly a decrease in TG levels (from -16 to -52.2 mg/dL; P≤0.05), was evidenced after dietary interventions, especially in the oily fish experimental groups. Given the lack of similarity between interventions and the studied lipid subclasses, it was not possible to perform a meta-analysis for dyslipidemia and circulating bioactive lipids, thus they were systematically reviewed (***Table 4***).

On the other hand, in 4 RCTs involving 1015 dyslipidemic subjects and 7 experimental arms, the effects of n-3 PUFA supplements on Lp-PLA2 mass were analyzed [40–43] (***Table 4***). The n-3 PUFAs were provided in capsules as highly bioavailable forms of carboxylic acids (CA), acylglycerols (AG) or ethyl esters (EE). Across the studies, the fixed-effects model meta-analysis revealed a significant decrease in Lp-PLA2 plasma mass [SMD (95% CI); -0.36 (-0.47, -0.25) ng/mL] with daily doses ranging from 1 to 5.56 g of EPA+DHA (***Figure 3***). The heterogeneity between the 4 studies was low (I^2=0.2%, P<0.00001), therefore, the duration of the intervention (between 1 and 3 months) was not expected to influence the treatment effects. Additionally, a significant increase in LDL and a decrease in HDL particle sizes was reported after n-3 PUFA interventions vs placebo [40]. Also, a significant reduction (by -10.9 %; P=0.01) in ox-LDL [41], and in non-fasting plasma TG levels (among -21.5 and -28 %; P<0.05 or less) was evidenced in the n-3 PUFA treatments versus placebo [42,43].

3.4.1.3. Metabolic syndrome

Four studies, involving 237 subjects, reported the effect of n-3 PUFA ED [28,44,45] or EF-C [51] on circulating bioactive lipids profile in MetS (***Table 5***). The MetS features were visceral obesity, dyslipidemia, hyperglycaemia and hypertension according to the International Diabetes Federation criteria [54]. In 3 RCTs [28,44,45], the effects of n-3 PUFA-rich Mediterranean-based dietary interventions on PC, PE, lyso-PC and lyso-PE subclasses were tested. In detail, a short-term significant increase in the plasma levels of LC- and VLC-PUFAs-containing bioactive lipids, including PC(38:4, 40:4, 40:6) (P≤0.001), PE(38:6, 40:4) (P<0.0005), and lyso-PC(20:5) (P<0.0005) subclasses, was evidenced after 2-month of n-3 PUFA ED providing from 4.2 to 6.2 % TEI as n-3. Particularly, PC(38:4) was revealed as discriminative marker between the RESMENA diet and the control diet at 2 months, and was positively associated to reduction of inflammatory markers [44]. In the long term, the plasma levels of most of the saturated and unsaturated-chain PC, PE, lyso-PC and lyso-PE subclasses were drastically reduced after 6-month intervention, except for some subclasses such as lyso-PCs(16:0, 18:1, 20:5 and 22:6), the levels of which significantly increased (P≤0.020). In 2 studies notable changes in the MetS features were reported at the end of n-3 PUFA ED [28,45]. Particularly, it was exhibited a significant reduction in fasting plasma TG levels (-15 to -38 mg/dL; P<0.05 or less).

On the other hand, one acute RT reported opposite changes in PC, lyso-PC and SM subclasses, especially on those containing saturated chains, after a 4-hour postprandial intervention based on high-fat breakfast meals providing dairy- and soy oil-based foods differing in their FAs content [51] (***Table 5***). In detail, a significant decrease was showed in PC(28:0, 29:0, 30:0, 31:0, 32:0), lyso-PC(14:0) and SM(32:0, 34:0) levels after 4-hour soy oil-based meal (-7.1 to -15 % median change, P<0.01), while a significant increase was noted after 4-hour dairy-based meal (+4.6 to

+43 % median change, P<0.01). In addition, mono and polyunsaturated-chain PC, PE, PI and lyso-PE subclasses were significantly increased after the high saturated fat dairy-based meal (P<0.001). Given the lack of similarity between the lipid subclasses studied in MetS, it was not possible to perform meta-analysis, thus they were systematically reviewed (***Table 5***).

3.4.1.4. Type 2 diabetes

Two RCTs, involving 86 subjects, reported the effect of MCFAs [29] and alpha lipoic acid [30] supplements on circulating bioactive lipids profile and plasma Lp-PLA2 mass in T2D (***Table 6***). In both studies the participants were non-insulin-dependent and met the diagnostic criteria for T2D according to the American Diabetes Association [55]. The baseline fasting blood glucose (FBG) levels ranged from 121 to 165 mg/dL, and the HbA1c was among 6.2 and 8.3%. In the first RCT [29], a significant decrease was reported in monounsaturated and saturated-chain SM, Cer and AC subclasses (-0.01 to -0.3 μM, P<0.01 or less) after the administration of a MCFAs-rich diet (providing 33-34 % SFAs and only 1-2 % PUFAs) compared to a LCFAs-rich diet. No lipid subclasses were different after the LCFAs diet. Moreover, the decreases in several SM subclasses, including SM(14:0, 15:0, 16:0, 20:0, 21:0, 23:1), were positively correlated with lower fasting plasma insulin and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) (P<0.05 or less).

In the second RCT [30], a significant decrease in plasma Lp-PLA2 mass was showed (-16.52 ng/mL, P=0.001) after a daily dose of 1200 mg alpha lipoic acid (***Table 6***). This decrease was related with a significant reduction in the percent of ApoB-associated Lp-PLA2 (P=0.001), and with a significant increase in the percent of HDL-associated Lp-PLA2 (P=0.03) compared to placebo. Also, there was a positive correlation between the reduction in the ox-LDL level and total plasma Lp-PLA2 mass in the alpha lipoic acid experimental group. Given the lack of similarity between the lipid subclasses and enzymatic precursor studied in T2D, it was not possible to perform meta-analysis, thus they were systematically reviewed (***Table 6***).

3.4.1.5. Hypertension

Two RCTs, involving 391 subjects, evaluated the effect of The Dietary Approaches to Stop Hypertension (DASH) diet, widely recommended for CVDs risk reduction [31], and n-3 PUFA supplements [32] on bioactive lipid concentrations and plasma Lp-PLA2 mass in hypertension (***Table 7***). In both studies the subjects presented grade 1 hypertension with baseline systolic BP mean values ranging from 130 to 133 mm Hg, and diastolic BP from 81 to 84 mm Hg. Among 52 and 85% of the participants used antihypertensive medication. In the first RCT [31], the DASH diet consisted of a high intake of fruit, vegetables, and low-fat dairy products, providing a high amount of fiber and protein and low amounts of saturated and monounsaturated fats (7.4 % TEI

as SFAs and 10.5 % as MUFAs) compared to the control diet (14.4 % TEI as SFAs and 12.6 % MUFAs). The results showed a broad array of serum bioactive lipids significantly associated with the DASH diet, mainly MUFAs- and LC-PUFAs-containing Cer, SM, PC, PE, lyso-PCs and lyso-PEs. Most of the lipid subclasses had negative correlation coefficients (β^2) ranging from -0.06 to -0.67, representing lower serum concentrations with the DASH diet relative to the control diet. Conversely, 3 bioactive lipids had positive correlation coefficients, significantly VLC-PUFAs-containing PC(40:6) and PE(40:6) (β^2 >0.15, P<0.00001), representing higher concentrations.

In the second RCT [32], a decrease in plasma Lp-PLA2 mass was evidenced after n-3 PUFAs supplementation at daily dose of 3.36 g EPA+DHA, however, the change was not significant relative to placebo (-18.1 ng/mL, P=0.08) (***Table 7***). Furthermore, no significant changes in BP or arterial stiffness were reported. Given the lack of similarity between the lipid subclasses and enzymatic precursor studied in hypertension, it was not possible to perform meta-analysis, thus they were systematically reviewed (***Table 7***).

3.4.1.6. Stable coronary artery disease

Two RCTs, involving 96 subjects, assessed the effect of supplemented n-3 and n-6 PUFA [34,41], on the plasma Lp-PLA2 mass in stable CAD (***Table 8***). In the 2 studies the participants were middle- to older-aged with poor dietary habits. Subjects who referred for coronary angiography or undergoing percutaneous coronary intervention (PCI) were included. The majority of subjects received usual statin therapy. All participants received dietary advice on a heart-healthy diet at baseline. Significant changes in Lp-PLA2 mass were reported in subjects with stable CAD after n-3 PUFAs [41] and n-6-rich canola oil [34] supplementation (***Table 8***). The fixed-effects model meta-analysis found a significant decrease in Lp-PLA2 mass [SMD (95% CI); -0.52 (-0.91, -0.12) ng/mL] after 4 to 6 weeks of n-3 PUFAs at daily doses of 1 g (0.84 g EPA+DHA), and n-6 PUFAs-rich canola oil at daily doses of 25 mL (providing 7.3 g PUFAs) (***Figure 4***). The heterogeneity between the 2 studies was moderate (I_2=41 %, P=0.01), which could be explained by the different administration forms. Moreover, the treatment with n-3 PUFAs was suggested as independent predictor of plasma Lp-PLA2 mass changes [41].

3.4.2. Effects of dietary fatty acid-based interventions on bioactive lipids profile in healthy subjects and comparison with subjects with CVD and with CVD risk factors

We systematically reviewed a total of 5 RCTs and 4 RTs that contained data on dietary FAs intake, circulating bioactive lipids, and Lp-PLA2 enzymatic precursor in healthy subjects (***Table 9***), and, if possible, compared the changes with those found in CVD and CVD risk subjects. Additionally, a set of fixed-effects model meta-analyses were performed in 4 RCTs, which

assessed the effects of n-3 PUFA-supplemented dietary interventions (***Figures 5; Supplemental Figure 2***).

3.4.2.1. Effects of enriched diets

Two cross-over RTs [50,52], involving 25 healthy individuals, reported the effect of short-term (4-day) [52] or postprandial (6-hour) [50] exposure to high saturated fat (HSF) meals compared to low saturated fat meals on lipemia (***Table 9***). The HSF meals were composed primarily of SFA-rich foods, such as heavy whipping cream and fast-food components (hamburgers, fries, frosted pop-tarts), providing from 24 to 44.7 g SFAs. Consumption of the HSF meals lead to plasma HDL enrichment in PLs, particularly LC-PUFAs-containing PC and PE subclasses, including PCs(32:2, 33:2, 34:2, 35:2, 35:3, 36:2, 36:3, 36:4, 38:4, 38:5) and PE(34:2, 36:2, 38:4), which were significantly increased in abundance (P from <0.05 to <0.0001). In contrast, PCs-containing VLC-PUFAs, including PCs(40:6 and 40:7), tended to increase only after low saturated fat meals (P<0.05 and P=0.001, respectively). Also, the abundance of saturated-chain SM(14:0) was significantly elevated after HSF meals (P=0.013), whereas the abundance of polyunsaturated-chain SM(34:2 and 42:3) was reduced (P<0.05). These multiple changes in the plasma HDL lipidome of healthy subjects indicate that bioactive lipid FAs composition, which is highly affected by dietary fats, is able to widely remodel the composition of plasma HDL particles at short-term.

Overall, PLs results, particularly the increased LC-PUFAs-containing PCs in healthy subjects, went in the same direction as those previously reported in MetS subjects after a postprandial intervention based on a high-fat breakfast dairy-based meal [51]. Interestingly, PC(38:4), which was pointed out as discriminative marker of a 6-month n-3 PUFAs-enriched dietary intervention in MetS subjects [44], resulted also significantly elevated in healthy subjects after HSF test meals (P≤0.001).

3.4.2.2. Effects of enriched food-components

Two studies [21,36], including 159 healthy individuals, assessed the effect of PUFA EF-C, provided as n-3 PUFAs-rich oily fish [21] and n-6 PUFAs-rich soy oil [36], on circulating bioactive lipids and Lp-PLA2 activity, respectively (***Table 9***). In the first study [21], a cross-over design RT was used to assign two different doses of Atlantic salmon (90g and 180g) twice weekly. The results showed significant selective increases of plasma VLC-PUFAs-containing PC, PE and lyso-PE subclasses, including PCs(36:5, 38:5, 38:6, 40:6), PE(38:6, 40:6, 40:7), and lyso-PE(22:6) (+0.20 to +18.5 μM, P between <0.05 and <0.0001) after 8-week 180 g salmon intervention (6.15 g/day EPA+DHA). Moreover, PCs(38:6 and 40:6) were elevated in a dose-dependent manner in the same way it was reported in MetS subjects after a diet rich in LC-n-3

PUFAs [45]. Remarkably, for the n-3 EPA and DHA-containing PCs, 16:0, 18:0 and 18:1 were observed as the major paired complementary FAs (e.g. the forms of PCs(18:1_20:5, 16:0_22:6 and 18:0_22:6)). Compared to CVD risk subjects, especially with dyslipidemic subjects [39], in healthy individuals we did not observe significant changes in VLC-PUFAs-containing lyso-PCs(20:5 and 22:6) after inclusion of dietary salmon. However, lyso-PE(22:6) showed opposed results than obese subjects after following an n-3 PUFAs ED [28].

In the second study [36], a significant increase in Lp-PLA2 activity (+1 nmol/mL/min, $P<0.01$) was evident after the intake of high n-6 linoleic acid (LA) enriched-soy oil at daily doses of 9.9 g (54.2% LA). Moreover, changes in plasma LA positively correlated with changes in Lp-PLA2 activity. In parallel, the high LA experimental group showed greater levels of serum ApoB and plasma ox-LDL.

3.4.2.3 Effects of dietary supplements

Five studies, involving 283 healthy adults, evaluated the effect of a postprandial test meal [49], and marine [22,35,37,47] or plant-derived [37] n-3 PUFA supplements, on circulating bioactive lipids profile and plasma Lp-PLA2 mass (***Table 9***).

One RT [49], identified specific PE, lyso-PE and Cer subclasses that were modulated by a postprandial test meal which consisted of a 5-hour oral lipid tolerance test (OLTT) based on a LCT emulsion providing 96 % of TEI as fat (31 g MUFAs and 16 g PUFAs). Particularly, PE(36:2, 36:3), lyso-PEs(18:1, 18:2) and Cer(16:1) showed a fold change > 1.5 at 2-hour ($P<0.05$), and remained elevated for the timecourse of the OLTT. In addition, saturated lyso-PCs(16:0 and 18:0) had a negative fold change at 5-hour, while the unsaturated lyso-PCs(18:1, 18:2 and 20:4) had a positive fold change at the final time point (***Table 9***). These results indicated that the dynamic changes in lyso-PCs highly depended on the saturation level of these lyso forms. In the same way, in a previous study in obese and normal-weight subjects [22], a depletion in the plasma concentrations of saturated lyso-PCs(16:0 and 18:0) was observed in response to a high-fat meal challenge (providing 85.8 g fat) only in normal-weight subjects who had previously chronically consumed n-3 PUFAs through fish oil.

Two RCTs [22,47], involving 3 experimental arms of n-3 PUFA marine fish oil supplements, analyzed acute [47] and sustained [22] changes in lyso-PC and lyso-PE concentrations (***Table 9***). The fixed-effects model meta-analysis evidenced a marked decrease in saturated lyso-PCs(16:0 and 18:0) [SMD (95% CI); -0.19 (-0.53, 0.16) and -0.17 (-0.51, 0.18) µM, respectively] and unsaturated lyso-PCs(18:1, 18:2 and 20:4) [SMD (95%); -0.13 (-0.47, 0.21), -0.14 (-0.48, 0.20) and -0.18 (-0.53, 0.16) µM, respectively] with EPA+DHA doses ranging from 1.9 to 4 g/day, although the model did not show significant results (***Supplemental Figure 2***). Despite this, in the qualitative synthesis (***Table 9***), a significant increase was observed in EPA-containing lyso-

PC(20:5) after acute EPA+DHA supplementation (+2.20 µM, P=0.002) [47]. Also, DHA-containing lyso-PE(22:6) was significantly increased after n-6 PUFA-rich corn oil provided as placebo at daily doses of 3 g (+0.10 µM, P<0.01) [22]. Compared to CVD risk subjects, opposed results were found in saturated lyso-PCs(16:0 and 18:0). Whereas these lyso forms tended to decrease in healthy subjects, a significant increase was reported in CVD risk, including subjects with obesity [22,46], dyslipidemia [46] and MetS [44], after following 1 to 6 months n-3 PUFA EF-C, ED and supplements. Nevertheless, VLC-PUFAs-containing Lyso-PCs, particularly lyso-PCs(20:5 and 22:6), remained increased in both CVD risk and healthy subjects, after both n-3 and n-6 PUFAs dietary interventions.

In the other 2 RCTs [35,37], with 5 experimental arms of n-3 PUFA-enriched marine or plant oil supplements, a significant decrease was found in plasma Lp-PLA2 mass after performing fixed-effects model meta-analysis [SMD (95% CI); -0.35 (-0.59, -0.10) ng/mL] (***Figure 5***). The effects were noted with daily doses ranging from 0.6 to 2 g of marine EPA and DHA, combined or alone, or ALA from flaxseed oil at daily doses of 0.57 g, for a period of 1.5 to 2 months. The heterogeneity between the 2 studies was low (I^2=19 %, P=0.006). In addition, a dose-concordant effect in plasma Lp-PLA2 mass was evidenced with EPA administered alone (600 to 1800 mg) [35]. Also, increases in ox-LDL were positively associated with increases in Lp-PLA2 mass (P<0.01) [37]. These effects of n-3 PUFAs on plasma Lp-PLA2 mass were concordant with those in dyslipidemic subjects.

4. DISCUSSION

The extent to which the dietary intake of FAs modulate circulating bioactive lipidome, and whether circulating bioactive lipids could be mediators of health, CVD or CVD risk factors, remains a gap in knowledge. Our systematic review and meta-analysis collect great evidence about the effects of main dietary FAs, involving different food sources and administration forms, on the circulating bioactive lipids profile of healthy subjects, with CVD and CVD risk factors. Overall, 21 RCTs and 6 RTs were available, identifying 11 bioactive lipid classes that represent more than 150 lipid subclasses differing in their fatty acyl chains, including LC-PUFAs-, VLC-PUFAS-, MUFAs-, SFAs- and TFAs-containing bioactive lipids. The results showed great versatility in bioactive lipid biochemical structures according to the type of FAs-intervention which reflects a highly dynamic lipidome with both concordant and discordant findings between healthy subjects, or with CVD or CVD risk factors.

The most direct effects on circulating bioactive lipids were attributed to the increased dietary intake of n-3 PUFAs, provided as marine fish or plant oil supplements; EF-C, including oily fish; and n-3 enriched Mediterranean-based diets. To a lesser extent, other interventions included n-6

PUFA as EF-C in the form of plant oils, as well as SFA and TFA which predominated in HSF test meals.

Our set of meta-analyses of RCTs found consistent changes especially in subjects with CVD risk factors. Notably, we evidenced a net increase in plasma concentrations of saturated-chain lyso-PCs(16:0 and 18:0) in obese subjects, about 0.50 µM, with n-3 PUFAs supplementation at daily doses ranging from 0.37 to 1.9 g EPA+DHA [22,46]. These increases were also reported in subjects with dyslipidemia and MetS. Nevertheless, the results were opposed in healthy subjects showing a decreased trend. Both lyso-PCs(16:0 and 18:0), which contain palmitic and stearic acid, have been recognized as major components of ox-LDL with potent pro-inflammatory activities, such as eosinophil adhesion, neutrophil priming, and cytokine secretion [46,56]. Their increased levels in obesity could be mediated by the state of low-grade chronic inflammation [57], but also could reflect a decrease in lyso-PCs catabolism under altered cardiometabolic conditions. As previously reported [22], obesity may impair the sensitivity of lyso-PLs metabolism to n-3 PUFAs. Unlike the healthy state, where free n-3 PUFAs from enriched diets can remodel the fatty acyl moieties of saturated PCs and lyso-PCs by incorporation of EPA and DHA into lipid pools [19,58], in obesity, this transformation process may not occur. However, there are still certain discrepancies regarding the pro-inflammatory or anti-inflammatory effects of saturated lyso-PCs, as reported in a recent review [11]. Thus we cannot draw firm conclusions about the effect of n-3 PUFAs on these outcomes.

Concomitant with increased saturated lyso-PCs, in subjects with CVD risk factors, particularly in dyslipidemia and MetS, high n-3 PUFA EF-C, including oily fish [39] and ALA-rich foods [33], resulted in long term (2 to 6 months) increased molar % of serum LDL surface or % of plasma EPA- and DHA-containing lyso-PCs(20:5 and 22:6). The greater effects were observed in dyslipidemic subjects when consuming oily fish at doses of at least 400 g per week (providing 1 to 1.6 g EPA+DHA)[39], constituting among 1x and 2x the mean recommended intake for fish in European countries [59]. In parallel, n-3 PUFA ED exhibited decreased serum TG levels, among other MetS features, and reduced LDL aggregation susceptibility. In agreement with these results, previous studies suggest that fish oil supplementation can alter the lipid metabolism of lyso-PLs, increasing the proportion of VLC-PUFAs-containing lyso-PLs [60]. Moreover, polyunsaturated lyso-PCs promote anti-inflammatory and anti-atherogenic actions against the saturated lyso-PCs-induced effects [56]. Thus, the dietary inclusion of oily fish at high doses improves the serum LDL surface and plasma FAs profile of lyso-PCs. Likewise, EPA- and DHA-containing lyso-PCs constitute promising bioactive lipids in the management of subjects at risk of dyslipidemia, and the changes in the LDL lipidome indicate reduced atherogenic effects.

The beneficial effects of n-3 PUFAs on bioactive lipids profile may relate in part to the lowering of Lp-PLA2 enzymatic precursor. Concordant decreases in plasma Lp-PLA2 mass, around -0.35 ng/mL, were found in both dyslipidemic and healthy subjects from our meta-

analysis. Also, in stable CAD, the meta-analysis showed more pronounced effects reaching decreases of -0.52 ng/mL. Although these effects were typically noted with n-3 PUFAs daily doses ranging from 1 to 5.56 g EPA+DHA, in two studies the highest effects were specific for EPA supplementation [35,43], which pointed out an independent effect. Contrary, n-6 LA provided as E-FC showed increased Lp-PLA2 activity in healthy subjects [36], which points out a detrimental effect compared to n-3 supplements. In agreement with these results, a recent meta-analysis of 15 prospective studies showed higher plasma Lp-PLA2 mass independently associated with increased risk of CVD events in patients with stable CAD [61]. Despite this, the effects of n-3 PUFAs on plasma Lp-PLA2 mass have not been fully reviewed to date. n-3 PUFAs treatment is the only lipid-altering therapy identified so far that lowers plasma Lp-PLA2 mass without lowering LDL-cholesterol, however, mechanisms by means n-3 PUFAs decrease Lp-PLA2 mass are unclear. Since Lp-PLA2 is a known vascular-specific inflammatory marker [15], it is quite likely that the greatest benefit of dietary n-3 on CVD risk may be related to its effects on decreasing the body's inflammatory responses, including the lowering of pro-inflammatory lyso-PCs. Additionally, the capacity of n-3 PUFAs to lowering ox-LDL might reduce the substrate for Lp-PLA2, being this another potential mechanism. In this regard, the reported decreases of Lp-PLA2 mass after alpha lipoic acid supplementation in T2D subjects might result from its reductive capacity on ox-LDL serum levels [30]. As previously reported, alpha lipoic acid, an essential cofactor of dehydrogenase enzymes, has antioxidant characteristics and reaches easily to the cardiovascular system [62].

Among the bioactive lipid categories identified in our qualitative synthesis, PLs and SPs, particularly PC, SM and Cer classes were the most representative, with 34, 41 and 29 different subclasses, respectively. Importantly, the major changes found on these lipid classes were in the postprandial period after HSF test meals. Throughout the day, people are primarily in a postprandial state; thus fasting bioactive lipid levels may not always reflect the risk of CVDs. In this sense, the postprandial response of circulating bioactive lipids after test meals may reveal more accurate information on the effects of dietary FAs than the fasting state in sustained interventions [63]. From our results, there was a wide range of net effects found in circulating PC subclasses, particularly those containing LC- and VLC-PUFAs and less double bonds such as PCs(36:2, 36:3, 36:4, 36:5, 38:4, 38:5, 38:6, 40:6, 40:7) among other. These effects highly depended on the dietary source of the FAs, and were concordant among healthy subjects and with CVD risk factors. Whereas a significant increase was found in unsaturated PCs after SFAs- and TFAs-rich sources, including partially hydrogenated soybean oil [38], whole dairy-based meals [51], and fast-food based meals [50,52], a generalized decrease was reported after high-n-3 PUFA ED [44,45,52], with exception of PCs(38:4, 38:6, 40:4 and 40:6) which remained increased after both n-3 PUFA and oily fish interventions, especially at long-term (1 to 6 months). These effects were especially noted with postprandial (4- to 6-hours) or short-term (4-day) doses of SFAs

ranging from 24 to 67 g; daily doses of TFAs of 15.7 g; and PUFAs representing from 4.2 to 6.2 % TEI in ED. In addition, significant effects were noted in the plasma HDL PCs content as HDL particles usually have the highest concentrations of PLs. n-3 LC-PUFA levels in plasma PLs have been suggested as potential biomarkers of disease [21]. In agreement with a previous dose-response study [19], our reported PLs results indicate that the PLs-FAs content exhibits increased unsaturation following SFAs and TFAs compared to n-3 PUFAs dietary interventions. These effects could be explained by a general up-regulation in the formation of LC- and VLC-PUFA-containing PLs through a preferential integration of SFAs and TFAs into the sn-1 position of PCs, all containing PUFAs in the sn-2 position [38]. This structural reconfiguration of PCs after SFAs and TFAs leads to shorter and fewer double bonded forms than n-3 PUFAs, which conform longer and double bonded forms. Therefore, the inclusion of an SFA on the sn-1 position yield a more condensed shape of the PC molecule which may influence the distribution of n-3 PUFAs in lipid rafts [64]. These effects could be contributing factors in modulating cholesterol homeostasis, mainly LDL-cholesterol raise, as PLs-containing TFAs and SFAs behave similar to free SFAs [38,65]. Conversely, the selective increases of DHA-containing PCs(38:6 and 40:6) with n-3 PUFA ED may support a protective role in CVDs as have shown the negative associations of large prospective studies [66]. Also, higher PC(38:4), which contain arachidonic acid (ARA), was positively associated to reduction of inflammatory markers in MetS subjects following n-3 PUFA ED [44]. Thus, these findings suggest that high n-3 PUFA ED could mediate a protective response against CVD risk through increasing select VLC-PUFAs-containing PCs. Despite this, more studies are needed to settle insights into the divergent effects for the different dietary FAs on PLs, and to corroborate whether their changes could be influenced by the duration of the intervention.

The effects of SFAs on circulating SM and Cer classes were, for the most part, consistent with that reported for PCs, especially in subjects with CVD risk factors. The most notable changes were found in saturated- and monounsaturated-chain SM and Cer subclasses, most frequently in SM(14:0, 15:0, 16:0, 18:0, 32:0, 34:0, 16:1, 18:1) and Cer(16:0, 18:0, 20:0, 24:1), after HSF postprandial test meals providing above 40 g SFAs [48,51]. Interestingly, significant increases were reported following SFAs EF-C, while a decrease was evident after n-6 PUFA EF-C. Both bioactive lipids, Cer and SM, are important signaling molecules linked with inflammation and the pathogenesis of CVDs [67]. As recently reviewed, serum Cer levels provide to be accurate biomarkers of adverse CVD outcomes [67]. Our reported effects are similar to that found in a recent large-scale lipidomic analysis where higher saturated fat intake, in contrast to a diet rich in polyunsaturated fat, was associated with higher concentrations of saturated- and monounsaturated-chain SPs [68]. On the other hand, in T2D subjects, the reported decreases in both saturated SM and Cer following a MCFAs-rich diet support a positive effect against the progression of insulin resistance [29]. Nonetheless, plasma SPs concentrations were not impaired in any of the n-3 PUFA dietary interventions.

Among the less frequent classes of circulating bioactive lipids in our qualitative synthesis, lyso-PE, lyso-PI, PE and PI were the most characteristic. Particularly, lyso-PEs and lyso-PIs, which encompass a smaller head group than lyso-PCs, were present at lower circulating levels. Plasma levels of VLC-PUFAs-containing lyso-PE subclasses, including lyso-PEs(20:4 and 22:6), have been increased about three-fold in acute coronary syndrome subjects [69]. However, as reported in lyso-PCs, they have also been shown to attenuate the inflammatory response [70]. Our findings evidenced discordant effects of n-3 PUFAs, n-6 PUFAs and SFAs on lyso-PEs(20:4 and 22:6) among CVD risk and healthy subjects. Whereas lyso-PE(22:6) significantly increased in healthy subjects following n-6 PUFAs and n-3 PUFAs-rich oily fish interventions [21,22], it was decreased in obese subjects after n-3 PUFA ED [28]. However, lyso-PE(20:4) was only increased after n-6 PUFAs in obese subjects. These findings, provide further insights into the versatile response of bioactive lyso-PLs to dietary FAs according to the cardiometabolic state.

The assessment of HDL and LDL lipidome could be a valuable approach to identify and characterize new biomarkers related to lipid metabolism. In 3 and 2 of our reviewed studies bioactive lipid concentrations were determined in the HDL and LDL particles surface, respectively, after different FA interventions. Particularly, significant increases were reported of unsaturated PCs in plasma HDL surface following HSF meals. As recently reported in hypercholesterolemic subjects, the inclusion of dietary FAs, mainly MUFAs, induce modulatory effects on HDL function through dysregulations in unsaturated bioactive lipid levels, including increased PCs [71]. Similarly, our reported increases in HDL unsaturated PCs indicate that SFAs may remodel the composition and function of HDL particles. In this context, although similar patterns of unsaturated PCs were reported among total plasma and HDL fraction in one of our reviewed studies [45], we cannot rule out a reporting bias for studies measuring changes only in the HDL fraction lipidome.

Importantly, in some of the FAs-supplemented studies n-3 PUFAs were complexed with CA [40,41]. Unlike EE forms (e.g. Lovaza), n-3-CA contain free FAs and do not require hydrolysis by lipase before they can be absorbed [72]. Thus, these formulations are expected to improve the systematic bioavailability of n-3 PUFAs. Moreover, the combination of n-3-CA with 2 g olive oil appeared to be more effective in the reduction of Lp-PLA2 mass in dyslipidemic subjects. It could be explained by a beneficial synergistic effect of n-3 and olive oil on lipid metabolism and oxidative stress, as previously reported in MetS [73]. On spite of these improved formulations, as foods are mixtures of different nutrients and other components, it is worth noting that dietary interventions involving FA EF-C and ED might be influenced by other compounds able to interfere the efficiency of n-3 absorption from the food sources. For instance, in 1 of our reviewed RCTs [33], the inclusion of a plant stanol-enriched rapeseed oil could have an important matrix effect mediated by phytosterols rather than n-6 and n-3 PUFAs. Hence, although our results

highlight the clear effects of different sources of FAs on bioactive lipids metabolism, the lack of adjustment for multiple testing of the included studies may suppose an overestimation of the observed effects. Therefore, results concerning single bioactive lipid subclasses should be interpreted with caution.

To our knowledge the circulating lipidome has not been extensively studied in relation to bioactive lipid classes and subclasses. Overall, the results from our systematic review and meta-analysis offer for the first time an overview on the effects of dietary FAs on particular bioactive lipids. Notably, marine n-3 PUFAs, provided as supplements or as oily fish, at doses from 0.30 to 3.4 g/day EPA+DHA, showed the most marked changes in circulating bioactive lipids profile in subjects with CVD risk factors. Also, doses from 0.6 to 5.56 g/day EPA+DHA, lead to decreased plasma Lp-PLA2 mass in healthy subjects, with CVD and CVD risk factors. These EPA+DHA doses, are above the last recommendation of the European Food Safety Authority (EFSA) based on cardiovascular risk considerations for European adults, which range from 250 to 500 mg/day of combined EPA+DHA [74].

The application of targeted and untargeted high-throughput lipidomics analysis through MS quantitative approaches, such as separation or direct infusion-MS, have allowed the characterization of a wide set of bioactive lipid moieties. Furthermore, the inclusion of the molecular formula provides information on the degree of complexity of the fatty acyl chains. Although our review focuses on the most prevalent bioactive lipid categories, future research should include other emergent bioactive lipid intermediates, such as lysophosphatidic acid, that has shown to play an important role in the initiation and progression of CVDs [75].

Our review and the different studies included had several limitations that need to be highlighted. First, several RCTs had small sample sizes, and varied significantly in terms of design, duration, FAs administration forms and dosages, which made difficult pooling all results in a meta-analysis. Second, the smaller number of studies included for certain CVD risk factors, such as T2D and hypertension, limited the establishment of rigorous conclusions. Third, the significant estimated effects could be influenced by potential confounders such as sex or ethnicity, since these factors could produce between-subject variations in circulating bioactive lipids [76]. Fourth, the choice of olive oil as placebo in some studies may have had non neutral effects on bioactive lipids. Fifth, in CVD risk factors, a large number of the study subjects were receiving statin therapy, which could limit the ability to discern a treatment effect.

5. CONCLUSIONS

The results of the present systematic review and meta-analysis support the conclusion that

marine n-3 PUFA supplementation, provided as EPA+DHA at doses from 1 to 5.56 g/day, and consumed from 1 to 6 months, exhibit positive effects reducing plasma Lp-PLA2 mass in healthy subjects, with CVD and CVD risk factors, suggesting an anti-inflammatory effect. The greater effects of decreased Lp-PLA2 mass are found in healthy, dyspidemic and stable CAD subjects, which represents improved circulating bioactive lipids profile by lowering pro-inflammatory lyso-PCs synthesis. However, further investigation is required to elucidate exact mechanisms by means n-3 PUFAs decrease Lp-PLA2.

From the meta-analysis of RCTs, the most consistent effects are evidenced on plasma pro-inflammatory lyso-PC(16:0 and 18:0) which is increased in obese subjects after supplementation of 0.37 to 1.9 g/day EPA+DHA, which suggests an impaired saturated lyso-PCs response to n-3 PUFAs. From the systematic review, the dietary intake of n-3 PUFAs-rich oily fish at weekly doses of at least 400g increase plasma EPA- and DHA-containing lyso-PC(20:5 and 22:6) in dyslipidemic subjects, reinforcing these unsaturated lyso-PCs promising role in the management of subjects at risk for dyslipidemia.

Larger meta-analysis with high-quality RCTs are required to reinforce the results and further inquire into the optimal dose of n-3 PUFAs and the treatment duration period. Lastly, whether the identified bioactive lipid moieties are involved in biological pathways of metabolic risk is unclear which warrants further future lipidomic research.

Acknowledgements

Each author (LC-P, JC, RS, AP and RMV) has made substantial contributions to the conception, design and investigation process as described in the CRediT author statement. All authors have read and approved the submitted version of the manuscript, and agrees to be personally accounted for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, are appropriately investigated, solved, and documented. NFOC-Salut group is a consolidated research group of Generalitat de Catalunya, Spain (2017 SGR522).

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

The authors declare no conflict of interest. Complete Declaration of Interest forms for each author has been uploaded at the time of manuscript submission.

ABBREVIATIONS

AC, acylcarnitine; ALA, alpha-linolenic acid; BMI, body mass index; CAD, coronary artery disease; Cer, ceramide; CVD, cardiovascular disease; DHA, docosahexaenoic acid; diCer, dihydroceramide; ED, enriched diet; EF-C, enriched food-component; EPA, eicosapentaenoic acids; FAs, fatty acids; gluCer, glucosylceramide; IHD, ischemic heart disease; LC-MS, liquid chromatography coupled to mass spectrometry; LA, linoleic acid; lacCer, lactosylceramide; LC-PUFAs, long chain polyunsaturated fatty acids; LCT, long chain triglyceride; Lp-PLA2, lipoprotein-associated PLA2; lyso-PCs, lysophosphatidylcholines; lyso-PEs, lysophosphatidylethanolamines; lyso-PIs, lysophosphatidylinositols; lyso-PLs, lysoglycerophospholipids; MCFAs, medium-chain fatty acids; MetS, metabolic syndrome; MUFAs, monounsaturated fatty acids; n-3, omega-3; n-6, omega-6; OLTT, oral lipid tolerance test; ox-LDL, oxidized LDL-cholesterol; PCs, phosphatidylcholines; PEs, phosphatidylethanolamines; PIs, phosphatidylinositols; PLs, glycerophospholipids; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids; RCTs, randomized controlled trial; RTs, non-controlled randomized trials; SM, sphingomyelin; SMD, standard mean difference; SPs, sphingolipids; TEI, total energy intake; TFAs, trans fatty acids; TG, triglycerides; T2D, type 2 diabetes; VLC-PUFAs, very long chain polyunsaturated fatty acids.

Figure captions

Figure 1 | PRISMA 2020 flow diagram of the systematic review and meta-analysis. PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis.

Figure 2 | Meta-analysis of RCTs of the effect of n-3 PUFA supplemented dietary interventions on saturated Lyso-PC levels in subjects with overweight and obesity. The squares and bars estimate of the net standard mean difference (SMD) (difference between the end and baseline concentrations of bioactive lipid subclasses) and the corresponding 95% CI for individual studies. The fixed effects model overall results are indicated by a rhombus symbol near the bottom of each graph. Lyso-PC, lysophosphatidylcholine; n-3, omega-3 polyunsaturated fatty acids.

Figure 3 | Meta-analysis of RCTSs of the effect of n-3 PUFA supplemented dietary interventions on Lp-PLA2 mass in subjects with dyslipidemia. The squares and bars estimate of the net standard mean difference (SMD) (difference in Lp-PLA2 concentrations compared to placebo) and the corresponding 95% CI for individual studies. The fixed effects model overall results are indicated by a rhombus symbol near the bottom of each graph. n-3, omega-3 polyunsaturated fatty acids.

Figure 4 | Meta-analysis of RCTs of the effect of PUFA supplemented dietary interventions (n-3 and n-6) on Lp-PLA2 mass in subjects with stable CAD. The squares and bars estimate of the net standard mean difference (SMD) (difference between the end and baseline concentrations of Lp-PLA2) and the corresponding 95% CI for individual studies. The fixed effects model overall results are indicated by a rhombus symbol near the bottom of each graph. n-3, omega-3 polyunsaturated fatty acids; n-6-, omega-6 polyunsaturated fatty acids.

Figure 5 | Meta-analysis of RCTs of the effect of n-3 PUFA supplemented dietary interventions on Lp-PLA2 mass in healthy subjects. The squares and bars estimate of the net standard mean difference (SMD) (difference between the end and baseline concentrations of Lp-PLA2) and the corresponding 95% CI for individual studies. The fixed effects model overall results are indicated by a rhombus symbol near the bottom of each graph. ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; n-3, omega-3 polyunsaturated fatty acids.

REFERENCES

[1] Roth GA, Mensah GA, Johnson CO, Addolorato G, Ammirati E, Baddour LM, et al. Global Burden of Cardiovascular Diseases and Risk Factors, 1990-2019: Update From the GBD 2019 Study. J Am Coll Cardiol 2020;76:2982–3021. https://doi.org/10.1016/j.jacc.2020.11.010.

[2] Joseph P, Leong D, McKee M, Anand SS, Schwalm J-D, Teo K, et al. Reducing the Global Burden of Cardiovascular Disease, Part 1. Circ Res 2017;121:677–94. https://doi.org/10.1161/CIRCRESAHA.117.308903.

[3] Wang J, Tan G-J, Han L-N, Bai Y-Y, He M, Liu H-B. Novel biomarkers for cardiovascular risk prediction. J Geriatr Cardiol 2017;14:135–50. https://doi.org/10.11909/j.issn.1671-5411.2017.02.008.

[4] Rai S, Bhatnagar S. Novel Lipidomic Biomarkers in Hyperlipidemia and Cardiovascular Diseases: An Integrative Biology Analysis. OMICS 2017;21:132–42. https://doi.org/10.1089/omi.2016.0178.

[5] Bari M, Bisogno T, Battista N. Bioactive Lipids in Health and Disease. Biomolecules 2020;10:1698. https://doi.org/10.3390/biom10121698.

[6] Silva IT, Mello APQ, Damasceno NRT. Antioxidant and inflammatory aspects of lipoprotein-associated phospholipase A_2 (Lp-PLA_2): a review. Lipids Health Dis 2011;10:170. https://doi.org/10.1186/1476-511X-10-170.

[7] Gomez-Larrauri A, Presa N, Dominguez-Herrera A, Ouro A, Trueba M, Gomez-Muñoz A. Role of bioactive sphingolipids in physiology and pathology. Essays Biochem 2020;64:579–89. https://doi.org/10.1042/EBC20190091.

[8] Sevastou I, Kaffe E, Mouratis M-A, Aidinis V. Lysoglycerophospholipids in chronic inflammatory disorders: The PLA2/LPC and ATX/LPA axes. Biochim Biophys Acta - Mol Cell Biol Lipids 2013;1831:42–60. https://doi.org/https://doi.org/10.1016/j.bbalip.2012.07.019.

[9] Matsumoto T, Kobayashi T, Kamata K. Role of lysophosphatidylcholine (LPC) in atherosclerosis. Curr Med Chem 2007;14:3209–20. https://doi.org/10.2174/092986707782793899.

[10] Meikle PJ, Wong G, Tsorotes D, Barlow CK, Weir JM, Christopher MJ, et al. Plasma Lipidomic Analysis of Stable and Unstable Coronary Artery Disease. Arterioscler Thromb Vasc Biol 2011;31:2723–32. https://doi.org/10.1161/ATVBAHA.111.234096.

[11] Knuplez E, Marsche G. An Updated Review of Pro- and Anti-Inflammatory Properties of Plasma Lysophosphatidylcholines in the Vascular System. Int J Mol Sci 2020;21:4501. https://doi.org/10.3390/ijms21124501.

[12] Law S-H, Chan M-L, Marathe GK, Parveen F, Chen C-H, Ke L-Y. An Updated Review of Lysophosphatidylcholine Metabolism in Human Diseases. Int J Mol Sci 2019;20:1149. https://doi.org/10.3390/ijms20051149.

[13] Suárez-García S, Caimari A, Del Bas JM, Suárez M, Arola L. Serum lysophospholipid levels are altered in dyslipidemic hamsters. Sci Rep 2017;7:10431. https://doi.org/10.1038/s41598-017-10651-0.

[14] Choi RH, Tatum SM, Symons JD, Summers SA, Holland WL. Ceramides and other sphingolipids as drivers of cardiovascular disease. Nat Rev Cardiol 2021. https://doi.org/10.1038/s41569-021-00536-1.

[15] Diaconu A, Coculescu B-I, Manole G, Vultur H, Coculescu EC, Stocheci CM, et al. Lipoprotein-associated phospholipase A2 (Lp-PLA2) – possible diagnostic and risk biomarker in chronic ischaemic heart disease. J Enzyme Inhib Med Chem 2021;36:68–73. https://doi.org/10.1080/14756366.2020.1839447.

[16] Davidson MH, Corson MA, Alberts MJ, Anderson JL, Gorelick PB, Jones PH, et al. Consensus Panel Recommendation for Incorporating Lipoprotein-Associated Phospholipase A_2 Testing into Cardiovascular Disease Risk Assessment Guidelines. Am J Cardiol 2008;101:S51–7. https://doi.org/10.1016/j.amjcard.2008.04.019.

[17] Rangel-Huerta OD, Gil A. Omega 3 fatty acids in cardiovascular disease risk factors: An updated systematic review of randomised clinical trials. Clin Nutr 2018;37:72–7. https://doi.org/10.1016/j.clnu.2017.05.015.

[18] EFSA Panel on Dietetic Products N and A (NDA). Scientific Opinion on the substantiation of health claims related to docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and brain, eye and nerve development (ID 501, 513, 540), maintenance of normal brain function (ID 497, 501, 510, 513, 519, 521, 53. EFSA J 2011;9:2078. https://doi.org/https://doi.org/10.2903/j.efsa.2011.2078.

[19] Browning LM, Walker CG, Mander AP, West AL, Madden J, Gambell JM, et al. Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. Am J Clin Nutr 2012;96:748–58. https://doi.org/10.3945/ajcn.112.041343.

[20] Raatz SK, Rosenberger TA, Johnson LK, Wolters WW, Burr GS, Picklo MJS. Dose-dependent consumption of farmed Atlantic salmon (Salmo salar) increases plasma phospholipid n-3 fatty acids differentially. J Acad Nutr Diet 2013;113:282–7. https://doi.org/10.1016/j.jand.2012.09.022.

[21] Žáček P, Bukowski M, Johnson L, SK R, Picklo M. Selective enrichment of n-3 fatty acids in human plasma lipid motifs following intake of marine fish. J Nutr Biochem 2018;54:57–65. https://doi.org/10.1016/j.jnutbio.2017.11.002.

[22] Del Bas JM, Caimari A, Rodriguez-Naranjo MI, Childs CE, Paras Chavez C, West AL,

et al. Impairment of lysophospholipid metabolism in obesity: altered plasma profile and desensitization to the modulatory properties of n-3 polyunsaturated fatty acids in a randomized controlled trial. Am J Clin Nutr 2016;104:266–79. https://doi.org/10.3945/ajcn.116.130872.

[23] Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JPA, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. BMJ 2009;339:b2700. https://doi.org/10.1136/bmj.b2700.

[24] Holčapek M, Liebisch G, Ekroos K. Lipidomic Analysis. Anal Chem 2018;90:4249–57. https://doi.org/10.1021/acs.analchem.7b05395.

[25] Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R, et al. HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res 2018;46:D608–17. https://doi.org/10.1093/nar/gkx1089.

[26] Higgins JPT, Thomas J, Chandler J, Cumpston M, Li T, Page MJ WV (editors). Cochrane Handbook for Systematic Reviews of Interventions version 6.2. Cochrane Handb Syst Rev Interv Version 62 (Updated Febr 2021) 2021. www.training.cochrane.org/handbook (accessed February 25, 2021).

[27] Higgins JPT, Altman DG, Gøtzsche PC, Jüni P, Moher D, Oxman AD, et al. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. BMJ 2011;343:d5928. https://doi.org/10.1136/bmj.d5928.

[28] Cantero I, Abete I, Del Bas JM, Caimari A, Arola L, Zulet MA, et al. Changes in lysophospholipids and liver status after weight loss: the RESMENA study. Nutr Metab (Lond) 2018;15:51. https://doi.org/10.1186/s12986-018-0288-5.

[29] Airhart S, WT C, Jiang H, AR C, SB R, Korenblat K, et al. A Diet Rich in Medium-Chain Fatty Acids Improves Systolic Function and Alters the Lipidomic Profile in Patients With Type 2 Diabetes: A Pilot Study. J Clin Endocrinol Metab 2016;101:504–12. https://doi.org/10.1210/jc.2015-3292.

[30] Baziar N, Nasli-Esfahani E, Djafarian K, Qorbani M, Hedayati M, Mishani MA, et al. The Beneficial Effects of Alpha Lipoic Acid Supplementation on Lp-PLA2 Mass and Its Distribution between HDL and apoB-Containing Lipoproteins in Type 2 Diabetic Patients: a Randomized, Double-Blind, Placebo-Controlled Trial 2020;2020. https://doi.org/10.1155/2020/5850865.

[31] Rebholz CM, Lichtenstein AH, Zheng Z, Appe LJ, Coresh J. Serum untargeted metabolomic profile of the Dietary Approaches to Stop Hypertension (DASH) dietary pattern 2018;108:243-255. https://doi.org/10.1093/ajcn/nqy099.

[32] MJ K, EP H, RI P, Beaty B, PS M, CS L. Effects of omega-3 fatty acids on arterial stiffness in patients with hypertension: a randomized pilot study. J Negat Results Biomed

2015;14:21. https://doi.org/10.1186/s12952-015-0040-x.

[33] Ruuth M, Äikäs L, Tigistu-Sahle F, Käkelä R, Lindholm H, Simonen P, et al. Plant Stanol Esters Reduce LDL (Low-Density Lipoprotein) Aggregation by Altering LDL Surface Lipids: The BLOOD FLOW Randomized Intervention Study. Arter Thromb Vasc Biol 2020;40:2310–21. https://doi.org/10.1161/ATVBAHA.120.314329.

[34] Khandouzi N, Zahedmehr A, Nasrollahzadeh J. Effects of canola or olive oil on plasma lipids, lipoprotein-associated phospholipase A(2) and inflammatory cytokines in patients referred for coronary angiography. Lipids Health Dis 2020;19:183. https://doi.org/10.1186/s12944-020-01362-z.

[35] IB A, JA G, Sever S, Gedik R, BF A, KV H, et al. Effects of eicosapentaenoic acid and docosahexaenoic acid on cardiovascular disease risk factors: a randomized clinical trial. Metabolism 2016;65:1636–45. https://doi.org/10.1016/j.metabol.2016.07.010.

[36] Kim M, Lee A, Yoo HJ, Her JS, Jee SH, Lee JH. Impact of 8-week linoleic acid intake in soy oil on Lp-PLA2 activity in healthy adults 2017;14. https://doi.org/10.1186/s12986-017-0186-2.

[37] TL N, JE H, MS H. Omega-3 fatty acids and lipoprotein associated phospholipase A(2) in healthy older adult males and females. Eur J Nutr 2011;50:185–93. https://doi.org/10.1007/s00394-010-0126-7.

[38] Gürdeniz G, Rago D, NT B, Savorani F, Astrup A, LO D. Effect of trans fatty acid intake on LC-MS and NMR plasma profiles. PLoS One 2013;8:e69589. https://doi.org/10.1371/journal.pone.0069589.

[39] Zhang J, Wang C, Li L, Man Q, Meng L, Song P, et al. Dietary inclusion of salmon, herring and pompano as oily fish reduces CVD risk markers in dyslipidaemic middle-aged and elderly Chinese women. Br J Nutr 2012;108:1455–65. https://doi.org/10.1017/S0007114511006866.

[40] RL D, SJ N, KC M, EM R, DG O, Curcio D, et al. Effects of omega-3 carboxylic acids on lipoprotein particles and other cardiovascular risk markers in high-risk statin-treated patients with residual hypertriglyceridemia: a randomized, controlled, double-blind trial. Lipids Heal Dis 2015;14:98. https://doi.org/10.1186/s12944-015-0100-8.

[41] Gajos G, Zalewski J, Konduracka E, Nessler J, Undas A. Polyunsaturated omega-3 fatty acids reduce lipoprotein associated phospholipase A2 (Lp-PLA2) levels in patients with stable angina undergoing percutaneous coronary intervention 2012;33:1078. https://doi.org/10.1093/eurheartj/ehs284.

[42] Hedengran A, Szecsi PB, Dyerberg J, Harris WS, Stender S. n-3 PUFA esterified to glycerol or as ethyl esters reduce non-fasting plasma triacylglycerol in subjects with hypertriglyceridemia: a randomized trial 2014;50:165-175. https://doi.org/10.1007/s11745-014-3968-6.

[43] Mosca L, Bays H, Philip S, Chowdhury S, Doyle R, Juliano R. Icosapent ethyl (eicosapentaenoic acid ethyl ester) in women with very high triglyceride levels: results from the marine study 2016;10:701-702.

[44] Bondia-Pons I, Martinez JA, de la Iglesia R, Lopez-Legarrea P, Poutanen K, Hanhineva K, et al. Effects of short- and long-term Mediterranean-based dietary treatment on plasma LC-QTOF/MS metabolic profiling of subjects with metabolic syndrome features: the Metabolic Syndrome Reduction in Navarra (RESMENA) randomized controlled trial 2015;59:711-728. https://doi.org/10.1002/mnfr.201400309.

[45] Bondia-Pons I, Pöhö P, Bozzetto L, Vetrani C, Patti L, AM A, et al. Isoenergetic diets differing in their n-3 fatty acid and polyphenol content reflect different plasma and HDL-fraction lipidomic profiles in subjects at high cardiovascular risk. Mol Nutr Food Res 2014;58:1873–82. https://doi.org/10.1002/mnfr.201400155.

[46] Padro T, Vilahur G, Sánchez-Hernández J, Hernández M, Antonijoan RM, Perez A, et al. Lipidomic changes of LDL in overweight and moderately hypercholesterolemic subjects taking phytosterol- and omega-3-supplemented milk 2015;56:1043-1056. https://doi.org/10.1194/jlr.P052217.

[47] RC B, Kakinami L, Jonovich M, Antonetti I, Lawrence P, Meednu N, et al. The combination of EPA+DHA and low-dose aspirin ingestion reduces platelet function acutely whereas each alone may not in healthy humans. Prostaglandins Leukot Essent Fat Acids 2012;87:143–51. https://doi.org/10.1016/j.plefa.2012.08.007.

[48] Rosqvist F, Kullberg J, Ståhlman M, Cedernaes J, Heurling K, HE J, et al. Overeating Saturated Fat Promotes Fatty Liver and Ceramides Compared With Polyunsaturated Fat: A Randomized Trial. J Clin Endocrinol Metab 2019;104:6207–19. https://doi.org/10.1210/jc.2019-00160.

[49] Morris C, O'Grada CM, Ryan MF, Gibney MJ, Roche HM, Gibney ER, et al. Modulation of the lipidomic profile due to a lipid challenge and fitness level: a postprandial study 2015;14:65. https://doi.org/10.1186/s12944-015-0062-x.

[50] Averill M, Rubinow KB, Cain K, Wimberger J, Babenko I, Becker JO, et al. Postprandial remodeling of high-density lipoprotein following high saturated fat and high carbohydrate meals 2019. https://doi.org/10.1016/j.jacl.2019.11.002.

[51] Meikle PJ, Barlow CK, Mellett NA, Mundra PA, Bonham MP, Larsen A, et al. Postprandial Plasma Phospholipids in Men Are Influenced by the Source of Dietary Fat 2015;145:2012-2018. https://doi.org/10.3945/jn.115.210104.

[52] Zhu Y, Bo Y, Liu Y. Dietary total fat, fatty acids intake, and risk of cardiovascular disease: a dose-response meta-analysis of cohort studies. Lipids Health Dis 2019;18:91. https://doi.org/10.1186/s12944-019-1035-2.

[53] Lopez-Legarrea P, De la Iglesia R, Abete I, Ibanez-Melo A, Navas-Carretero S, Bondia-

Pons I, et al. The resmena diet: a new effective dietary strategy for reducing metabolic syndrome. Obes Facts 2012;5:230-231. https://doi.org/10.1159/000258190.

[54] Alberti KGMM, Zimmet P, Shaw J. The metabolic syndrome--a new worldwide definition. Lancet (London, England) 2005;366:1059–62. https://doi.org/10.1016/S0140-6736(05)67402-8.

[55] 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2021. Diabetes Care 2021;44:S15–33. https://doi.org/10.2337/dc21-S002.

[56] Hung ND, Sok D-E, Kim MR. Prevention of 1-palmitoyl lysophosphatidylcholine-induced inflammation by polyunsaturated acyl lysophosphatidylcholine. Inflamm Res Off J Eur Histamine Res Soc . [et Al] 2012;61:473–83. https://doi.org/10.1007/s00011-012-0434-x.

[57] Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity and inflammation: the linking mechanism and the complications. Arch Med Sci 2017;13:851–63. https://doi.org/10.5114/aoms.2016.58928.

[58] Balogun KA, Albert CJ, Ford DA, Brown RJ, Cheema SK. Dietary omega-3 polyunsaturated fatty acids alter the fatty acid composition of hepatic and plasma bioactive lipids in C57BL/6 mice: a lipidomic approach. PLoS One 2013;8:e82399. https://doi.org/10.1371/journal.pone.0082399.

[59] Lofstedt A, de Roos B, Fernandes PG. Less than half of the European dietary recommendations for fish consumption are satisfied by national seafood supplies. Eur J Nutr 2021. https://doi.org/10.1007/s00394-021-02580-6.

[60] Ottestad I, Hassani S, Borge GI, Kohler A, Vogt G, Hyötyläinen T, et al. Fish oil supplementation alters the plasma lipidomic profile and increases long-chain PUFAs of phospholipids and triglycerides in healthy subjects. PLoS One 2012;7:e42550. https://doi.org/10.1371/journal.pone.0042550.

[61] Li D, Zhao L, Yu J, Zhang W, Du R, Liu X, et al. Lipoprotein-associated phospholipase A2 in coronary heart disease: Review and meta-analysis. Clin Chim Acta 2017;465:22–9. https://doi.org/10.1016/j.cca.2016.12.006.

[62] Skibska B, Goraca A. The protective effect of lipoic acid on selected cardiovascular diseases caused by age-related oxidative stress. Oxid Med Cell Longev 2015;2015:313021. https://doi.org/10.1155/2015/313021.

[63] Pellis L, van Erk MJ, van Ommen B, Bakker GCM, Hendriks HFJ, Cnubben NHP, et al. Plasma metabolomics and proteomics profiling after a postprandial challenge reveal subtle diet effects on human metabolic status. Metabolomics 2012;8:347–59. https://doi.org/10.1007/s11306-011-0320-5.

[64] Wassall SR, Leng X, Canner SW, Pennington ER, Kinnun JJ, Cavazos AT, et al. Docosahexaenoic acid regulates the formation of lipid rafts: A unified view from

experiment and simulation. Biochim Biophys Acta Biomembr 2018;1860:1985–93. https://doi.org/10.1016/j.bbamem.2018.04.016.

[65] Niu S-L, Mitchell DC, Litman BJ. Trans fatty acid derived phospholipids show increased membrane cholesterol and reduced receptor activation as compared to their cis analogs. Biochemistry 2005;44:4458–65. https://doi.org/10.1021/bi048319+.

[66] Stegemann C, Pechlaner R, Willeit P, Langley SR, Mangino M, Mayr U, et al. Lipidomics Profiling and Risk of Cardiovascular Disease in the Prospective Population-Based Bruneck Study. Circulation 2014;129:1821–31. https://doi.org/10.1161/CIRCULATIONAHA.113.002500.

[67] Borodzicz S, Czarzasta K, Kuch M, Cudnoch-Jedrzejewska A. Sphingolipids in cardiovascular diseases and metabolic disorders. Lipids Health Dis 2015;14:55. https://doi.org/10.1186/s12944-015-0053-y.

[68] Seah JYH, Chew WS, Torta F, Khoo CM, Wenk MR, Herr DR, et al. Dietary Fat and Protein Intake in Relation to Plasma Sphingolipids as Determined by a Large-Scale Lipidomic Analysis. Metabolites 2021;11. https://doi.org/10.3390/metabo11020093.

[69] Kurano M, Suzuki A, Inoue A, Tokuhara Y, Kano K, Matsumoto H, et al. Possible involvement of minor lysophospholipids in the increase in plasma lysophosphatidic acid in acute coronary syndrome. Arterioscler Thromb Vasc Biol 2015;35:463–70. https://doi.org/10.1161/ATVBAHA.114.304748.

[70] Park S-J, Im D-S. 2-Arachidonyl-lysophosphatidylethanolamine Induces Anti-Inflammatory Effects on Macrophages and in Carrageenan-Induced Paw Edema. Int J Mol Sci 2021;22. https://doi.org/10.3390/ijms22094865.

[71] Fernández-Castillejo S, Pedret A, Catalán Ú, Valls R-M, Farràs M, Rubió L, et al. Virgin Olive Oil Phenolic Compounds Modulate the HDL Lipidome in Hypercholesterolaemic Subjects: A Lipidomic Analysis of the VOHF Study. Mol Nutr Food Res 2021;65:2001192. https://doi.org/https://doi.org/10.1002/mnfr.202001192.

[72] Lapointe J-F, Harvey L, Aziz S, Jordan H, Hegele RA, Lemieux P. A Single-dose, Comparative Bioavailability Study of a Formulation containing OM3 as Phospholipid and Free Fatty Acid to an Ethyl Ester Formulation in the Fasting and Fed States. Clin Ther 2019;41:426–44. https://doi.org/10.1016/j.clinthera.2019.01.017.

[73] Venturini D, Simão ANC, Urbano MR, Dichi I. Effects of extra virgin olive oil and fish oil on lipid profile and oxidative stress in patients with metabolic syndrome. Nutrition 2015;31:834–40. https://doi.org/10.1016/j.nut.2014.12.016.

[74] EFSA Panel on Dietetic Products N and A (NDA). Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). EFSA J 2012;10:2815. https://doi.org/https://doi.org/10.2903/j.efsa.2012.2815.

[75] Zhao Y, Hasse S, Zhao C, Bourgoin SG. Targeting the autotaxin – Lysophosphatidic acid receptor axis in cardiovascular diseases. Biochem Pharmacol 2019;164:74–81. https://doi.org/https://doi.org/10.1016/j.bcp.2019.03.035.

[76] Begum H, Li B, Shui G, Cazenave-Gassiot A, Soong R, Ong RT-H, et al. Discovering and validating between-subject variations in plasma lipids in healthy subjects. Sci Rep 2016;6:19139. https://doi.org/10.1038/srep19139.

TABLE 1 | PICOS criteria for inclusion and exclusion of studies

Criteria	Inclusion reason	Exclusion reason
Population	- Adult humans (age 18 years or older) - All sexes and races - Healthy subjects, or subjects with associated cardiovascular or cardiometabolic risk factors (type 2 diabetes, obesity, overweight, dyslipidemia, metabolic syndrome, hypertension), or with established CVD (stable CAD)	- Pregnant women - Bariatric surgery - Acute coronary syndrome - Obstructive sleep apnea syndrome - Non-alcoholic fatty liver disease - Chronic kidney disease - Renal disease - Prediabetes - Diabetic nephropathy - Prehypertension - Fabry's disease - Alzheimer's disease
Intervention	- Studies with fatty acid-based dietary interventions. - Any fatty acid type is permitted (SFAs, PUFAs, MUFAs, TFAs) - Fatty acids administered as supplements (capsule), or enriched food-components (dairy- and cereal-based or oily matrixes) and enriched diets - Sustained, postprandial or short-term interventions	- Studies not involving fatty acid-based dietary interventions - Studies assessing the effects of pharmacological treatment only. E.g. statin, insulin sensitizers, Lp-PLA2 inhibitors, ACE inhibitors - Pharmacological and medicinal plant interventions
Comparison	- Placebo capsule - Control arms with dietary components, meals or diets	
Outcomes	- Differences in bioactive lipid levels in terms of lipid class (PCs, PEs, PIs, lyso-PCs, lyso-PEs, lyso-PIs, Cer, diCer, gluCer, lacCer, SM, AC), in terms of lipid subclass (particular bioactive lipids conformed by monounsaturated, polyunsaturated or saturated acyl chains), or in terms of enzymatic precursor, after exposure to dietary fatty acids in healthy subjects or subjects with CVD or different cardiovascular/cardiometabolic risk factors	- Studies that not specify bioactive lipids class or subclass
Study design	- Randomized clinical trials involving controlled and not controlled interventions (RCTs and RTs) - Parallel or cross-over design	- Non-randomized clinical trials - Systematic reviews - Meta-analysis - Case-control studies - Cohort studies
Meta-analysis	- RCTs providing the net difference (molar concentration or abundance) of circulating bioactive lipids or enzymatic precursors after fatty acid intervention compared to placebo or controlled group - Supplemented fatty acid interventions - At least 2 studies for each bioactive lipid or enzymatic precursor in the different clustered cardiovascular/ cardiometabolic risk factors or healthy subjects*	- RCTs or RTs not providing the mean difference, SD or SE values - RCTs or RTs involving interventions with hypocaloric or fatty acid-enriched diets

Abbreviations: ACE, angiotensin-converting enzyme; AC, acylcarnitines; CAD, coronary artery disease; Cer, ceramides; CVD, cardiovascular disease; diCer, dihydroceramides; gluCer, glucosylceramides; lacCer, lactosylceramides; Lp-PLA2, lipoprotein-associated phospholipase A2; lyso-PCs, lysophosphatidylcholines; lyso-PEs, lysophosphatidylethanolamines; lyso-PIs, lysophosphatidylinositols; MUFAs, monounsaturated fatty acids; PCs, phosphatidylcholines; PEs, phosphatidylethanolamines; PIs, phosphatidylinositols; PUFAs, polyunsaturated fatty acids; RCTs, randomized controlled trials; RTs, randomized trials; SD, standard deviation; SE, standard error; SFAs, saturated fatty acids; SM, sphingomyelin; TFAs, trans fatty acids. *Meta-analysis could be performed minimally with two RCTs whenever those two can be meaningfully pooled and provided their results as sufficiently similar.

TABLE 2 | Summary of evaluated evidence for the effect of fatty acid-based dietary interventions on circulating bioactive lipid levels

Outcome	No. of randomized trials	Fatty-acid intervention (n)			[§]Dose range according to fatty-acid intervention	Study subjects, n *(%)*		Overall study quality (n)		
		S	EF-C	ED		Healthy	CVD risk factors	Low	High	Unclear
Phosphatidylcholines, PCs										
All subclasses	11	1	6	4	**S:** SFAs, 7 g/d; MUFAs, 3 g/d; PUFAs, 16 g/d **EF-C:** TFAs, 15.7 g/d; n-3 EPA+DHA, 0.3-0.6 g/d; n-6 LA, 3.3 g/d; n-3 ALA, 1.3 g/d **ED:** PUFAs, 3.3-7.6% TEI; 1.5-24g/d MUFAs, 10.5 % TEI; 1.8-40 g/d SFAs, 7.4 % TEI; 13-67 g/d	74 *(9.9%)*	674 *(90.1%)*	4	1	6
[a] *Containing VLC-PUFAs*	*11*									
[c] *Containing MUFAs*	*6*									
[d] *Containing SFAs*	*4*									
Phosphatidylethanolamines, PEs										
All subclasses	7	1	2	4	**S:** SFAs, 7 g/d; MUFAs, 3 g/d; PUFAs, 16 g/d **EF-C:** PUFAs, 5-24 g/d; MUFAs, 23-40 g/d; SFAs, 37-67 g/d; n-3 EPA+DHA, 0.30-0.61 g/d **ED:** PUFAs, 3.3-7.6 % TEI; 3.2-15.6 g/d; MUFAs, 10.5 % TEI; 1.8-41 g/d; SFAs, 7.4 % TEI; 13-44.6 g/d	59 *(10.5%)*	500 *(89.5%)*	1	1	5
[a] *Containing VLC-PUFAs*	*6*									
[c] *Containing MUFAs*	*3*									
[d] *Containing SFAs*	*1*									
Phosphatidylinositols, PIs										
All subclasses	1	0	1	0	**EF-C:** PUFAs, 5-24 g/d; MUFAs, 23-40 g/d; SFAs, 37-67 g/d	0 *(0%)*	21 *(100%)*	0	0	1
[a] *Containing VLC-PUFAs*	*1*									
[c] *Containing MUFAs*	*1*									
Lysophosphatidylcholines, Lyso-PCs										
All subclasses	14	3	6	5	**S:**	165 *(16.7%)*	823 *(83.3%)*	5	1	8
[a] *Containing VLC-PUFAs*	*3*									

[b] *Containing LC-PUFAs*	*11*				n-3 EPA+DHA, 1.9-3.4 g/d; SFAs, 7 g/d; MUFAs, 31 g/d; PUFAs, 16 g/d **EF-C:** TFAs, 15.7 g/d; n-3 EPA+DHA, 0.3-0.6 g/d; PUFAs, 17.6-24 g/d; n-3 PUFAs, 2.1-3.2 g/d MUFAs, 23-40 g/d; SFAs, 37-67 g/d; n-6 LA, 3.3 g/d; n-3 ALA, 1.3 g/d **ED:** PUFAs, 0.4-7.6 % TEI; MUFAs, 10.5 % TEI; SFAs, 7.4 % TEI					
[c] *Containing MUFAs*	*11*									
[d] *Containing SFAs*	*11*									
Lysophosphatidylethanolamines, Lyso-PEs										
All subclasses	7	2	2	3	**S:** n-3 EPA+DHA, 1.9 g/d **EF-C:** n-3 EPA+DHA, 1-1.6 g/d; PUFAs, 5-24 g/d; MUFAs, 23-40 g/d; SFAs, 37-67 g/d **ED:** PUFAs, 0.4-7.6 % TEI; MUFAs, 10.5 % TEI; SFAs, 7.4 % TEI	49 *(8.5%)*	526 *(91.5%)*	2	1	4
[a] *Containing VLC-PUFAs*	*5*									
[b] *Containing LC-PUFAs*	*5*									
[c] *Containing MUFAs*	*4*									
[d] *Containing SFAs*	*6*									
Lysophosphatidylinositols, Lyso-PIs										
All subclasses	2	1	0	1	**S:** n-3 EPA+DHA, 1.9 g/d **ED:** PUFAs, 0.4-6.2 % TEI	0 *(0%)*	104 *(100%)*	1	0	1
[c] *Containing MUFAs*	*2*									
[d] *Containing SFAs*	*2*									
Ceramide, Cer										
All subclasses	7	2	2	3	**S:** PUFAs, 16 g/d; MUFAs, 31 g/d; SFAs, 7 g/d; SFAs, 33-34 % as MCFAs **EF-C:** n-6 PUFAs, 8.9-64.5 % of FC; PUFAs, 5-24 g/d; MUFAs, 23-40 g/d; SFAs, 37-67 g/d **ED:** PUFAs, 1.5-15.6 g/d; 7.6 % TEI; MUFAs, 1.8-41 g/d; 10.5 % TEI; SFAs, 13-44.6 g/d; 7.4 % TEI	65 *(13.2%)*	426 *(86.8%)*	2	0	5
[a] *Containing VLC-PUFAs*	*1*									
[b] *Containing LC-PUFAs*	*3*									
[c] *Containing MUFAs*	*4*									
[d] *Containing SFAs*	*6*									
Sphingomyelin, SM										
All subclasses	11	2	5	4	**S:**	74 *(12.7%)*	510 *(87.3%)*	5	0	6

[b] *Containing LC-PUFAs*	*3*				PUFAs, 16 g/d; MUFAs, 31 g/d; SFAs, 7 g/d; SFAs, 33-34 % as MCFAs **EF-C:** TFAs, 15.7 g/d; n-3 EPA+DHA, 0.3-0.6 g/d; n-6 PUFAs, 8.9-64.5 % of FC; PUFAs, 5-24 g/d; MUFAs, 23-40 g/d; SFAs, 37-67 g/d; n-6 LA, 3.3 g/d; n-3 ALA, 1.3 g/d **ED:** PUFAs, 1.5-15.6 g/d; 7.6 % TEI; MUFAs, 1.8-41 g/d; 10.5 % TEI; SFAs, 13-44 g/d; 7.4 % TEI					
[c] *Containing MUFAs*	*8*									
[d] *Containing SFAs*	*9*									
Acylcarnitine, AC										
All subclasses	1	1	0	0	**S:** SFAs, 33-34 % as MCFAs	0 *(0%)*	16 *(100%)*	0	0	1
[b] *Containing LC-PUFAs*	*1*									
[d] *Containing SFAs*	*1*									
Lipoprotein-associated phospholipase A2, Lp-PLA2										
	10	8	2	0	**S:** n-3 EPA+DHA, 0.6-5.56 g/d; n-3 ALA, 0.57 g/d Alpha lipoic acid, 1.2 g/d **EF-C:** n-6 LA, 54.2 % of EF-C; PUFAs, 29.2 g/d; MUFAs, 61.3 g/d; SFAs, 6.4 g/d	330 *(21.7%)*	1189 *(78.3%)*	6	1	3

Abbreviations: ALA, alpha-linolenic acid; CVD, cardiovascular disease; DHA, docosahexaenoic acid; ED, enriched diet; EF-C, enriched food component; EPA, eicosapentaenoic acid; LA, linoleic acid; MCFAs, medium-chain fatty acids; MUFAS, monounsaturated fatty acids; PUFAs, saturated fatty acids; S, supplement; SFAs saturated fatty acids; TEI, total energy intake; TFAs, trans-fatty acids.

n: absolute number of studies

a, b, c, d: Fatty acid contained in the sn-2 position of the acyl chain of bioactive lipids: VLC-PUFAs, very long chain polyunsaturated fatty acids; LC-PUFAs, long chain polyunsaturated fatty acids.

[§] The reported dose range depends on the type of intervention and on the units the results are expressed: grams/day, % total energy intake, % of EF-C.

TABLE 3 | Description of included studies assessing the effects of fatty acid-based interventions on bioactive lipid levels in subjects with overweight and obesity

Author, year (reference)	Country	Study design	Population	Fatty acid intervention and daily dose	Comparator	Duration of exposure	Participants *N* (I/C)	Intention-to-treat
Cantero et al. 2018 [28]	Spain	Randomized, open-label, controlled trial. Two sequential periods: 2-month nutritional-learning and 4-month dietary self-control	35-70 y men and women *BMI:* >27 kg/m^2	RESMENA diet based on the Mediterranean dietary pattern reinforcing high n-3 PUFAs. 7 meals/day providing 6.2±0.4 % TEI as PUFAs	Control diet based on the American Heart Association (AHA) guidelines. 3 to 5 meals/day providing 5±0.2 % TEI as PUFAs	6 months	66 (33/33)	No
Del Bas et al. 2016 [22]	United Kingdom	Randomized, double-blind, placebo-controlled parallel trial	18-65 y men and women *BMI:* 30-40 kg/m^2	Marine n-3 PUFAs capsule providing 1.1:0.8 g of EPA:DHA. Daily dose: 1.9 g	Placebo corn-oil capsule Daily dose: 3 g	3 months	38 (19/19)	No
Gürdeniz et al.2013 [38]	Denmark	Randomized, double-blind, controlled trial	45-70 y postmenopausal women *BMI:* 25-32 kg/m^2	Partially hydrogenated soybean oil provided into frozen bread rolls containing 15.7 g of trans fatty acids (TFAs, *trans* 18:1) Daily dose: 26 g oil	Control oil provided intro frozen bread rolls containing a mix of palm oil and high oleic sunflower oil	4 months	52 (25/27)	No
Padro et al. 2015 [46]	Spain	Randomized, double-blind, two-arm cross-over trial. 4-week washout period	25-70 y men and women *BMI:* 25-35 Kg/m^2	• *Intervention sequence 1:* Phytosterol (phyS)-enriched milk + n-3 PUFAs enriched milk Daily dose: 0.37 g EPA +DHA/250 mL • *Intervention sequence 2:* n-3 PUFAs enriched milk + PhyS-enriched milk Daily dose: 0.37 g EPA +DHA/250 mL	Run-in period. Plain low-fat milk free of PhyS and n-3 PUFAs Daily dose: 250 mL	Two 28 days study periods	32	No
Rosqvist et al. 2019 [48]	Sweden	Randomized, double-blind, parallel-assignment trial	20-55 y men and women *BMI:* 25-32 kg/m^2	• *Group 1:* Muffins containing sunflower oil providing 65.4 % of 18:2 n-6 PUFAs Daily dose: 40 g oil • *Group 2:* Muffins containing palm oil providing 53.5 % of 16:0 SFAs (palmitate) and only 8.9 % of n-6 PUFAs Daily dose: 40 g oil	Not controlled	2 months	60 (30/30)	No

Table 3. continued

	Bioactive lipid						
	Assessment method	**Identification reference**	**Class**	**Subclass[$]**	**Molecular formula**	**Changes in concentration attributed to intervention and direction of change**	
Cantero et al. 2018 [28]	Targeted LC-MS (Agilent 6550 Accurate-Mass Quadrupole-Time of Flight (Q-TOF) mass spectrometer (MS))	Calibration curves and internal standards	Lyso-PC	14:0	$C_{22}H_{46}NO_7P$	B: 1.70 (0.1); F: 1.20 (0.1)*, P=0.008	Decreased
				15:0	$C_{23}H_{48}NO_7P$	B: 0.80 (0.05); F: 0.70 (0.04)*, P=0.024	
				16:0	$C_{24}H_{50}NO_7P$	B: 147 (6); F: 136 (8)*, P=0.231	
				16:1	$C_{24}H_{48}NO_7P$	B: 3.70 (0.2); F: 3.0 (0.19)*, P=0.009	
				18:0	$C_{26}H_{54}NO_7P$	B: 78.6 (4.8); F: 65.5 (5)*, P=0.060	
				18:1	$C_{26}H_{52}NO_7P$	B: 55.8 (4.1); F: 49.8 (3.3)*, P=0.189	
				18:2	$C_{26}H_{50}NO_7P$	B: 56.8 (3.2); F: 57.0 (4)*, P=0.856	
				18:4	$C_{26}H_{46}NO_7P$	B: 1.10 (0.06); F: 0.89 (0.05)*, P=0.006	Decreased
				20:1	$C_{28}H_{56}NO_7P$	B: 0.84 (0.06); F: 0.74 (0.04)*, P=0.124	
				20:2	$C_{28}H_{54}NO_7P$	B: 0.83 (0.06); F: 0.70 (0.04)*, P=0.090	
				20:3	$C_{28}H_{52}NO_7P$	B: 9.94 (0.6); F: 8.77 (0.56)*, P=0.214	
				20:4	$C_{28}H_{50}NO_7P$	B: 36.5 (2); F: 31.0 (1.7)*, P=0.026	
			Lyso-PE	18:0	$C_{23}H_{48}NO_7P$	B: 25.7 (1.7); F: 23.0 (1.5)*, P=0.230	
				18:1	$C_{23}H_{46}NO_7P$	B: 3.80 (0.36); F: 3.57 (0.3)*, P=0.627	
				18:2	$C_{23}H_{44}NO_7P$	B: 4.10 (0.3); F: 3.90 (0.4)*, P=0.803	
				20:2	$C_{27}H_{44}NO_7P$	B: 5.20 (0.39); F: 5.10 (0.4)*, P=0.911	
				20:4	$C_{25}H_{50}NO_7P$	B: 3.37 (0.16); F: 3.15 (0.21)*, P=0.427	Decreased
				20:5	$C_{25}H_{42}NO_7P$	B: 0.75 (0.06); F: 0.77 (0.16)*, P=0.917	
				22:5	$C_{27}H_{46}NO_7P$	B: 0.27 (0.0); F: 0.27 (0.0)*, P=0.931	
				22:6	$C_{25}H_{48}NO_7P$	B: 1.90 (0.13); F: 1.66 (0.1)*, P=0.054	
			Lyso-PI	18:0	$C_{27}H_{53}O_{12}P$	B: 2.50 (0.2); F: 2.20 (0.23)*, P=0.367	
				18:1	$C_{27}H_{51}O_{12}P$	B: 0.61 (0.06); F: 0.50 (0.03)*, P=0.105	Decreased
Del Bas et al. 2016 [22]	Targeted LC-MS (Agilent 6550 Accurate-Mass Quadrupole-Time of Flight (Q-TOF) mass spectrometer (MS))	Calibration curves and internal standards	Lyso-PC	16:0	$C_{24}H_{50}NO_7P$	B: 125.7; F: 131.9 (6.74)*, P<0.05	
				18:0	$C_{26}H_{54}NO_7P$	B: 22.11; F: 25.50 (4.41)*, `P<0.05	
				18:1	$C_{26}H_{52}NO_7P$	B: 11.88 ; F: 19.90 (9.76)*, P>0.05	
						B: 13.12; F: 18.00 (8.71)**, P<0.05	
				18:2	$C_{26}H_{50}NO_7P$	B: 0.770; F: 1.790 (0.82)*, P>0.05	
						B: 1.070; F: 1.760 (0.82)**, P<0.05	Increased
				20:1	$C_{28}H_{56}NO_7P$	B: 0.177; F: 0.186 (0.07)*, P<0.05	
				20:2	$C_{28}H_{54}NO_7P$	B: 0.470; F: 0.524 (0.11)*, P>0.05	
						B: 0.525; F: 0.550 (0.10)**, P<0.05	
				20:3	$C_{28}H_{52}NO_7P$	B: 1.446; F: 1.500 (0.52)*, P>0.05	
						B: 1.260; F: 1.760 (0.56)**, P<0.05	
				20:4	$C_{28}H_{50}NO_7P$	B: 2.870; F: 4.070 (1.52)*, P>0.05	
			Lyso-PE	18:0	$C_{23}H_{48}NO_7P$	B: 0.056; F: 0.089 (0.08)*, P>0.05	
				18:1	$C_{23}H_{46}NO_7P$	B: 0.277; F: 0.421 (0.11)*, P>0.05	
				18:2	$C_{23}H_{44}NO_7P$	B: 0.394; F: 0.802 (0.19)*, P>0.05	Increased
				20:2	$C_{27}H_{44}NO_7P$	B: 0.766; F: 1.130 (0.21)*, P>0.05	
				20:4	$C_{25}H_{50}NO_7P$	B: 0.277; F: 0.421 (0.05)*, P>0.05	

				20:5		B: 0.357; F: 0.467 (0.10)**, P<0.05	
				22:5	$C_{25}H_{42}NO_7P$	B: 0.118; F: 0.135 (0.08)*, P>0.05	Increased
				22:6	$C_{27}H_{46}NO_7P$	B: 0.084; F: 0.097 (0.009)*, P>0.05	
					$C_{25}H_{48}NO_7P$	B: 0.355; F: 0.379 (0.04)*, P>0.05	
				18:0			
			Lyso-PI	18:1	$C_{27}H_{53}O_{12}P$	B: 0.101; F: 0.109 (0.02)*, P>0.05	Increased
					$C_{27}H_{51}O_{12}P$	B: 0.081; F: 0.109 (0.01)*, P>0.05	
Gürdeniz et al.2013 [38]	Untargeted UPLC-MS (coupled to Quadrupole-Time of Flight (Q-TOF) mass spectrometer (MS)) combined with NMR analysis	MZmine (version 2.7) MATLAB (version 7.2)	PC	40:7	$C_{48}H_{82}NO_8P$	Not reported	
				40:6	$C_{48}H_{84}NO_8P$		
				44:9	$C_{52}H_{86}NO_8P$		
				38:4	$C_{46}H_{84}NO_8P$		
				38:5	$C_{46}H_{82}NO_8P$		Increased
				trans18:1/20:3	$C_{46}H_{84}NO_8P$		
				trans18:1/22:4	$C_{48}H_{86}NO_8P$		
				trans18:1/22:5	$C_{48}H_{84}NO_8P$		
				trans18:1/22:6	$C_{48}H_{82}NO_8P$		
			SM	36:3 (d18:2/18:1)	$C_{41}H_{79}N_2O_6P$		Increased
			Lyso-PC	18:1	$C_{26}H_{52}NO_7P$		Increased
Padro et al. 2015 [46]	Targeted LC/MS/MS (Agilent 1200) coupled to a AB Sciex 3200 Qtrap triple quadrupole mass spectrometer. On isolated LDL fraction	LipidView (version 1.1)	Lyso-PC	16:0	$C_{24}H_{50}NO_7P$	B: 39.40; F: 47.20 (32.06)#, P=0.346	Increased
				18:0	$C_{26}H_{56}NO_6P$	B: 12.50; F: 23.50 (19.56)#, P=0.040	
			PC	36:5	$C_{44}H_{78}NO_8P$	B: 52.3; F: 90.5 (49.34)#, P=0.008	Increased
			SM	38:1	$C_{43}H_{87}N_2O_6P$	B: 43.4; F: 72.0 (39.45)#, P=0.011	Increased
Rosqvist et al. 2019 [48]	Targeted UPLC-MS	Not described	Cer	18:0	$C_{36}H_{71}NO_3$	-3.27 (28.31)¥, p<0.05 (n-6 PUFA)	
				20:0	$C_{38}H_{75}NO_3$	-9.25 (11.02)¥, P<0.05 (n-6 PUFA)	Decreased
				24:1	$C_{42}H_{81}NO_3$	-213.14 (232.9)¥, P<0.01 (n-6 PUFA)	
			DiCer	16:0	$C_{34}H_{69}NO_3$	+1.96 (5.50)¥, P<0.05 (SFA)	Increased
						-1.54 (8.43) ¥,P<0.05 (n-6 PUFA)	
				18:0	$C_{36}H_{73}NO_3$	-1.80 (5.19)¥, P<0.05 (n-6 PUFA)	Decreased
				20:0	$C_{38}H_{77}NO_3$	-1.40 (4.81)¥, P<0.05 (n-6 PUFA)	
				24:1	$C_{42}H_{83}NO_3$	-18.70 (23.12)¥, P<0.01 (n-6 PUFA)	
			GluCer	16:0	$C_{44}H_{87}NO_3$	+72.22 (62.68)¥, P<0.05 (SFA)	Increased
						-16.94 (57.05)¥, P<0.05 (n-6 PUFA)	
				18:0	$C_{42}H_{81}NO_8$	-5.34 (5.48)¥ ,P<0.05 (n-6 PUFA)	Decreased
				20:0	$C_{44}H_{85}NO_8$	-9.28 (5.84)¥, P<0.05 (n-6 PUFA)	
				24:0	$C_{48}H_{93}NO_8$	+48.47 (135.84)¥, P<0.05 (SFA)	Increased
						-55.44 (86.13)¥, P<0.05 (n-6 PUFA)	Decreased
				24:1	$C_{48}H_{91}NO_8$	-118.7 (65.92)¥, P<0.01 (n-6 PUFA)	
			LacCer	18:0	$C_{48}H_{91}NO_{13}$	-2.20 (2.80)¥, P<0.05 (n-6 PUFA)	

	20:0	$C_{50}H_{95}NO_{13}$	-0.93 (1.14)[¥], P<0.05 (n-6 PUFA)	Decreased
	24:1	$C_{54}H_{101}NO_{13}$	-87.50 (142.5)[¥], P<0.01 (n-6PUFA)	
SM	16:0	$C_{39}H_{79}N_2O_6P$	+10.68 (12.23)[¥], P<0.05 (SFA)	
	16:1	$C_{21}H_{43}N_2O_6P$	+1.31 (1.64)[¥], P<0.05 (SFA)	Increased
	18:0	$C_{41}H_{83}N_2O_6P$	+0.44 (2.76) [¥], P<0.05 (SFA)	
	18:1	$C_{41}H_{81}N_2O_6P$	+0.30 (1.25)[¥], P<0.05 (SFA)	

[$] Only significant subclasses or those that are comparable among the included studies are shown. [*] Mean expressed as µM (SD), effect after n-3 PUFAs intervention or [**] after corn oil intervention. [#] Mean of LDL lipid-specimens expressed as µg/100mL (SD), effect after n-3 PUFAs-enriched milk intervention. [¥] Mean expressed as nM (SD), reported change after-before n-6 PUFAs or SFAs intervention. Abbreviations: B, baseline value; BMI, body mass index; C, control group; Cer, ceramide; DHA, docosahexaenoic acid; DiCer, dihydroceramide; EPA, eicosapentaenoic acid; F; final value; GluCer; glucosylceramide; I, intervention group; LacCer, lactosylceramide; Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine; Lyso-PI, lysophosphatidylinositol; PC, phosphatidylcholine; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids; SM, sphingomyelin; TEI, total energy intake; TFAs, trans fatty acids.

TABLE 4 | Description of included studies assessing the effects of fatty acid-based interventions on bioactive lipid levels in subjects with dyslipidemia

Author, year (reference)	**Country**	**Study design**	**Population**	**Fatty acid intervention and daily dose**	**Comparator**	**Duration of exposure**	**Participants** *N* (I/C)	**Intention-to-treat**
Padro et al. 2015 [46]	Spain	Randomized, double-blind, two-arm cross-over trial. 4-week washout period	25-70 y men and women *High-LDL group:* 160.4 (4.7) mg//dL *Low-LDL group:* 113.3 (4.2) mg/dL *AT-DYSL group:* TG: 150 - 290 mg/dL HDL: <40 in male or <50 in female	• *Intervention sequence 1:* Phytosterol (phyS)-enriched milk + n-3 PUFAs enriched milk Daily dose: 0.37 g EPA +DHA/250 mL • *Intervention sequence 2:* n-3 PUFAs enriched milk + PhyS-enriched milk Daily dose: 0.37 g EPA +DHA/250 mL	Run-in period. Plain low-fat milk free of PhyS and n-3 PUFAs Daily dose: 250 mL	Two 28 days study periods	32	No
Ruuth et al. 2020 [33]	Finland	Randomized, double-blind, placebo-controlled parallel trial	24-66 y men and women *LDL aggregate size:* 1490 (3000) nm *LDL particle concentration:* 1355 (1939) nmol/L *LDL:* 135 (31) mg/dL *HDL:* 69.6 (19) mg/dL	Plant stanol ester-enriched rapeseed oil-based spread (STAEST) *(Raisio Nutrition Ltd)* Daily dose: 20 g (3 g plant stanols + 3.3 g n-6 LA + 1.3 g n-3 ALA)	Control spread without added plant stanols Daily dose: 20 g	6 months	90 (44/16)	No
Zhang et al. 2012 [39]	China	Randomized, open-label, parallel-group controlled dietary intervention study	35-70 y women *TG:* 200 - 216 mg/dL *LDL:*148 - 154 mg/dL *HDL:* 48 - 52 mg/dL	• *Group 1:* Experimental lunch containing Norwegian salmon (SM), provided in servings of 80 g, 5 d/wk Daily dose: 17.6 g PUFAs (1.6 g EPA+DHA) • *Group 2:* Experimental lunch containing herring (HR), provided in servings of 80 g, 5 d/wk Daily dose: 17.5 g PUFAs (1.6 g EPA+DHA) • *Group 3:* Experimental lunch containing local farmed pompano (PP), provided in servings of 80 g, 5 d/wk Daily dose: 17.6 g PUFAs (1 g EPA+DHA)	Commonly eaten control meals mix containing pork/chicken/beef/lean fish, provided in servings of 80 g, 5 d/wk Daily dose: 16.6 g PUFAs (1.1 g n-3)	2 months	126 (32/39/33/32)	No
Dunbar et al. 2015 [40]	Philadelphia, USA	Randomized, double-blind, placebo-controlled trial	≥18 y men and women *TG:* 200 - 500 mg/dL	• *Group 1:* n-3 PUFA carboxylic acids plus olive oil capsule (OM3-	Placebo olive oil capsule combined with usual statin therapy	6 weeks	627 (209/207/211)	Yes

				CA+OO) combined with usual statin therapy Daily dose: 4 g (2 g OM3+2 g OO) • *Group 2:* n-3 PUFAs capsule (OM3-CA) combined with usual statin therapy Daily dose: 4 g	Daily dose: 4 g			
Gajos et al. 2013 [41]	Poland	Randomized, double-blind, placebo-controlled study	30-80 y men and women *TG:* ≥ 150 mg/dL *LDL:* ≥ 140 mg/dL *HDL:* < 40 mg/dL	n-3 PUFA carboxylic acids capsule providing 460:380 mg of EPA:DHA *(Omacor; Pronova Biocare)* combined with usual Aspirin and Clopidogrel therapy Daily dose: 1 g	Placebo soybean oil capsule combined with usual Aspirin and Clopidogrel therapy Daily dose: 1 g	1 month	54 (30/24)	No
Hedengran et al. 2014 [42]	Denmark	Randomized, double-blind, placebo-controlled parallel-arm interventional trial	≥18 y men and women *TG:* 264 (97) mg/dL *HDL:* 44 (11) mg/dL	• *Group 1:* AG n-3 PUFAs soft capsule providing 767:1930 mg of EPA:DHA *(Lipomar®, Marine Ingredients, Mount Bethel)* Daily dose: 5.56 g • *Group 2:* EE n-3 PUFAs soft capsule providing 1702:1382 mg of EPA:DHA *(Omacor®/Lovaza®,Pronova BioPharma)* Daily dose: 4 g	Placebo olive oil capsule Daily dose: 4.6 g	2 months	119 (39/40/40)	Yes
Mosca et al. 2017 [43]	New York, USA	Randomized, double-blind, placebo-controlled trial	49-73 y women *HDL:* 18 - 65 mg/dL *LDL:* 55 - 196 mg/dL *High TG group:* 200 - 500 mg/dL *Very high TG group:* 500 - 2000 mg/dL	n-3 EPA-EE capsule *(AMR101)* containing ≥96 % EPA ethyl ester and no DHA Daily dose: 4 g	Placebo capsule containing light liquid paraffin Daily dose: 2 g	3 months	215 (109/106)	Yes

Table 4. continued	**Bioactive lipids**					
	Assessment method	**Identification reference**	**Class**	**Subclass[$]**	**Molecular formula**	**Changes in concentration attributed to intervention and direction of change**

Padro et al. 2015 [46]	Targeted LC/MS/MS (Agilent 1200) coupled to an AB Sciex 3200 Qtrap mass spectrometer. On isolated LDL fraction	LipidView (version 1.1)	Lyso-PC	16:0	$C_{24}H_{50}NO_7P$	B: 39.40; F: 47.20 (32.06), P=0.346	Increased
				18:0	$C_{26}H_{56}NO_6P$	B: 12.50; F: 23.50 (19.56), P=0.040	
			PC	36:5	$C_{44}H_{78}NO_8P$	B: 52.3; F: 90.5 (49.34), P=0.008	Increased
			SM	38:1	$C_{43}H_{87}N_2O_6P$	B: 43.4; F: 72.0 (39.45), P=0.011	Increased
Ruuth et al. 2020 [33]	Targeted LC/MS/MS combined with electrospray ionization (Agilent 6410 Triple Quad LC/MS; Agilent Technologies). On isolated LDL particles	Lipid Mass Spectrum Analysis software and internal standards to quantify the molar % of H surface lipids	SM	14:0	$C_{37}H_{75}N_2O_6P$	B: 2.11 (0.46); F: 2.16 (0.57)*, P<0.05	
				15:0	$C_{38}H_{77}N_2O_6P$	B: 0.80 (0.30); F: 0.89 (0.29)*, P<0.01	
				16:0	$C_{39}H_{79}N_2O_6P$	B: 29.83 (1.94); F: 29.94 (2.28)*,P<0.01	Increased
				16:1	$C_{39}H_{77}N_2O_6P$	B: 2.79 (0.46); F: 2.89 (0.48)*, P<0.01	
				18:0	$C_{41}H_{83}N_2O_6P$	B: 6.02 (0.89); F: 6.05 (0.98)*, P<0.05	
				18:1	$C_{41}H_{83}N_2O_6P$	B: 2.04 (0.41); F: 2.00 (0.46)*, P<0.01	Decreased
				23:0	$C_{46}H_{95}N_2O_6P$	B: 8.04 (1.28); F: 8.20 (1.43)*, P<0.05	Increased
				23:1	$C_{46}H_{93}N_2O_6P$	B: 6.06 (1.01); F: 5.97 (0.86)*, P<0.05	Decreased
				24:0	$C_{47}H_{95}N_2O_6P$	B: 7.75 (1.47); F: 7.82 (1.74)*, P<0.01	Increased
				24:1	$C_{47}H_{93}N_2O_6P$	B: 25.85 (2.12); F: 25.88 (2.47)*,P<0.01	
			PC	24:2	$C_{47}H_{91}N_2O_6P$	B: 8.02 (2.07); F: 7.97 (2.17)*, P<0.01	
				34:2	$C_{42}H_{80}NO_8P$	B: 7.20 (1.32); F: 7.36 (1.44)*, P<0.01	
				34:3	$C_{42}H_{78}NO_8P$	B: 3.26 (1.42); F: 3.55 (1.33)*, P<0.01	Increased
				36:3	$C_{44}H_{82}NO_8P$	B: 3.94 (0.79); F: 4.14 (0.87)*, P<0.01	
				36:5	$C_{44}H_{78}NO_8P$	B: 4.19 (1.36); F: 4.36 (1.38)*, P<0.01	
				38:5	$C_{46}H_{82}NO_8P$	B: 8.11 (1.54); F: 8.57 (2.00)*, P<0.01	
				38:6	$C_{46}H_{80}NO_8P$	B: 3.64 (1.57); F: 3.63 (1.40)*, P<0.01	Decreased
				40:7	$C_{48}H_{82}NO_8P$	B: 0.23 (0.10); F: 0.22 (0.07)*, P<0.05	
			Lyso-PC	16:0	$C_{24}H_{50}NO_7P$	B: 55.00 (5.27); F: 55.09 (5.73)*,P<0.05	Increased
				18:0	$C_{26}H_{54}NO_7P$	B: 25.22 (3.67); F: 24.84 (4.42)*,P<0.05	Decreased
				18:1	$C_{26}H_{52}NO_7P$	B: 8.50 (2.69); F: 8.30 (2.03)*, P<0.05	
				18:2	$C_{26}H_{50}NO_7P$	B: 9.78 (2.85); F: 10.09 (3.92)*, P<0.05	
				20:3	$C_{28}H_{52}NO_7P$	B: 0.07 (0.19); F: 0.08 (0.22)*, P<0.01	Increased
				20:4	$C_{28}H_{50}NO_7P$	B: 1.40 (2.00); F: 1.57 (2.14)*, P<0.05	
				20:5	$C_{28}H_{48}NO_7P$	B: 0.03 (0.09); F: 0.04 (0.10)*, P<0.05	
Zhang et al. 2012 [39]	TLC silica gel plates spraying with a methanolic solution and visualized under UV light. Combined with GC (Shimadzu GC 14B) fitted with a capillary column (CP-SIL 88 (VARIAN)) to separate fatty acid methyl esters.	Comparison with retention times of commercially available fatty acid standards, and quantification of peak areas with a computer data system (CBM-101	Lyso-PC	18:2	$C_{26}H_{50}NO_7P$	B: 24.5 (0.4); F: 21.6 (0.4)**, P=0.05 after SM B: 24.1 (0.7); F: 22.3 (0.4))**,P=0.05 after HR B: 24.9 (0.5); F: 22.4 (0.6)**, P=0.05 after PP	
				20:4	$C_{28}H_{50}NO_7P$	B: 9.9 (0.4); F: 8.7 (0.3)**, P<0.05 after SM B: 8.9 (0.4); F: 8.1 (0.4)**, P=0.05 after HR B: 10.0 (0.4); F: 9.0 (0.3)**, P=0.05 after PP	Decreased
				20:5	$C_{28}H_{48}NO_7P$	B: 2.6 (0.2); F: 3.6 (0.3)**, P<0.05 after SM B: 2.4 (0.2); F: 3.6 (0.3)**, P<0.01 after HR B: 2.0 (0.1); F: 2.9 (0.2)**, P=0.05 after PP	Increased
				22:6	$C_{30}H_{50}NO_7P$	B: 2.3 (0.2); F: 3.8 (0.2)**, P<0.01 after SM B: 2.2 (0.2); F: 3.5 (0.3)**, P<0.01 after HR B: 2.4 (0.1); F: 3.6 (0.2)**, P<0.01 after PP	Increased

workstation; Shimadzu).

Table 4. continued

	Bioactive lipid enzymatic precursors					
	Assessment method	**Class**	**Subclass**		**Changes in mass attributed to intervention and direction of change**	
Dunbar et al. 2015 [40]	Latex particle-enhanced turbidimetric immunoassay on a Roche-P modular analyzer, PLAC™ test (diaDexus, Inc. South San Francisco, CA, USA)	Platelet-activating factor	Lipoprotein-associated phospholipase A2 enzyme	Lp-PLA2	B: 218 (54.7); F: 205 (52.5)[#], P<0.001 after OM3-CA+OO B: 216 (50.1); F: 194 (51.4)[#], P<0.001 after OM3-CA	Decreased
Gajos et al. 2013 [41]	PLAC ELISA kit (diaDexus, Inc. South San Francisco, CA, USA)				B: 260 (68); F: 240 (64)[†], P=0.026 and P=0.041	Decreased
Hedengran et al. 2014 [42]	Colorimetric activity method, CAM assay (diaDexus, San Francisco, CA, USA)				B: 260 (102); F: 248 (104)[‡], P=0.563 after AG n-3 PUFA B: 247 (101); F: 228 (109)[‡], P<0.001 after EE n-3 PUFA	Decreased
Mosca et al. 2017 [43]	PLAC ELISA kit (diaDexus, Inc. South San Francisco, CA, USA)				B: 177 (53); F: 154 (44)[⁋], P<0.0001 ANCHOR experimental group B: 234 (177); F: 201 (88)[⁋], P=0.652 MARINE experimental group	Decreased

[$] Only significant subclasses or those that are comparable among the included studies are shown. [⁋] Mean of LDL lipid-specimens expressed as µg/100mL (SD), effect after n-3 PUFAs-enriched milk intervention. [*] Mean expressed as molar % of LDL surface lipid subclasses (SD), effect after STAEST intervention [**] Mean of plasma Lyso-PC subclasses expressed as % of fatty acids 18:2n-6, 20:4n-6, 20:5n-3 and 22:6n-3 (SEM), effect after the three oily fish interventions. [#] Mean expressed as ng/mL (SD), effect of OM3-CA 2g and 4g compared to placebo. [†] Mean expressed as ng/mL (SD), effect after n-3 PUFAs intervention and effect compared to placebo. [‡] Mean expressed as nmol/mL (SD), effect of AG and EE n-3 PUFAs compared to placebo. [⁋] Mean expressed as ng/mL (SD), effect of n-3 eicosapentaenoic EE intervention compared to placebo. Abbreviations: AG, acylglycerol; AT-DYSL, atherogenic dyslipidemia; B: baseline value; C, control group; DHA; docosahexaenoic acid; EE, ethyl esters; EPA, eicosapentaenoic acid; F, final value; HDL, high-density lipoprotein cholesterol; I, intervention group; LDL; low-density lipoprotein cholesterol; Lyso-PC, lysophosphatidylcholine; PC, phosphatidylcholine; PUFAs, polyunsaturated fatty acids; SM, sphingomyelin; TG, triglyceride. Baseline dyslipidemia features are described in the Population column (mean ± SD or range of mean values).

TABLE 5 | Description of included studies assessing the effects of fatty acid-based interventions on bioactive lipid levels in subjects with metabolic syndrome

Author, year (reference)	**Country**	**Study design**	**Population**	**Fatty acid intervention and daily dose**	**Comparator**	**Duration of exposure**	**Participants** *N* (I/C)	**Intention-to-treat**
Bondia-Pons et al. 2014 [45]	Italy	Randomized, open-label, parallel-group controlled trial. Three different isoenergetic diets. Preceded by a 3-week run-in stabilization period with the habitual diet	35-75 y men and women *BMI:* 27 - 35 Kg/m^2 *WC:* 101 - 105 cm *TG:* 120 - 138 mg/dL *HDL:* 41 - 44 mg/dL *FBG:* 100 – 104 mg/dL *SBP:* 119 - 126 mmHg *DBP:* 73 - 76 mmHg	• *Group 1:* Diet rich in LCn-3 PUFAs and low in polyphenols providing 4.2±0.0 % TEI as PUFAs (0.43/0.58 % TEI as EPA/DHA) • *Group 2:* Diet rich in polyphenols and low in LCn-3 PUFAs providing 3.3±0.2 % TEI as PUFAs (0.04/0.01 % TEI as EPA/DHA) • *Group 3:* Diet rich in LCn-3 PUFAs and polyphenols providing 4.2±0.1 % TEI as PUFAs (0.47/0.60 % TEI as EPA/DHA)	Diet low in LCn-3 PUFAs and polyphenols providing 3.6±0.1 % TEI as PUFAs (0.02/0.01 % TEI as EPA/DHA)	2 months	78 (19/20/19/20)	Yes
Bondia-Pons et al. 2015 [44]	Spain	Randomized, open-label, controlled trial. Two sequential periods: 2-month nutritional-learning and 4-month dietary self-control	35-70 y men and women *BMI:* 35.4 (4.4) Kg/m^2 *WC:* 110.9 (12) cm *TG:* 194 (124) mg/dL *HDL:* 43 (10) mg/dL *FBG:* 124 (38) mg/dL *SBP:* 147.2 (20.7) mmHg *DBP:* 84.2 (9) mmHg	RESMENA diet based on the Mediterranean dietary pattern reinforcing high n-3 PUFAs. 7 meals/day providing 6.2±0.4 % TEI as PUFAs	Control diet based on the American Heart Association (AHA) guidelines. 3 to 5 meals/day providing 5±0.2 % TEI as PUFAs	6 months	66 (33/33)	No
Cantero et al. 2018 [28]	Spain	Randomized, open-label, controlled trial. Two sequential periods: 2-month nutritional-learning and 4-month dietary self-control	35-70 y men and women *BMI:* 34.8 (4) Kg/m^2 *WC:* 110 (13) cm *TG:* 198.6 (120) mg/dL *HDL:* 46.5 (10) mg/dL *FBG:* 121.5 (37) mg/dL *SBP:* 153.3 (21) mmHg *DBP:* 84.4 (9) mmHg	RESMENA diet based on the Mediterranean dietary pattern reinforcing high n-3 PUFAs. 7 meals/day providing 6.2±0.4 % TEI as PUFAs	Control diet based on the American Heart Association (AHA) guidelines. 3 to 5 meals/day providing 5±0.2 % TEI as PUFAs	6 months	66 (33/33)	No
Meikle et al. 2015 [51]	Australia	Randomized, postprandial, cross-over trial 4 to 6-week washout period	40-60 y men *BMI:* 30.1 (5.7) Kg/m^2 *WC:*104 (13) cm *TG:* 1.6 (0.5) mg/dL *HDL:* 1.2 (0.2) mg/dL *FBG:* 97 (11) mg/dL	• *Intervention sequence 1:* High-fat breakfast meal consisting on dairy-based foods (cheddar cheese, butter, extra creamy milk) Daily dose: 67 g:23 g:5 g of SFAs:MUFAs:PUFAs • *Intervention sequence 2:* High-fat breakfast meal consisting on soy oil-based foods	Not controlled	Two 4-hour postprandial test meals	21	No

(cheddar flavoured soy cheese, soy beverage, non-dairy spread) Daily dose: 37 g:40 g:24 g of SFAs:MUFAs:PUFAs

Table 5. continued

	Bioactive lipid						
	Assessment method	**Identification reference**	**Class**	**Subclass[$]**	**Molecular formula**	**Changes in concentration attributed to intervention and direction of change**	
Bondia-Pons et al. 2014 [45]	Untargeted UPLC-MS (coupled to Quadrupole-Time of Flight mass spectrometer (Q-TOF/MS)). On plasma and isolated HDL fraction	MZmine 2 combined with an internal spectral library	PC	32:2	$C_{40}H_{76}NO_8P$	-0.10-0.45*, P<0.005 after control diet	Decreased
				36:4	$C_{44}H_{80}NO_8P$		
				38:3	$C_{46}H_{86}NO_8P$		
				40:4	$C_{48}H_{88}NO_8P$	+0.10-0.70*, P<0.0005 after high LCn-3 PUFA diets	Increased
				40:6	$C_{48}H_{84}NO_8P$		
			PE	36:2	$C_{41}H_{80}NO_7P$	-0.10-0.45*, P<0.005 after control diet	Decreased
				40:3	$C_{45}H_{84}NO_8P$		
				38:6	$C_{43}H_{84}NO_8P$	+0.10-0.70*, P<0.0005 after high LCn-3 PUFA diets	Increased
				40:4	$C_{45}H_{84}NO_7P$		
			LysoPC	18:0	$C_{26}H_{56}NO_6P$	-0.10-0.45*, P<0.005 after control diet	Decreased
				20:4	$C_{28}H_{50}NO_7P$		
				20:5	$C_{28}H_{48}NO_7P$	+0.70*, P<0.0005 after high LCn-3 PUFA diets	Increased
Bondia-Pons et al. 2015 [44]	Untargeted UPLC-MS (coupled to Quadrupole-Time of Flight mass spectrometer (Q-TOF/MS), Agilent 1290/6540)	Human Metabolome Database METLIN Metabolite database Mass-Bank database	PC	34:4	$C_{42}H_{76}NO_8P$	P=0.024** after 2- and 6-month	
				32:2	$C_{40}H_{76}NO_8P$	P<0.0001** after 2- and 6-month	
				32:1	$C_{40}H_{78}NO_8P$	P=0.0001** after 2- and 6-month	
				36:4	$C_{44}H_{80}NO_8P$	P=0.036** after 2-month	Decreased
				34:2	$C_{42}H_{80}NO_8P$	P=0.028** after 2- and 6-month	
				38:3	$C_{46}H_{36}NO_8P$	P=0.0001** after 2-month	
				36:2	$C_{44}H_{84}NO_8P$	P=0.040** after 2- and 6-month	
				38:4	$C_{46}H_{84}NO_7P$	P=0.001** after 2-month	Increased
			PE	32:2	$C_{37}H_{74}NO_8P$	P=0.021** after 2- and 6-month	Decreased
			LysoPC	14:0	$C_{22}H_{46}NO_7P$	P<0.0001** after 2-month	
				15:0	$C_{23}H_{48}NO_7P$	P<0.0001** after 6-month	
				16:1	$C_{24}H_{48}NO_7P$	P=0.0003** after 2-month	Decreased
				20:3	$C_{28}H_{52}NO_7P$	P=0.002** after 2-month	
				20:4	$C_{28}H_{50}NO_7P$	P=0.002** after 2-month	
				16:0	$C_{24}H_{50}NO_7P$	P=0.023** after 6-month	
				18:0	$C_{26}H_{54}NO_7P$	P=0.027** after 6-month	
				18:1	$C_{26}H_{52}NO_7P$	P=0.003** after 6-month	Increased
				20:5	$C_{28}H_{48}NO_7P$	P=0.026** after 2- and 6-month	
				22:6	$C_{30}H_{50}NO_7P$	P=0.009** after 6-month	
			LysoPE	18:1	$C_{23}H_{46}NO_7P$	P=0.0004** after 2-month	Decreased

				18:2	$C_{23}H_{44}NO_7P$	P<0.0001** after 2-month	
Cantero et al. 2018 [28]	Targeted LC-MS (Agilent 6550 Accurate-Mass Quadrupole-Time of Flight (Q-TOF) mass spectrometer (MS))	Calibration curves and internal standards	LysoPC	14:0	$C_{22}H_{46}NO_7P$	B: 1.70 (0.1); F: 1.20 (0.1)#, P=0.008	
				15:0	$C_{23}H_{48}NO_7P$	B: 0.80 (0.05); F: 0.70 (0.04)#, P=0.024	
				16:0	$C_{24}H_{50}NO_7P$	B: 147 (6.00); F: 136 (8)#, P=0.231	
				16:1	$C_{24}H_{48}NO_7P$	B: 3.70 (0.20); F: 3.00 (0.19)#, P=0.009	Decreased
				18:0	$C_{26}H_{54}NO_7P$	B: 78.6 (4.80); F: 65.5 (5)#, P=0.060	
				18:1	$C_{26}H_{52}NO_7P$	B: 55.8 (4.10); F: 49.8 (3.3)#, P=0.189	
				18:2	$C_{26}H_{50}NO_7P$	B: 56.8 (3.20); F: 57.0 (4)#, P=0.856	
				18:4	$C_{26}H_{46}NO_7P$	B: 1.10 (0.06); F: 0.89 (0.05)#, P=0.006	
				20:1	$C_{28}H_{56}NO_7P$	B: 0.84 (0.06); F: 0.74 (0.04)#, P=0.124	
				20:2	$C_{28}H_{54}NO_7P$	B: 0.83 (0.06); F: 0.70 (0.04)#, P=0.090	
				20:3	$C_{28}H_{52}NO_7P$	B: 9.94 (0.6); F: 8.77 (0.56)#, P=0.214	Decreased
				20:4	$C_{28}H_{50}NO_7P$	B: 36.5 (2.00); F: 31.0 (1.70)#, P=0.026	
				22:5	$C_{30}H_{52}NO_7P$	B: 1 (0.07); F: 0.98 (0.05)#, P=0.256	
				22:6	$C_{30}H_{50}NO_7P$	B: 7.8 (0.50); F: 6.6 (0.50)#, P=0.093	
			LysoPE	18:0	$C_{23}H_{48}NO_7P$	B: 25.7 (1.7); F: 23.0 (1.5)#, P=0.230	
				18:1	$C_{23}H_{46}NO_7P$	B: 3.80 (0.36); F:.57 (0.3)#, P=0.627	
				18:2	$C_{23}H_{44}NO_7P$	B: 4.10 (0.3); F: 3.90 (0.4)#, P=0.803	Decreased
				20:2	$C_{27}H_{44}NO_7P$	B: 5.20 (0.39); F: 5.10 (0.4)#, P=0.911	
				20:4	$C_{25}H_{50}NO_7P$	B: 3.37 (0.16); F: 3.15 (0.21)#, P=0.427	
				20:5	$C_{25}H_{42}NO_7P$	B: 0.75 (0.06); F: 0.77 (0.16)#, P=0.917	
				22:5	$C_{27}H_{46}NO_7P$	B: 0.27 (0.0); F: 0.27 (0.0)#, P=0.931	Increased
				22:6	$C_{25}H_{48}NO_7P$	B: 1.90 (0.13); F:1.66 (0.1)#, P=0.054	Decreased

Meikle et al. 2015 [51]	Targeted LC/MS (Agilent 1200 LC system combined with an Applied Biosystems API 4000 Q/TRAP mass spectrometer) Semi-quantitative	Analyst (version 1.5) LIPID MAPS library	Cer	18:0	$C_{36}H_{71}NO_3$	-16 (-27, -3.6)†, P=0.070 after soy	Decreased
			DiCer	18:0	$C_{36}H_{73}NO_3$	-20 (-36, 2.9)†, P=0.030 after soy	
			SM	32:0	$C_{37}H_{77}N_2O_6P$	-12 (-19, -5.5)†, P=0.007 after soy +8.1 (-3.4, 18)†, P<0.001 after dairy	
				32:1	$C_{37}H_{75}N_2O_6P$	-3.6 (-12, 4.4)†, P=0.022 after soy +5.4 (-0.4, 13)†, P<0.001 after dairy	
				32:2	$C_{37}H_{73}N_2O_6P$	-5.7 (-13, 1.4)†, P=0.016 after soy +3.4 (-2, 10)†, P<0.001 after dairy	
				34:0	$C_9H_{19}N_2O_6P$	-15 (-21, -4.8)†, P=0.007 after soy +14 (-0.9, 30)†, P<0.001 after dairy	Decreased/ Increased
				35:1	$C_{40}H_{81}N_2O_6P$	-8.5 (-16, 2.4)†, P=0.05 after soy +2.5 (-4.7, 11)†, P<0.001 after dairy	
				36:2	$C_{41}H_{81}N_2O_6P$	-6.1 (-16, 2)†, P=0.021 after soy +6.9 (-5.7, 14)†, P<0.001 after dairy	
				38:1	$C_{43}H_{87}N_2O_6P$	-9.3 (-20, 0)†, P=0.030 after soy +1.3 (-5.7, 12)†, P<0.001 after dairy	
			PC	28:0	$C_{36}H_{72}NO_8P$	-13 (-22, -3.1)†, P=0.007 after soy +43 (13, 94)†, P<0.001 after dairy	Decreased/ Increased
				29:0	$C_{37}H_{74}NO_8P$	-14 (-25, 4.6)†, P=0.010 after soy +21 (0.0, 69)†, P<0.001 after dairy	
				30:0	$C_{38}H_{76}NO_8P$	-12 (-24, -4.4)†, P=0.007 after soy +18 (0.5, 32)†, P<0.001 after dairy	
				31:0	$C_{39}H_{78}NO_8P$	-7.1 (-16, 1.9)†, P=0.022 after soy +7.3 (-3.2, 23)†, P<0.001 after dairy	Decreased/ Increased
				32:0	$C_{40}H_{80}NO_8P$	-9.7 (-21, 4.1)†, P=0.012 after soy +10 (-20.4, 23)†, P<0.001after dairy	
				32:1	$C_{40}H_{78}NO_8P$	+11 (1.0, 22)†, P<0.001 after dairy	
				36:3	$C_{44}H_{82}NO_8P$	+13 (2.9, 20)†, P<0.001 after dairy	
				36:5	$C_{44}H_{78}NO_8P$	+14 (3.1, 24)†, P<0.001 after dairy	
				38:3	$C_{46}H_{86}NO_8P$	+16 (2.3, 22)†, P<0.001 after dairy	Increased
				38:5	$C_{46}H_{82}NO_8P$	+9.3 (2.0, 15)†, P<0.001 after dairy	
				38:6	$C_{46}H_{80}NO_8P$	+7.1 (-1.6, 13)†, P<0.001 after dairy	
				40:5	$C_{48}H_{86}NO_8P$	+4.7 (-1.8, 12)†, P<0.001 after dairy	
				40:6	$C_{48}H_{84}NO_8P$	+6.3 (0.3, 12)†, P<0.001 after dairy	
			PE	35:1	$C_{40}H_{78}NO_8P$	+0.6 (0.0, 58)†, P<0.001 after dairy	
				36:5	$C_{41}H_{72}NO_8P$	+38 (8.0, 83)†, P<0.001 after dairy	
				38:3	$C_{43}H_{80}NO_8P$	+37 (11, 65)†, P<0.001 after dairy	Increased
				38:6	$C_{43}H_{74}NO_8P$	+10 (-1.4, 26)†, P<0.001 after dairy	
				40:6	$C_{45}H_{78}NO_8P$	+14 (3.1, 25)†, P<0.001 after dairy	
			PI	34:1	$C_{43}H_{81}O_{13}P$	+22 (8, 30)†, P<0.001 after dairy	
				36:1	$C_{45}H_{85}O_{13}P$	+14 (4.2, 25)†, P<0.001 after dairy	
				36:2	$C_{45}H_{83}O_{13}P$	+18 (8.5, 27)†, P<0.001 after dairy	Increased

		36:4	$C_{45}H_{79}O_{13}P$	+23 811, 38)†, P<0.001 after dairy	
		38:3	$C_{47}H_{85}O_{13}P$	+17 (0.7, 37)†, P<0.001 after dairy	
		38:4	$C_{47}H_{83}O_{13}P$	+18 (2.9, 28)†, P<0.001 after dairy	
	LysoPC	14:0	$C_{22}H_{46}NO_7P$	-11 (-17, 0.1)†, P=0.016 after soy	Decreased
				+4.6 (-1.6, 11)†, P<0.001 after dairy	Increased
		15:0	$C_{23}H_{48}NO_7P$	-8.2 (-14, -0.8)†, P=0.042 after soy	
		16:0	$C_{24}H_{50}NO_7P$	-4.1 (-16, 4.1)†, P=0.240 after soy	
		16:1	$C_{24}H_{48}NO_7P$	-9.6 (-17, -0.3)†, P=0.150 after soy	Decreased
		17:0	$C_{25}H_{52}NO_7P$	-10 (-17, 1.3)†, P=0.070 after soy	
		18:0	$C_{26}H_{54}NO_7P$	-11 (-17, 0.1) †, P=0.080 after soy	
		18:1	$C_{26}H_{52}NO_7P$	-9.3 (-19, -3.5)†, P=0.210 after soy	
		18:2	$C_{26}H_{50}NO_7P$	+7.3 (-7.0, 21)†, P=0.409 after soy	Increased
		20:1	$C_{28}H_{56}NO_7P$	-22 (-30, -1.0)†, P=0.100 after soy	
		20:2	$C_{28}H_{54}NO_7P$	-17 (-26, -6.1)†, P=0.049 after soy	
		20:4	$C_{28}H_{50}NO_7P$	-10 (-17, 0.7)†, P=0.290 after soy	Decreased
		22:5	$C_{30}H_{52}NO_7P$	-8.8 (-20, 8.3)†, P=0.820 after soy	
		22:6	$C_{30}H_{50}NO_7P$	-9.2 (-16, 4.2)†, P=0.140 after soy	
	LysoPE	18:0	$C_{23}H_{48}NO_7P$	+5.1 (-6.6, 18)†, P<0.001 after dairy	
		18:1	$C_{23}H_{46}NO_7P$	+35 (11, 66)†, P<0.001 after dairy	
		18:2	$C_{23}H_{44}NO_7P$	+40 (22, 65)†, P<0.001 after dairy	Increased
		20:4	$C_{25}H_{50}NO_7P$	+13 (-5.4, 38)†, P<0.001 after dairy	
		22:6	$C_{25}H_{48}NO_7P$	+3.3 (-8.4, 18)†, P<0.001 after dairy	

$Only significant subclasses or those that are comparable among the included studies are shown. *Mean levels expressed as average lipid concentration (LDL amount), reported change intervals after LCn-3 PUFAs and control interventions. ** P for changes in detected mass at 2-month vs baseline, and at 6-month vs baseline in RESMENA diet intervention. # Mean expressed as µM (SD), effect after n-3 PUFAs intervention.† Mean expressed as Median % change (25th, 75th percentile) from baseline, reported change after 4-hour dairy and soy oil-based meals. Abbreviations: B, baseline value; BMI; body mass index; C, control group; Cer, ceramide; DBP; diastolic blood pressure; DHA; docosahexaenoic acid; DiCer, dihydroceramide; EPA, eicosapentaenoic acid; F, final value; FBG, fasting blood glucose; GluCer, glucosylceramide; HDL; high-density lipoprotein cholesterol; I, intervention group; LC, long chain; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; LysoPI, lysophosphatidylinositol; MUFAs, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFAs, polyunsaturated fatty acids; SBP, systolic blood pressure; SFAs, saturated fatty acids; SM, sphingomyelin; TEI: total energy intake; TG; triglyceride. Baseline MetS features are described in the Population column (mean ± SD or range of mean values).

TABLE 6 | Description of included studies assessing the effects of fatty acid-based interventions on bioactive lipid levels in subjects with type 2 diabetes

Author, year (reference)	**Country**	**Study design**	**Population**	**Fatty acid intervention and daily dose**	**Comparator**	**Duration of exposure**	**Participants** *N* (I/C)	**Intention-to-treat**
Airhart et al. 2016 [29]	Missouri, USA	Randomized, double-blind, controlled feeding pilot study	37-65 y men and women *FBG:* 121 - 165 mg/dL *Insulin:* 17 - 27 µU/mL *HbA1c:* 7.1 - 8.3 %	Medium-chain fatty acids (MCFAs)-rich diet *(Delios S, Cognis Corporation)* Daily dose: 33-34 % SFAs, 1-2 % MUFAs, 1-2 % PUFAs	Long-chain fatty acids (LCFAs)-rich diet administered as vegetable oil Daily dose: 18-21 % SFAs, 7-11 % MUFAs, 3-8 % PUFAs	2 weeks	16 (9/7)	No
Baziar et al. 2020 [30]	Iran	Randomized, double-blind, placebo-controlled trial	40-60 y men and women *FBG:* 122.5 - 145.6 mg/dL *Insulin:* 9.3 - 12.4 mU/L *HbA1c:* 6.2 - 7 % *HOMA-IR:* 3 - 4.1	Alpha lipoic acid capsule Daily dose: 1200 mg	Placebo capsule containing maltodextrin Daily dose: 1200 mg	2 months	70 (35/35)	Yes

Table 6. continued

Bioactive lipids

Assessment method	**Identification reference**	**Class**	**Subclass**[$]	**Molecular formula**	**Changes in concentration attributed to intervention and direction of change**	
Targeted LC-MS	Analyst (version 1.5)	SM	14:0	$C_{37}H_{75}N_2O_6P$	B: 0.083 (0.002); F: 0.071 (0.003)[*], P<0.01	Decreased
			15:0	$C_{38}H_{77}N_2O_6P$	B: 0.257 (0.002); F: 0.250 (0.002)[*], P<0.01	
			16:0	$C_{39}H_{79}N_2O_6P$	B: 1.169 (0.040); F: 1.066 (0.039)[*], P<0.01	
			16:1	$C_{39}H_{77}N_2O_6P$	B: 0.157 (0.006); F: 0.141 (0.006)[*], P<0.01	
			20:0	$C_{43}H_{87}N_2O_6P$	B: 0.194 (0.005); F: 0.165 (0.009)[*], P<0.01	
			21:0	$C_{44}H_{89}N_2O_6P$	B: 0.071 (0.001); F: 0.058 (0.002)[*],P<0.005	
			22:0	$C_{45}H_{91}N_2O_6P$	B: 0.324 (0.019); F: 0.267 (0.018)[*], P<0.01	
			22:1	$C_{45}H_{89}N_2O_6P$	B: 0.214 (0.006); F: 0.191 (0.008)[*], P<0.01	
			23:0	$C_{46}H_{93}N_2O_6P$	B: 0.139 (0.005); F: 0.117 (0.004)[*], P<0.01	
			23:1	$C_{46}H_{91}N_2O_6P$	B: 0.081 (0.002); F: 0.072 (0.002)[*], P<0.01	
		Cer	23:0	$C_{41}H_{81}NO_3$	B: 0.047 (0.007); F: 0.345 (0.005) [*], P<0.01	Decreased
		AC	18:0	$C_{25}H_{49}NO_4$	B: 0.175 (0.013); F: 0.127 (0.013)[*], P<0.01	Decreased
			18:3	$C_9H_{18}NO_4$	B: 0.040 (0.003); F: 0.029 (0.002)[*], P<0.05	
			20:0	$C_{27}H_{53}NO_4$	B: 0.013 (0.001); F: 0.009 (0.001)[*],P<0.001	

Table 6. continued

Bioactive lipid enzymatic precursors

Assessment method	**Class**	**Subclass**		**Changes in mass attributed to intervention and direction of change**	
PLAC ELISA kit (diaDexus, Inc. South San Francisco, CA, USA)	Platelet-activating factor acetylhydrolases	Lipoprotein-associated phospholipase A2 enzyme	Lp-PLA2	B: 194.30 (1.60); F: 177.18 (1.56)[**], P=0.001	Decreased

[$]Only significant subclasses or those that are comparable among the included studies are shown. [*]Mean expressed as µM (SD), effect after MCFAs intervention.[**]Mean expressed as ng/mL (SD), effect after alpha lipoic acid intervention. Abbreviations: -AC, acylcarnitine; B, baseline value; C, control group; Cer, ceramide; F, final value; FBG, fasting blood glucose; HbA1C, glycated haemoglobin; HOMA-IR, homeostasis model of assessment index; I, intervention group; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids; SM, sphingomyelin. Baseline T2D features are described in the Population column (range of mean values).

TABLE 7 | Description of included studies assessing the effects of fatty acid-based interventions on bioactive lipid levels in subjects with hypertension

Author, year (reference)	Country	Study design	Population	Fatty acid intervention and daily dose	Comparator	Duration of exposure	Participants *N* (I/C)	Intention-to-treat
Rebholz et al. 2018 [31]	Baltimore, USA	Multicenter, randomized, single-blind, controlled feeding trial	≥ 22 y men and women *SBP:* 130.1 (12.6) mmHg *DBP:* 84.4 (6.9) mmHg	• *Group 1:* DASH diet consisting on high intake of fruits, vegetables and low-fat dairy providing 7.4 % of TEI as SFAs; 10.5 % as MUFAs; and 7.6 % as PUFAs • *Group 2:* Diet rich in fruits and vegetables providing 13 % of TEI as SFAs; 14 % as MUFAs; and 6.9 % as PUFAs	Control diet low in fruits, vegetables and dairy products providing 14.4 % of TEI as SFAs; 12.6 % as MUFAs; and 7.1 % as PUFAs	2 months	329 (110/111/108)	Yes
Krantz et al. 2015 [32]	Denver, USA	Randomized, double-blind, placebo-controlled pilot study	≥ 18 y men and women *SBP:* 133 (16) mmHg *DBP:* 81 (10) mmHg *PWV:* 1652 (330) cm/s	n-3 PUFA capsules providing 465:375 mg of EPA:DHA *(Lovaza™ Glaxo Smith Kline)* Daily dose: 3.36 g	Matched corn-oil placebo capsule Daily dose: 3.36 g	3 months	62 (27/35)	No

Table 7. continued

	Bioactive lipids					
	Assessment method	**Identification reference**	**Class**	**Subclass[$]**	**Molecular formula**	**Changes in concentration attributed to intervention and direction of change**
Rebholz et al. 2018 [31]	Untargeted GC-MS and LC-MS (Thermo Scientific Orbitrap mass spectrometers)	Characterization in an extensive chemical library Validation with reference standards	Cer	34:1	$C^{34}H^{67}NO^{3}$	-0,142 (0.029)
				35:1	$C_{35}H_{69}NO_3$	-0.349 (0.059)
				36:1	$C_{36}H_{71}NO_3$	-0.323 (0.039)*
				38:1	$C_{50}H_{95}NO_{13}$	-0.211 (0.037)*
				40:2	$C_{40}H_{77}NO_3$	-0.230 (0.040)*
				41:2	$C_{41}H_{79}NO_3$	-0.107 (0.020)*
			SM	32:0	$C_{37}H_{77}N_2O_6P$	-0.213 (0.034)*
				32:1	$C_{37}H_{75}N_2O_6P$	-0.112 (0.023)
				32:2	$C_{37}H_{73}N_2O_6P$	-0.207 (0.038)*
				33:1	$C_{38}H_{77}N_2O_6P$	-0.148 (0.025)*
				33:2	$C_{38}H_{75}N_2O_6P$	-0.179 (0.038)
				34:1	$C_{39}H_{79}N_2O_6P$	-0.067 (0.016)*
				34:2	$C_{39}H_{77}N_2O_6P$	-0.095 (0.018)*
				35:1	$C_{40}H_{81}N_2O_6P$	-0.160 (0.029)*
				36:0	$C_{41}H_{85}N_2O_6P$	-0.679 (0.107)*
				36:1	$C_{41}H_{83}N_2O_6P$	-0.133 (0.029)*
				36.2	$C_{41}H_{81}N_2O_6P$	-0.182 (0.028)*
				36:3	$C_{41}H_{79}N_2O_6P$	-0.258 (0.039)*
				37:1	$C_{42}H_{85}N_2O_6P$	-0.170 (0.035)*
				38:0	$C_{43}H_{89}N_2O_6P$	-0.306 (0.067)*

Decreased

Decreased

			41:1	$C_{46}H_{93}N_2O_6P$	-0.169 (0.030)*	Decreased
			41:2	$C_{46}H_{91}N_2O_6P$	-0.171 (0.029)*	
		Lyso-PC	16:0	$C_{24}H_{50}NO_7P$	-0.167 (0.034)*	Decreased
			18:1	$C_{26}H_{52}NO_7P$	-0.117 (0.021)	
		Lyso-PE	18:0	$C_{23}H_{48}NO_7P$	-0.239 (0.049)	Decreased
			20:4	$C_{25}H_{50}NO_7P$	-0.135 (0.030)	
		PC	30:0	$C_{38}H_{76}NO_8P$	-0.254 (0.059)	
			34:0	$C_{42}H_{84}NO_8P$	-0.120 (0.024)	Decreased
			34:1	$C_{42}H_{82}NO_8P$	-0.103 (0.022)	
			36:1	$C_{44}H_{86}NO_8P$	-0.225 (0.030)	
			40:6	$C_{48}H_{84}NO_8P$	+0.151 (0.031)*	Increased
		PE	40:6	$C_{45}H_{76}NO_8P$	+0.301 (0.062)*	Increased
			40:7	$C_{45}H_{78}NO_8P$	+0.567 (0.112)	

Table 7. continued

	Bioactive lipid enzymatic precursors				
	Assessment method	**Class**	**Subclass**		**Changes in mass/activity attributed to intervention and direction of change**
Krantz et al. 2015 [32]	PLAC ELISA kit (diaDexus, Inc. South San Francisco, CA, USA)	Platelet-activating factor acetylhydrolases	Lipoprotein-associated phospholipase A2 enzyme	Lp-PLA2	B: 252.0 (62); F: 233.9 (41.1)#, P>0.05 B: -18.1 (41.1); F: -6.1 (31.7)†, P=0.08 — Decreased

$ Only significant subclasses or those that are comparable among the included studies are shown. * Significant change in β_2 correlation coefficient and standard error (SE) among those assigned to the DASH diet compared with those assigned to the control diet (P<0.00001). Negative/Positive β_2-coefficients indicate that the lipid metabolite was lower/higher among those randomly assigned to the DASH diet compared with those assigned to the control diet. # Mean expressed as ng/mL (SD), effect after n-3 PUFAs intervention, and † effect compared to the placebo group. Abbreviations: B, baseline value; C, control group; Cer, ceramide; DBP, diastolic blood pressure; DHA; docosahexaenoic acid; EPA, eicosapentaenoic acid; F, final value; I, intervention group; Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine; MUFAs, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFAs, polyunsaturated fatty acids; PWV; pulse wave velocity; SBP; systolic blood ressure; SFAs, saturated fatty acids; SM, sphingomyelin. Baseline Hypertension features are described in the Population column (mean ± SD).

TABLE 8 | Description of included studies assessing the effects of fatty acid-based interventions on bioactive lipid levels in subjects with stable coronary artery disease

Author, year (reference)	**Country**	**Study design**	**Population**	**Fatty acid intervention and daily dose**	**Comparator**	**Duration of exposure**	**Participants** *N* (I/C)	**Intention-to-treat**
Gajos et al. 2013 [41]	Poland	Prospective, randomized, double-blind, placebo-controlled study	30-80 y men and women *Ox-LDL:* 83 (141) µg/L *MPO:* 245.6 (13.5) ng/mL *IL-6:* 23.2 (9) pg/mL	n-3 PUFA carboxylic acids capsule providing 460:380 mg of EPA:DHA *(Omacor; Pronova Biocare)* combined with usual Aspirin and Clopidogrel therapy Daily dose: 1 g	Placebo soybean oil capsule combined with usual Aspirin and Clopidogrel therapy Daily dose: 1 g	1 month	54 (30/24)	No
Khandouzi et al. 2020 [34]	Iran	Randomized, open-label, controlled parallel-arm trial	51-64 y men and postmenopausal women *IL-6:* 19.5 (10.5) pg/mL *LDL:* 42.9 (6.5) mg/dL *HDL:* 74.6 (30.5) mg/dL	Canola oil *(CanaPlus, British Columbia)* providing 6.40 g SFAs, 61.37 g MUFAs, 29.20 g PUFAs (mainly n-6 LA, and n-3 ALA) per 100 g Daily dose: 25 mL	Refined olive oil providing 17.87 g SFAs, 66.68 g MUFAs, 11.75 g PUFAs per 100 g Daily dose: 25 mL	6 weeks	42 (20/22)	No

Table 8. continued

	Bioactive lipid enzymatic precursors					
	Assessment method	**Class**	**Subclass**		**Changes in mass attributed to intervention and direction of change**	
Gajos et al. 2013 [41]	PLAC ELISA kit (diaDexus, Inc. South San Francisco, CA, USA)	Platelet-activating factor acetylhydrolases	Lipoprotein-associated phospholipase A2 enzyme	Lp-PLA2	B: 260 (68); F: 240 (64)[**], P=0.026	Decreased
Khandouzi et al. 2020 [34]	ELISA kit (ZellBio GmbH, Ulm, Germany)				B: 3.96 (2.45); F: 2.99 (1.65)[#], P=0.008	Decreased

[$] Only significant subclasses or those that are comparable among the included studies are shown. [**] Mean expressed as ng/mL (SD), effect after n-3 PUFAs intervention. [#] Mean expressed as ng/mL (SD), effect after canola oil intervention. Abbreviations: ALA, alpha linoleic acid; B, baseline value; C, control group; DHA; docosahexaenoic acid; EPA, eicosapentaenoic acid; F, final value; HDL; high-density lipoprotein; I, intervention group; IL-6, interleukine-6; LA, linoleic acid; LDL, low-density lipoprotein; LysoPC, lysophosphatidylcholine; MPO, myeloperoxidase; MUFAs, monounsaturated fatty acids; Ox-LDL, oxidized LDL; PC, phosphatidylcholine; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids; SM, sphingomyelin. Baseline stable CAD features are described in the Population column (mean ± SD).

TABLE 9 | Description of included studies assessing the effects of fatty acid-based interventions on bioactive lipid levels in healthy subjects

Author, year (reference)	**Country**	**Study design**	**Population**	**Fatty acid intervention and daily dose**	**Comparator**	**Duration of exposure**	**Participants** *N*, (I/C)	**Intention-to-treat**
Averill et al. 2020 [50]	Washington, USA	Randomized, postprandial, cross-over trial. ≥1-week washout period	19-49 y men and women	• *Intervention sequence 1:* High-saturated fat meal (HSF) providing 20 % TEI, and 80 % fat: 24 g SFAs, 4 g MUFAs and 2 g PUFAs • *Intervention sequence 2:* High-carbohydrate meal (HC) providing 20 % TEI, and 10 % fat: 0 g SFAs, 3 g MUFAs and 1.5 g PUFAs	Not controlled	Two 6-hour postprandial test meals	15	No
Block et al. 2012 [47]	New York, USA	Randomized, double-blind, placebo-controlled, postprandial cross-over trial. 1-month washout period	18-50 y men and women	• *Intervention sequence 1:* Marine n-3 PUFAs capsule *(Lovaza®, Reliant Pharmaceuticals)* Daily dose: 4 g (3.4 g EPA+DHA) • *Intervention sequence 2:* Aspirin (ASA) tablet Daily dose: 81 mg • *Intervention sequence 3:* Marine n3 PUFAs capsule + ASA tablet Daily dose: 4 g + 81 mg	Placebo and 30-day run-in period free of fish oil before each visit	Four 4-hour postprandial sessions	25	No
Del Bas et al. 2016 [22]	United Kingdom	Randomized, double-blind, placebo-controlled parallel trial	18-65 y men and women	Marine n-3 PUFAs capsule providing 1.1:0.8 g of EPA:DHA Daily dose: 1.9 g	Placebo corn-oil capsule Daily dose: 3g	3 months	38 (19/19)	No
Morris et al. 2015 [49]	Ireland (The Metabolic Challenge Study (MECHE))	Randomized, open-label, postprandial parallel trial	21-49 y men and women	• *Group 1:* Oral lipid tolerance test (OLTT), 100 mL Calogen *(Nutricia)* combined with 50mL Liquid Duocal *(SHS Nutrition)* providing 7 g SFAs, 31 g MUFAs and 16 g PUFAs • *Group 2:* Oral glucose tolerance test (OGTT), 75 mL anhydrous glucose plus 100 mLwater combined with a Physical tests session	Not controlled	5-hour postprandial test	40	No

— Zacek et al. 2018 [21]	Grand Forks, USA (The Salmo Salar study)	Randomized, open-label, cross-over trial. 4 to 8-week washout period	48-52 y men and women	• *Intervention sequence 1:* n-3 PUFAs-rich farmed Atlantic salmon provided in servings of 90 g, 2 d/wk Daily dose: 0.30 g EPA+DHA • *Intervention sequence 2:* n-3 PUFAs-rich farmed Atlantic salmon provided in servings of 180 g, 2 d/wk Daily dose: 0.61 g EPA+DHA	Not controlled	Two 4-week study periods	9	No
Zhu et al. 2019 [52]	California, USA	Randomized, open-label, cross-over trial. 4-day washout period	18-25 y men and women	• *Intervention sequence 1:* Fast food or western diet (FF) enriched in red meat, simple sugars, cholesterol and SFAs, providing 44.67 g SFAs (29.16 % TEI), 1.89 g MUFAs and 3.24 g PUFAs • *Intervention sequence 2:* Mediterranean diet (Med) enriched in fruits, vegetables, fiber, MUFAs and PUFAs providing 13.24 g SFAs (5.27 % TEI), 41.28 g MUFAs and 15.63 g PUFAs	Not controlled	Two 4-day study periods	10	No
Asztalos et al. 2016 [35]	Boston, USA	Randomized, double-blind, parallel-assignment, placebo-controlled trial	21-70 y men and women	• *Group 1:* n-3 EPA-enriched oil capsule providing 627 mg EPA Daily dose: 0.60 g EPA • *Group 2:* n-3 EPA-enriched oil capsule providing 1689:5 mg of EPA:DHA Daily dose: 1.8 g EPA • *Group 3:* n-3 DHA-enriched oil capsule providing 1.6:758 mg of EPA:DHA. Daily dose: 0.60 g DHA	Placebo olive oil capsule providing 3954mg of oleic acid Daily dose: 6 g	6 weeks	121 (30/31/30/30)	Yes
Kim et al. 2017 [36]	Korea	Randomized, open-label, controlled trial	30-65 y men and women	• *Group 1:* Low linoleic acid (LA); replacement of 10 mL/day soy oil with one apple • *Group 2:* High LA; reduction of 1/3 cup of cooked refined rice and addition of 9.9 g/d of soy oil as a supplement (54.2 % LA)	Medium LA control; maintenance of the usual food intake	2 months	150 (50/50/50)	No

Nelson et al. 2011 [37]	Fort Collins, USA	Randomized, open-label, controlled trial	≥50 y men and women	• *Group 1:* Isocaloric diet supplemented with fish oil capsule providing 180:120 mg of EPA:DHA plus olive oil Daily dose: 2 g EPA+DHA + 9 g OO • *Group 2:* Isocaloric diet supplemented with flaxseed oil capsule Daily dose: 11 g (0.57 g ALA)	Isocaloric diet supplemented with olive oil capsule providing Daily dose: 11 g (800 mg MUFAs from oleic acid)	2 months	59 (20/20/19)	No

Table 9. continued

	Bioactive lipids						
	Assessment method	**Identification reference**	**Class**	**Subclass[$]**	**Molecular formula**	**Changes in concentration attributed to intervention and direction of change**	
Averill et al. 2020 [50]	Untargeted LC-MS on isolated HDL particles	Not reported	Cer	40:1	$C_{40}H_{79}NO_3$	-0.052 (0.06)[*], P=0.005	Decreased
				42:1	$C_{42}H_{83}NO_3$	+0.163 (0.18)[*], P=0.004	Increased
				42:2	$C_{42}H_{81}NO_3$	+0.191 (0.22)[*], P=0.005	
			LacCer	34:1	$C_{34}H_{67}NO_3$	+0.194 (0.65)[*], P=0.023	Increased
			PC	32:2	$C_{40}H_{76}NO_8P$	+0.147 (0.08)[*], P<0.0001	
				34:1	$C_{42}H_{82}NO_8P$	+0.124 (0.08)[*], P=0.0001	
				34:2	$C_{42}H_{80}NO_8P$	+0.079 (0.07)[*], P=0.001	
				36:2	$C_{44}H_{84}NO_8P$	+0.081 (0.05)[*], P=0.0001	Increased
				36:3	$C_{44}H_{82}NO_8P$	+0.083 (0.07)[*], P=0.001	
				36:4	$C_{44}H_{80}NO_8P$	+0.115 (0.06)[*], P<0.0001	
				38:4	$C_{46}H_{84}NO_8P$	+0.086 (0.06)[*], P=0.0002	
				38:5	$C_{46}H_{82}NO_8P$	+0.099 (0.07)[*], P=0.0002	
			SM	14:0	$C_{37}H_{75}N_2O_6P$	+0.108 (0.43)[*], P=0.013	Increased
Block et al. 2012 [47]	Direct infusion electrospray mass spectrometry (QTrap 2000 MS/MS using an Advion Inc. (Ithaca, NY) Nanomate nanospray (ESI) source)	Calibration curves and internal standards	Lyso-PC	16:0	$C_{24}H_{50}NO_7P$	-2.10 (63.3)[**], P=0.390 after n-3 -2.70 (72.3)[**], P=0.840 after n-3+ASA	
				18:0	$C_{26}H_{56}NO_6P$	+3.59 (25.3)[**], P=0.390 after n-3 +0.32 (21.4)[**], P=0.810 after n-3+ASA	
				18:1	$C_{26}H_{52}NO_7P$	-7.50 (25.0)[**], P=0.750 after n-3 -11.40 (32.3)[**], P=0.270 after n-3+ASA	Decreased/
				18:2	$C_{26}H_{50}NO_7P$	-16.0 (69.9)[**], P=0.540 after n-3 -34.6 (117.0)[**], P=0.700 after n-3+ASA	Increased
				20:4	$C_{28}H_{50}NO_7P$	+2.40 (67.4)[**], P=0.310 after n-3 -13.20 (95.3)[**], P=0.910 after n-3+ASA	
				20:5	$C_{28}H_{48}NO_7P$	+2.20 (5.2)[**], P=0.002 after n-3 +0.86 (7.7)[**], P=0.130 after n-3+ASA	
Del Bas et al. 2016 [22]	Targeted LC-MS (Agilent 6550	Calibration curves and internal standards	Lyso-PC	16:0	$C_{24}H_{50}NO_7P$	B: 266.50; F: 199.20 (74.95)[†], P<0.01	Decreased
				18:0	$C_{26}H_{54}NO_7P$	B: 84.00; F: 60.20 (35.30)[†], P<0.01	

	Accurate-Mass Quadrupole-Time of Flight (Q-TOF) mass spectrometer (MS))			18:1	$C_{26}H_{52}NO_7P$	B: 45.53; F: 51.70 (24.84)†, P>0.05 B: 49.70; F: 72.30 (30.20)‡, P<0.05	Increased Increased
				18:2	$C_{26}H_{50}NO_7P$	B: 3.25; F: 4.28 (2.04)†, P<0.05 B: 3.38; F: 5.89 (2.09)‡, P<0.01	
				20:4	$C_{28}H_{50}NO_7P$	B: 6.86; F: 6.55 (2.70)†, P>0.05 B: 7.80; F: 10.51 (4.41)‡, P<0.05	Decreased Increased
			Lyso-PE	16:0	$C_{21}H_{44}NO_7P$	B: 0.097; F: 0.103 (0.022)†, P>0.05	
				18:0	$C_{23}H_{48}NO_7P$	B: 0.374; F: 0.393 (0.131)†, P>0.05 B: 0.344; F: 0.433 (0.151)‡, P<0.05	
				18:1	$C_{23}H_{46}NO_7P$	B: 0.259; F: 0.634 (0.248)†, P<0.0001 B: 0.320; F: 0.723 (0.248)‡, P<0.0001	
				18:2	$C_{23}H_{44}NO_7P$	B: 0.497; F: 1.277 (0.641)†, P<0.0001 B: 0.648; F: 1.528 (0.538)‡, P<0.0001	Increased
				20:4	$C_{25}H_{44}NO_7P$	B: 0.299; F: 0.517 (0.144)†, P<0.0001 B: 0.394; F: 0.740 (0.267)‡, P<0.0001	
				22:6	$C_{27}H_{44}NO_7P$	B: 0.414; F: 0.455 (0.148)†, P>0.05 B: 0.307; F: 0.408 (0.120)‡, P<0.01	
Morris et al. 2015 [49]	Untargeted high throughput flow injection ESI-MS/MS (4000 QTrap® tandem mass spectrometer, AB SCIEX)	Internal standards and BIOCRATES in-house software MetIDQ™	Lyso-PC	16:0	$C_{24}H_{50}NO_7P$	Fold change < 1.5#, P<0.05 at 300 min	Decreased
				18:0	$C_{26}H_{54}NO_7P$		
				18:1	$C_{26}H_{52}NO_7P$	Fold change > 1.5#, P<0.05 at 300 min	
				18:2	$C_{26}H_{50}NO_7P$		Increased
				20:4	$C_{28}H_{50}NO_7P$		
			Lyso-PE	16:0	$C_{21}H_{44}NO_7P$	Fold change > 1.5#, P<0.05 at 120 min	
				18:0	$C_{23}H_{48}NO_7P$		
				18:1	$C_{23}H_{46}NO_7P$		
				18:2	$C_{23}H_{44}NO_7P$		Increased
				20:4	$C_{25}H_{44}NO_7P$		
				22:6	$C_{27}H_{44}NO_7P$		
			PC	34:1	$C_{42}H_{82}NO_8P$	Fold change > 1.5#, P<0.05 at 180 min	
				34:2	$C_{42}H_{80}NO_8P$	AND	
				36:2	$C_{44}H_{84}NO_8P$	Fold change < 1.5#, P<0.05 at 300 min	
				36:3	$C_{44}H_{82}NO_8P$		
				36:4	$C_{44}H_{80}NO_8P$		Increased/
				38:4	$C_{46}H_{84}NO_8P$		Decreased
				38:5	$C_{46}H_{82}NO_8P$		
				38:6	$C_{46}H_{80}NO_8P$		
				40:5	$C_{48}H_{86}NO_8P$		
				40:6	$C_{48}H_{84}NO_8P$		
			PE	36:2	$C_{41}H_{78}NO_8P$	Fold change > 1.5#, P<0.05 at 300 min	Increased
				36:3	$C_{41}H_{76}NO_8P$		
			Cer	16:1	$C_{34}H_{67}NO_3$	Fold change > 1.5#, P<0.05 at 300 min Fold change > 1.5#, P<0.05 at 180 min	Increased

			DiCer	24:0 26:0	$C_{42}H_{85}NO_3$ $C_{44}H_{89}NO_3$	Fold change > 1.5[#], P<0.05 at 60 min	Increased
			SM	16:0 16:1 18:0 18:1 20:0 20:1 20:2 21:0 22:0 23:0 24:2	$C_{39}H_{79}N_2O_6P$ $C_{39}H_{77}N_2O_6P$ $C_{41}H_{83}N_2O_6P$ $C_{41}H_{83}N_2O_6P$ $C_{43}H_{87}N_2O_6P$ $C_{43}H_{85}N_2O_6P$ $C_{43}H_{83}N_2O_6P$ $C_{44}H_{89}N_2O_6P$ $C_{45}H_{91}N_2O_6P$ $C_{46}H_{93}N_2O_6P$ $C_{47}H_{89}N_2O_6P$	Fold change >1.5[#], P<0.05 at 180 min	Increased
Žáček et al. 2018 [21]	Targeted LC-MS by using an AB Sciex 5500 QTRAP hybrid quadrupole ion-trap mass spectrometer with a Turbo Spray source (AB Sciex) operating at unit mass resolution	LIPID MAPS MS internal standards LipidView™ software	PC	36:5 38:5 38:6 40:6 40:7	$C_{44}H_{78}NO_8P$ $C_{46}H_{82}NO_8P$ $C_{46}H_{80}NO_8P$ $C_{48}H_{84}NO_8P$ $C_{48}H_{82}NO_8P$	B: 12.0 (4.1); F: 30.5 (18.8)[F], P=0.027 B: 7.6 (2.6); F: 18.3 (9.0)[F], P=0.006 B: 2.0 (0.7); F: 4.2 (2.3)[F], P=0.049 B: 22.5 (4.8); F: 39.9 (10.3)[F], P<0.001 B: 6.7 (1.0); F: 9.2 (1.7)[F], P=0.057	Increased
			PE	36:0 38:0 38:1 38:6 40:0 40:6 40:7	$C_{41}H_{82}NO_8P$ $C_{41}H_{82}NO_7P$ $C_{43}H_{86}NO_7P$ $C_{43}H_{76}NO_7P$ $C_{45}H_{90}NO_8P$ $C_{45}H_{80}NO_7P$ $C_{45}H_{76}NO_8P$	B: 0.39 (0.09); F: 0.67 (0.10)[F], P<0.001 B: 0.64 (0.14); F: 1.01 (0.25), P=0.0003 B: 0.27 (0.05); F: 0.42 (0.04)[F],P<0.0001 B: 3.97 (2.17); F: 6.12 (3.17)[F], P=0.001 B: 0.04 (0.04); F: 0.10 (0.01)[F], P=0.001 B: 2.57 (1.35); F: 3.94 (1.89)[F],P=0.0003 B: 0.67 (0.35); F: 0.90 (0.50)[F], P=0.007	Increased
			Lyso-PC	16:0 18:0 18:1 18:2 20:4	$C_{24}H_{50}NO_7P$ $C_{26}H_{54}NO_7P$ $C_{26}H_{52}NO_7P$ $C_{26}H_{50}NO_7P$ $C_{28}H_{50}NO_7P$	B: 100.7 (10.7); F: 102.9 (21.9)[F], P=0.850 B: 36.4 (4.2); F: 37.5 (8.0)[F], P=0.850 B: 20.9 (4.2); F: 19.0 (4.2)[F], P=0.850 B: 37.9 (10.0); F: 41.3 (18.4)[F], P=0.140 B: 5.1 (3.6); F: 5.1 (4.0)[F], P=0.440	Increased
			Lyso-PE	18:0 22:6 34:1 34:2 36:1 36:2	$C_{23}H_{48}NO_7P$ $C_{27}H_{44}NO_7P$ $C_{39}H_{79}N_2O_6P$ $C_{39}H_{77}N_2O_6P$ $C_{41}H_{83}N_2O_6P$ $C_{41}H_{81}N_2O_6P$	B: 0.06 (0.01); F: 0.08 (0.02)[F], P=0.042 B: 0.33 (0.10); F: 0.53 (0.17)[F], P=0.0003 B: 120.1 (11.1); F: 129.5 (16.5)[F], P=0.580 B: 17.5 (3.1); F: 18.2 (2.6)[F], P=0.780 B: 25.0 (3.7); F: 27.4 (3.3)[F], P=0.410 B: 13.5 (2.5); F: 14.5 (1.9)[F],P=0.370	Increased
			SM	40:1 42:1 42:2	$C_{45}H_{91}N_2O_6P$ $C_{47}H_{95}N_2O_6P$ $C_{47}H_{93}N_2O_6P$	B: 41.1 (5.5); F: 43.8 (11.8)[F], P=0.810 B: 39.0 (4.9); F: 40.6 (9.5)[F], P=0.890 B: 78.9 (11.1); F: 90.3 (13.1)[F], P=0.130	Increased

Zhu et al. 2019 [52]	Untargeted LC-MS using an Agilent 6530 QTOF with positive mode, and an Agilent 6550 QTOF with negative mode. On isolated HDL fractions	Internal standards and calibration curves In house rt-mz library and in silico LipidBlast library	PC	30:0	$C_{41}H_{82}NO_8P$	B: 6.1e-04; F: 6.5e-04§, P<0.05 after FF	Increased
						B: 9.9e-04; F: 5.3e-04§, P<0.05 after MED	Decreased
				32:2	$C_{40}H_{76}NO_8P$	B: 5.1e-04; F: 5.7e-04§, P<0.05 after FF	Increased
						B: 6.6e-04; F: 4.1e-04§, P<0.05 after MED	Decreased
				33:0	$C_{41}H_{82}NO_8P$	B: 8.1e-05; F: 4.9e-05§P<0.05 after MED	Decreased
				33:1	$C_{41}H_{80}NO_8P$	B: 3.1e-04; F: 3.6e-04§, P<0.05 after FF	Increased
						B: 3.9e-04; F: 2.7e-04§P<0.05 after MED	Decreased
				33:2	$C_{41}H_{78}NO_8P$	B: 5.1e-04; F: 6.5e-04§, P<0.0001 after FF	Increased
						B: 5.4e-04; F: 4.6e-04§, P<0.0001 after MED	Decreased
				34:1	$C_{42}H_{82}NO_8P$	B: 7.7e-04; F: 0.001§, P<0.0001 after FF	Increased
						B: 8.5e-04; F: 6.4e-04§, P<0.0001 after MED	Decreased
				34:2	$C_{42}H_{80}NO_8P$	B: 0.001; F: 0.002§, P<0.0001 after FF	Increased
				35:1	$C_{43}H_{84}NO_8P$	B: 2.9e-04; F: 3.5e-04§, P=0.001 after FF	Increased
						B: 3.2e-04; F: 2.5e-04§, P=0.001 after MED	Decreased
				35:2	$C_{43}H_{82}NO_8P$	B: 9.9e-04; F: 0.002§, P<0.0001 after FF	Increased
						B: 0.001; F: 9.4e-04§, P<0.0001 after MED	Decreased
				35:3	$C_{43}H_{80}NO_8P$	B: 2.9e-04; F: 4.2e-04§, P<0.0001 after FF	Increased
						B: 3.2e-04; F: 2.8e-04§, P<0.0001 after MED	Decreased
				36:2	$C_{44}H_{84}NO_8P$	B: 4.2e-04; F: 5.1e-04§, P<0.0001 after FF	Increased
						B: 4.1e-04; F: 3.6e-04§, P<0.0001 after MED	Decreased
				36:4	$C_{44}H_{80}NO_8P$	B: 0.004; F: 0.006§, P<0.0001 after FF	Increased
						B: 0.005; F: 0.004§, P<0.0001 after MED	Decreased
				38:4	$C_{46}H_{84}NO_8P$	B: 3.3e-04; F: 4.7e-04§, P=0.001 after FF	Increased
						B: 3.4e-04; F: 3.1e-04§, P=0.001 after MED	Decreased
				40:6	$C_{48}H_{84}NO_8P$	B: 0.002; F: 0.002§, P<0.05 after MED	Unchanged
				40:7	$C_{48}H_{82}NO_8P$	B: 0.002; F: 0.002§, P=0.001 after MED	
			PE	34:2	$C_{39}H_{74}NO_7P$	B: 9.0e-04; F: 0.001§, P<0.0001 after FF	Increased
						B: 9.5e-04; F: 6.5e-04§, P<0.0001 after MED	Decreased
				36:2	$C_{41}H_{78}NO_8P$	B: 0.001; F: 0.002§, P<0.0001 after FF	Increased
						B: 0.001; F: 8.0e-04§, P<0.0001 after MED	Decreased
				38:4	$C_{43}H_{78}NO_8P$	B: 0.003; F: 0.005§, P<0.0001 after FF	Increased
						B: 0.04; F: 0.002§, P<0.0001 after MED	Decreased
			Lyso-PC	16:0	$C_{24}H_{50}NO_7P$	B: 0.001; F: 0.001§,P>0.05 after FF and MED	Unchanged
				18:0	$C_{26}H_{54}NO_7P$	B: 6.7e-04; F: 6.4e-04§, P>0.05 after FF	Decreased
						B: 6.5e-04; F: 5.7e-04§, P>0.05 after MED	
				18:1	$C_{26}H_{52}NO_7P$	B: 4.0e-04; F: 3.3e-04§, P<0.05 after FF	Decreased
						B: 3.8e-04; F: 4.1e-04§, P<0.05 after MED	Increased
				18:2	$C_{26}H_{50}NO_7P$	B: 5.5e-04; F: 6.3e-04§, P>0.05 after FF	Increased
						B: 5.1e-04; F: 5.2e-04§, P>0.05 after MED	
				20:4	$C_{28}H_{50}NO_7P$	B: 7.9e-05; F: 6.7e-05§, P>0.05 after FF	Decreased
						B: 8.5e-05; F: 7.0e-05§, P>0.05 after MED	
			SM	34:0	$C_{39}H_{81}N_2O_6P$	B: 5.2e-04; F: 4.9e-04§, P<0.05 after FF	Decreased
						B: 5.0e-04; F: 5.4e-04§, P<0.05 after MED	Increased
				34:2	$C_{39}H_{77}N_2O_6P$	B: 0.002; F: 0.001§,P<0.05 after FF and MED	Decreased
				40:2	$C_{45}H_{89}N_2O_6P$	B: 0.004; F: 0.005§, P<0.05 after MED	Increased

			42:3	$C_{47}H_{91}N_2O_6P$	B: 0.003; F: 0.002[§], P<0.0001 after FF	Decreased
					B: 0.002; F: 0.003[§], P<0.0001 after MED	Increased
		Cer	40:1	$C_{40}H_{79}NO_3$	B: 2.0e-05; F: 2.1e-05[§], P>0.05 after FF	Increased
					B: 1.9e-05; F: 1.9e-05[§], P>0.05 after MED	Decreased
			42:1	$C_{42}H_{83}NO_3$	B: 1.1e-04; F: 1.1e-04[§], P>0.05 after FF	Increased
					B: 1.1e-04; F: 1.1e-04[§], P>0.05 after MED	Decreased
			42:2	$C_{42}H_{81}NO_3$	B: 2.3e-05; F: 2.3e-05[§], P>0.05 after FF	Increased
					B: 1.7e-05; F: 1.3e-05[§], P>0.05 after MED	Decreased

Table 9. continued

Bioactive lipid enzymatic precursors					
	Assessment method	**Class**	**Subclass**		**Changes in mass/activity attributed to intervention and direction of change**
Asztalos et al. 2016 [35]	PLAC ELISA kit (diaDexus, Inc. South San Francisco, CA, USA)	Platelet-activating factor acetylhydrolases	Lipoprotein-associated phospholipase A2 enzyme	Lp-PLA2	B: 170 (51.7); F: 157 (59.7)[†], P=0.004 after EPA 600mg B: 145.5 (29.9); F: 124.1 (33.7)[†], P=0.003 after EPA 1800mg B: 167.5 (41.3); F: 167.2 (49.9)[†], P=0.970 after DHA 600mg
					Decreased
Kim et al. 2017 [36]	PAF-AH activity assay kit (Biovision, Milpitas, CA)				B: 15.2 (4.24); F: 14.9 (3.53)[†], P=0.587 after low LA
					Decreased
					B: 14.7 (3.53); F: 15.7 (2.82)[†], P<0.01 after high LA
					Increased
Nelson et al. 2011 [37]	PLAC ELISA kit (diaDexus, Inc. South San Francisco, CA, USA)				B: 221.8 (62.1); F: 216.1 (63.5)[⊥], P=0.910 after EPA+DHA
					Decreased
					B: 223.3 (63.5); F: 213.8 (64.4)[⊥], P=0.910 after ALA
					Decreased

[§] Only significant subclasses or those that are comparable among the included studies are shown. [*] Average relative abundance (%) change (SD) in HDL-associated lipid subclasses relative to baseline, reported change after 6-hour HSF meal test.[**] Mean change expressed as µM (SD), reported change after 4-hour n-3 PUFAs or n-3 PUFAs + ASA intervention.[†] Mean expressed as µM (SD), effect after n-3 PUFAs intervention or [‡] after corn oil intervention.[#] Fold change of the lipid subclasses which change significantly from baseline during the different timepoints of the 5-hour OLTT. [‖] Mean expressed as µmol/L (SD), effect after 180g Atlantic salmon intake.[§] Relative abundance (mg %) of lipid subclasses after FF and MED interventions.[†] Mean expressed as ng/mL (SD), effect after EPA and DHA interventions compared to placebo.[†] Mean expressed as nmol/mL/min (SD), effect after LA interventions.[⊥] Mean expressed as ng/mL (SD), effect within groups after fish oil and flaxseed oil interventions. Abbreviations: ALA, alpha-linolenic acid; B, baseline value; C, control group; Cer, ceramide; DHA, docosahexaenoic acid; DiCer, dihydroceramide; EPA, eicosapentaenoic acid; F, final value; I, intervention group; LA, linoleic acid; LacCer, lactosylceramide; Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine; MUFAs, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids; SM, sphingomyelin; TEI, total energy intake.

Figure 1.

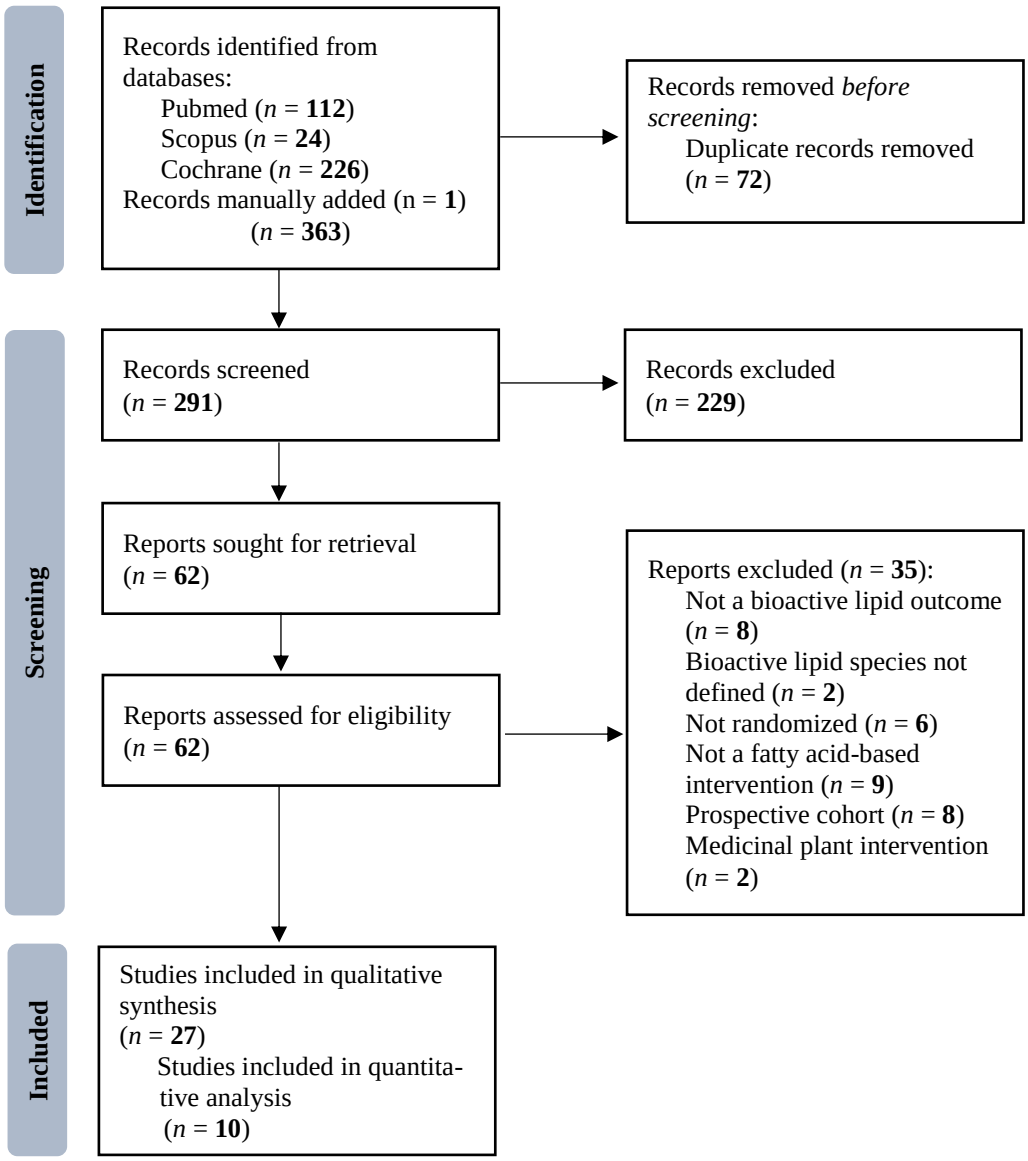

Identification
Records identified from databases:
Pubmed (n = 112)
Scopus (n = 24)
Cochrane (n = 226)
Records manually added (n = 1)
(n = 363)
Records removed before screening:
Duplicate records removed (n = 72)
Screening
Records screened (n = 291)
Records excluded (n = 229)
Reports sought for retrieval (n = 62)
Reports assessed for eligibility (n = 62)
Reports excluded (n = 35):
Not a bioactive lipid outcome (n = 8)
Bioactive lipid species not defined (n = 2)
Not randomized (n = 6)
Not a fatty acid-based intervention (n = 9)
Prospective cohort (n = 8)
Medicinal plant intervention (n = 2)
Included
Studies included in qualitative synthesis (n = 27)
Studies included in quantitative analysis (n = 10)

Figure 2.

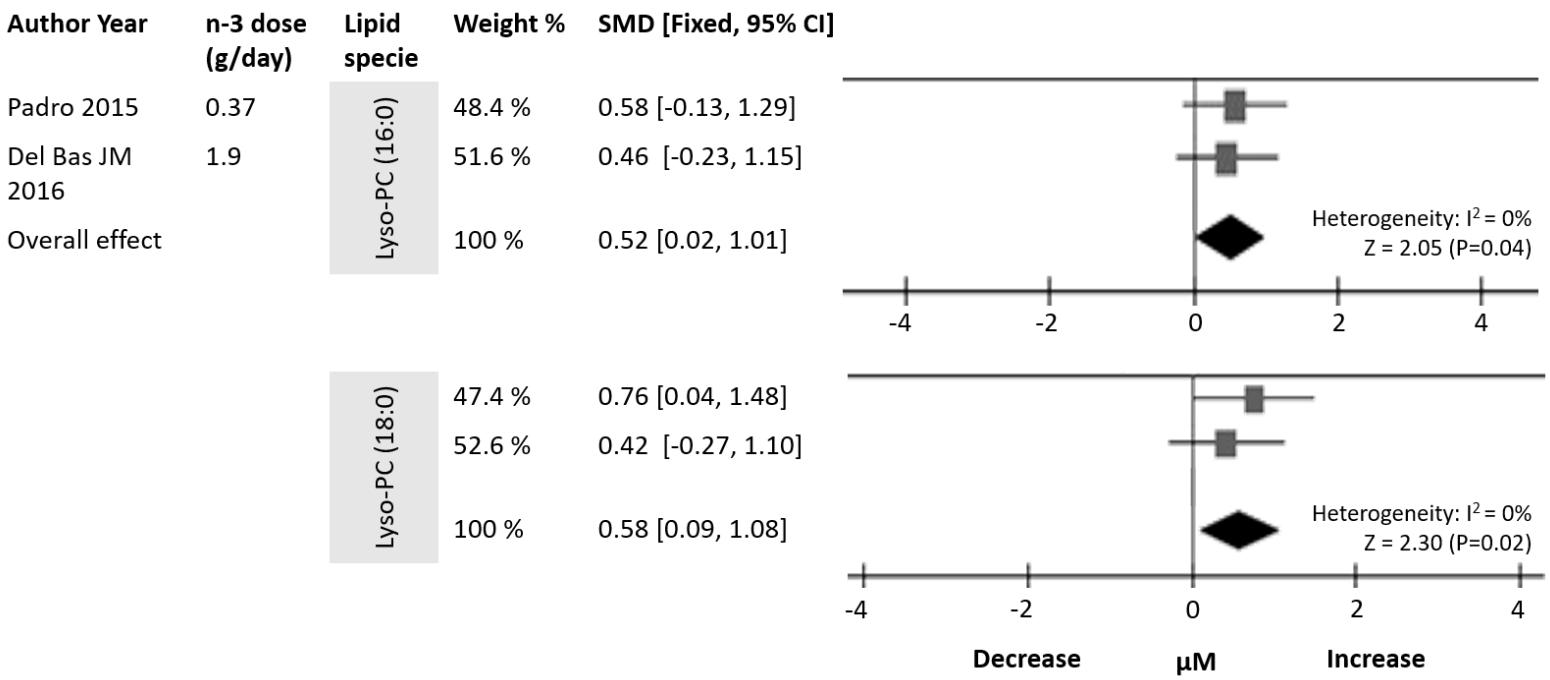

Author Year
n-3 dose (g/day)
Lipid specie
Weight %
SMD [Fixed, 95% CI]
Padro 2015
0.37
48.4 %
0.58 [-0.13, 1.29]
Del Bas JM 2016
1.9
51.6 %
0.46 [-0.23, 1.15]
Overall effect
100 %
0.52 [0.02, 1.01]
Lyso-PC (16:0)
Heterogeneity: I² = 0%
Z = 2.05 (P=0.04)
47.4 %
0.76 [0.04, 1.48]
52.6 %
0.42 [-0.27, 1.10]
100 %
0.58 [0.09, 1.08]
Lyso-PC (18:0)
Heterogeneity: I² = 0%
Z = 2.30 (P=0.02)
-4
-2
0
2
4
Decrease
µM
Increase

Figure 3.

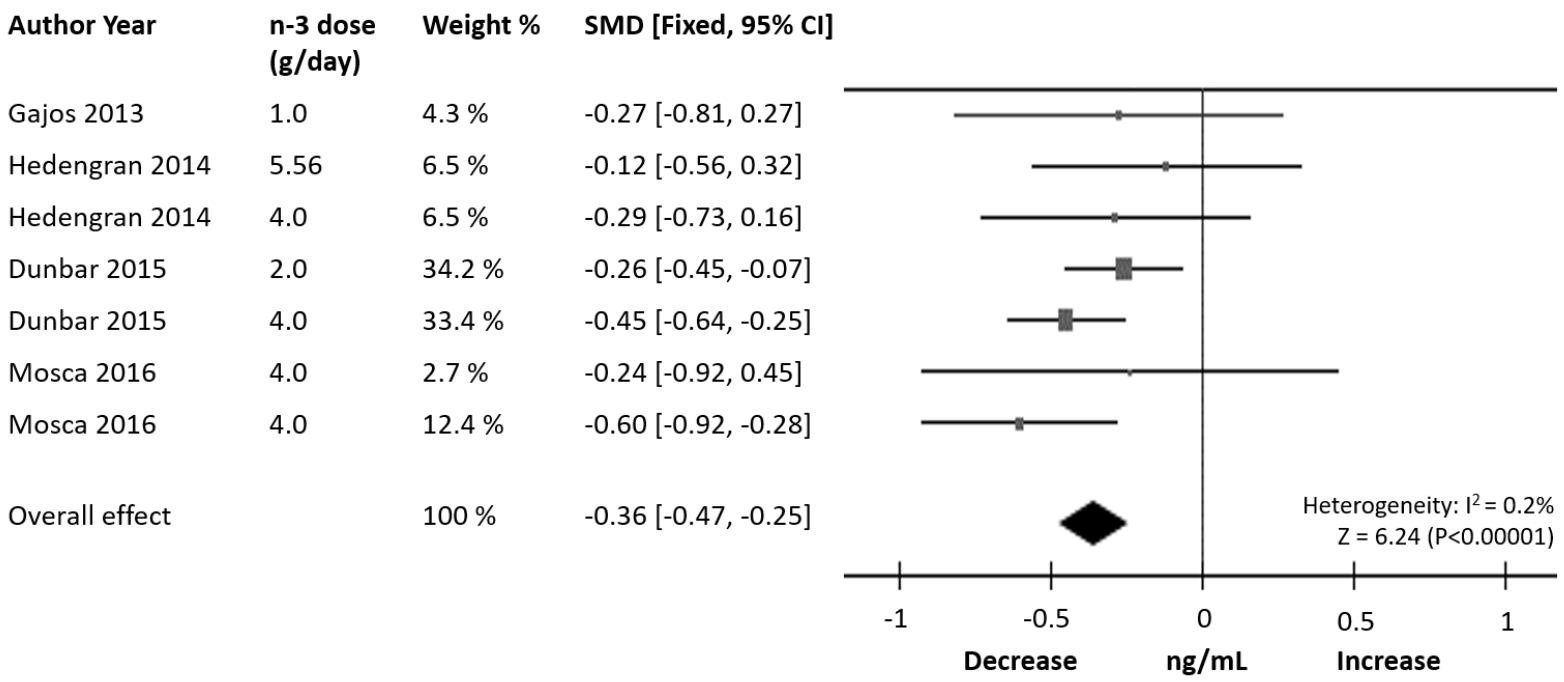

Author Year
n-3 dose (g/day)
Weight %
SMD [Fixed, 95% CI]
Gajos 2013 1.0 4.3 % -0.27 [-0.81, 0.27]
Hedengran 2014 5.56 6.5 % -0.12 [-0.56, 0.32]
Hedengran 2014 4.0 6.5 % -0.29 [-0.73, 0.16]
Dunbar 2015 2.0 34.2 % -0.26 [-0.45, -0.07]
Dunbar 2015 4.0 33.4 % -0.45 [-0.64, -0.25]
Mosca 2016 4.0 2.7 % -0.24 [-0.92, 0.45]
Mosca 2016 4.0 12.4 % -0.60 [-0.92, -0.28]
Overall effect 100 % -0.36 [-0.47, -0.25]
Heterogeneity: I² = 0.2%
Z = 6.24 (P<0.00001)
-1
-0.5
0
0.5
1
Decrease
ng/mL
Increase

Figure 4.

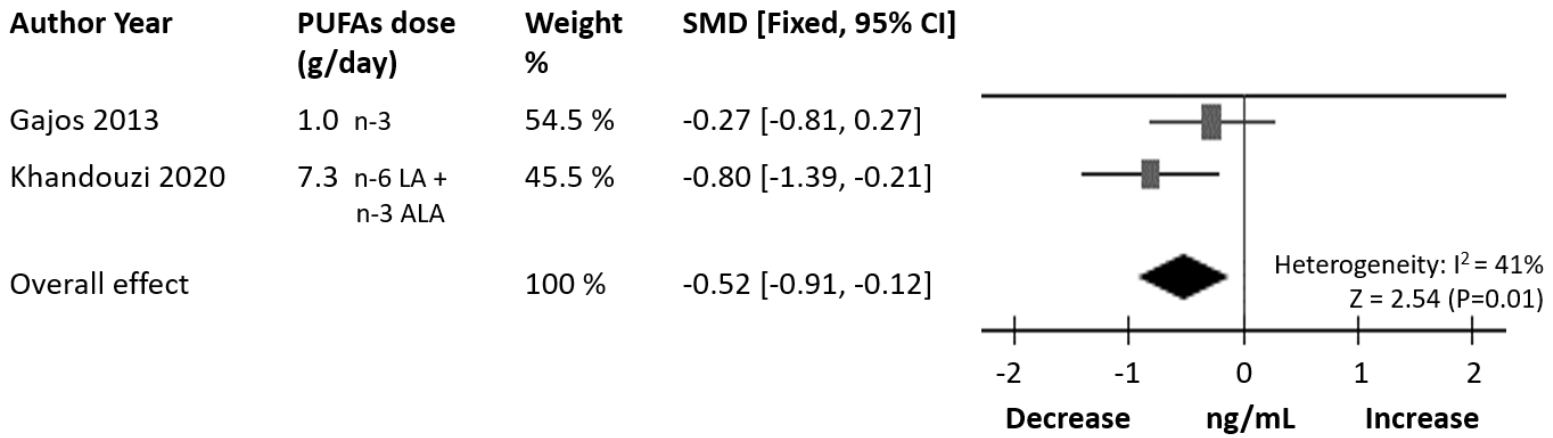
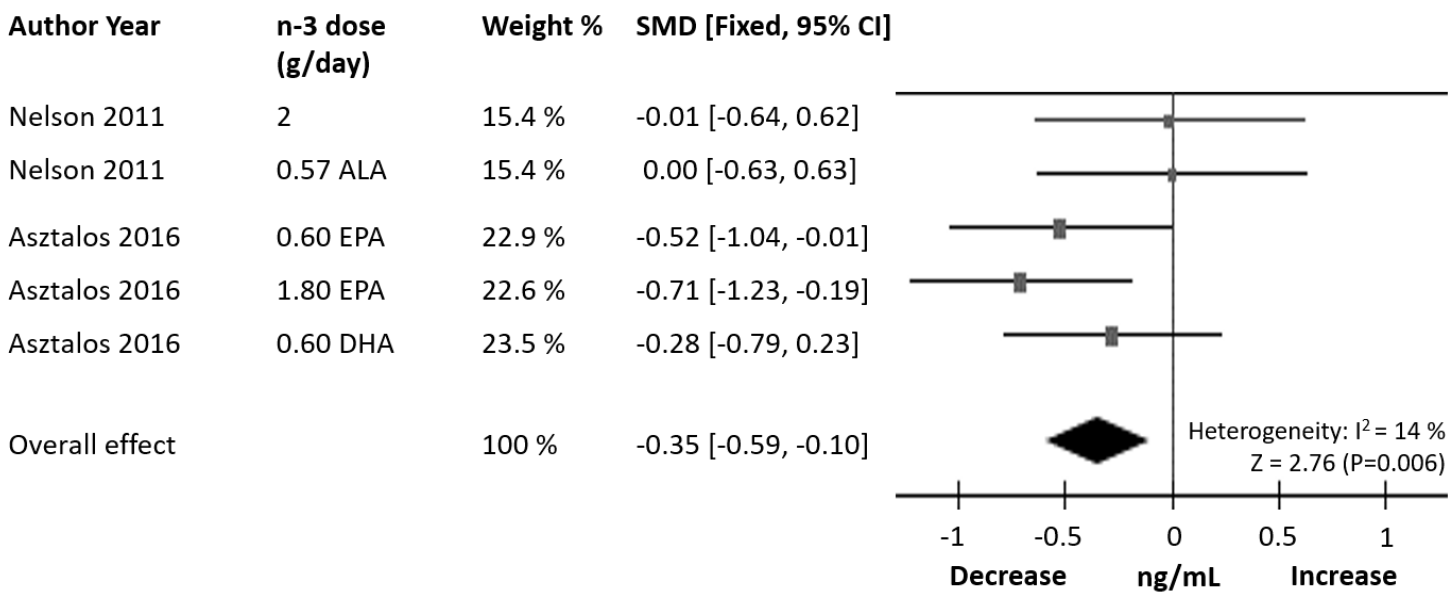

Author Year
PUFAs dose (g/day)
Weight %
SMD [Fixed, 95% CI]
Gajos 2013
1.0 n-3
54.5 %
-0.27 [-0.81, 0.27]
Khandouzi 2020
7.3 n-6 LA + n-3 ALA
45.5 %
-0.80 [-1.39, -0.21]
Overall effect
100 %
-0.52 [-0.91, -0.12]
Heterogeneity: I² = 41%
Z = 2.54 (P=0.01)
-2
-1
0
1
2
Decrease
ng/mL
Increase

Figure 5.


Author Year
n-3 dose (g/day)
Weight %
SMD [Fixed, 95% CI]
Nelson 2011
2
15.4 %
-0.01 [-0.64, 0.62]
Nelson 2011
0.57 ALA
15.4 %
0.00 [-0.63, 0.63]
Asztalos 2016
0.60 EPA
22.9 %
-0.52 [-1.04, -0.01]
Asztalos 2016
1.80 EPA
22.6 %
-0.71 [-1.23, -0.19]
Asztalos 2016
0.60 DHA
23.5 %
-0.28 [-0.79, 0.23]
Overall effect
100 %
-0.35 [-0.59, -0.10]
Heterogeneity: I² = 14 %
Z = 2.76 (P=0.006)
-1
-0.5
0
0.5
1
Decrease
ng/mL
Increase

Supplemental File 1 | Search strategy

We searched in PubMed, SCOPUS, and Cochrane Library, for eligible indexed articles published in English from October 2010 to October 2020. The following search strategy was applied:

#1. (lysophospholipids OR glycerophospholipids OR lysoglycerophospholipids OR sphingolipids OR lysophosphatidic acid OR lipoprotein-associated phospholipase A2 OR Lp-PLA2)

#2. (cardiovascular disease OR cardiometabolic disease OR cardiovascular risk NOT Fabry's disease)

#3. (fatty acid OR omega-3 fatty acids OR omega-6 fatty acids OR polyunsaturated fatty acids OR monounsaturated fatty acids OR saturated fatty acids)

#4. (RCT OR randomized clinical trial OR randomized control* trial NOT case-control NOT cohort NOT review)

PubMed

#A. #1 and #2 and #4

#B. #1 and #2 and #3 and #4

Additional limits:

#C. Limit to humans

#D. Limit to English language

#E. Limit to Controlled Clinical Trial; Randomized Controlled trial

SCOPUS

#A. (TITLE-ABS-KEY (#1)) AND (TITLE-ABS-KEY(#2)) AND (TITLE-ABS-KEY(#4)) AND (LANGUAGE (english)) AND (LIMIT-TO (humans))

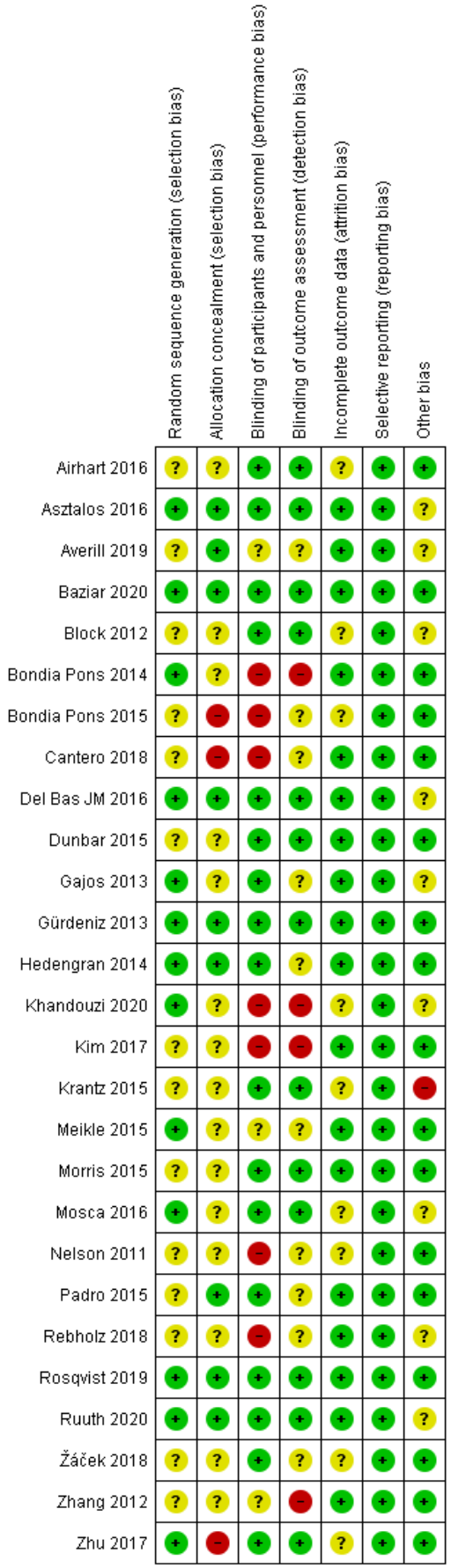
#B. (TITLE-ABS-KEY (#1)) AND (TITLE-ABS-KEY(#2)) AND (TITLE-ABS-KEY(#3)) AND (TITLE-ABS-KEY(#4)) AND (LANGUAGE (english)) AND (LIMIT-TO (humans))

Cochrane Library

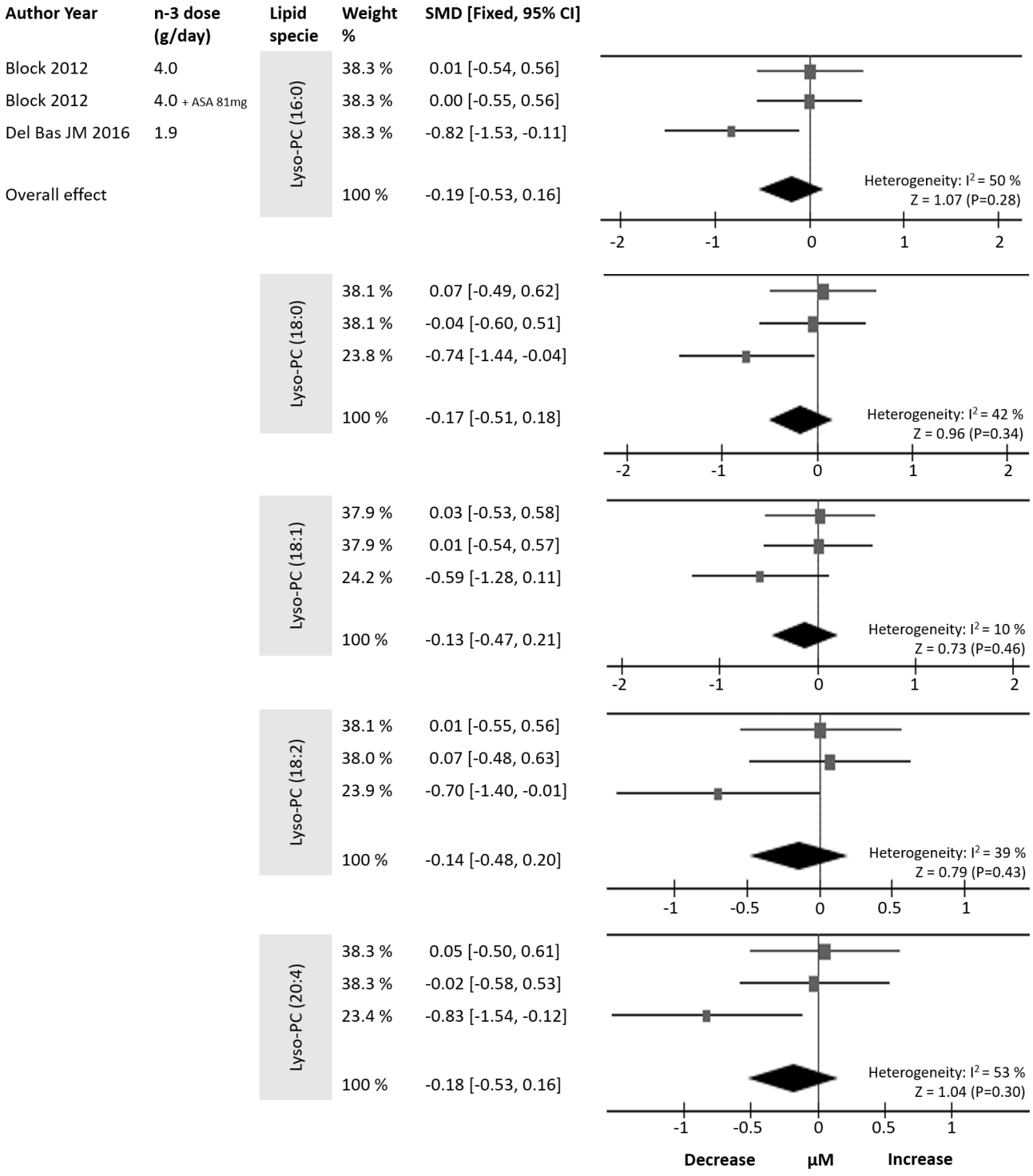
#A. #1 AND #2 Refined by: DOCUMENT TYPE: (TRIALS) AND LANGUAGES (ENGLISH)

#B. #1 AND #2 AND #3 Refined by: DOCUMENT TYPE: (TRIALS) AND LANGUAGES (ENGLISH)

Supplemental Figure 1 | Risk of bias summary: review authors' judgements about each risk of bias item for each included study.



Supplemental Figure 2 | Meta-analysis of RCTs of the effect of n-3 PUFA supplemented dietary interventions on saturated and unsaturated Lyso-PC levels in healthy subjects. The squares and bars estimate of the net standard mean difference (SMD) (difference between the end and baseline concentrations of bioactive lipid species) and the corresponding 95% CI for individual studies. The fixed effects model overall results are indicated by a rhombus symbol near the bottom of each graph. Lyso-PC, lysophosphatidylcholine; n-3, omega-3 polyunsaturated fatty acids.



SUMMARY OF RESULTS: PART II.II

Summary of results – Article 4. The effects of fatty acid-based dietary interventions on circulating bioactive lipid levels as intermediate biomarkers of health, cardiovascular disease and cardiovascular disease risk factors: A systematic review and meta-analysis of randomized clinical trials

From the 362 citations yielded from the literature search, 291 articles were retrieved and reviewed of which 229 were excluded for not meeting the eligibility criteria. Finally, 62 full-text RCTs and RTs were assessed for eligibility of which 27 were included in the overall systematic review, with 10 RCTs selected for meta-analysis. The complete PRISMA 2020 flow diagram is shown in page 266 of the present thesis.

Characteristics of the included studies

Of the 27 included studies, 21 were RCTs and 6 were RTs with sustained and postprandial interventions ranging from 1 to 6 months and 4 to 6 hours, respectively. These studies represented about 2560 subjects (men and women) aged between 18 and 75 years. Over 78% of the enrolled subjects presented at least one associated CVD or cardiometabolic disease risk factor, while less than 22% were healthy.

All the studies reported the effects of FA-based dietary interventions on circulating bioactive lipid levels, among other biochemical compounds, after lipidomic profiling. The most identified bioactive lipid classes were PLs, lyso-PLs and SLs (n = 17 studies), and their corresponding subclasses which were diverse according to the configuration and the degree of saturation of their acyl chain. Enzymatic intermediates, such as Lp-PLA2, were also identified

through immunoassay methods and assessed their changes after FA interventions (n = 10 studies).

The dietary interventions were variable between the studies, and FAs were administered in three different forms: as supplements (n = 12), EF-C (n = 9), or ED (n = 6). The supplemented interventions mainly consisted of marine fish oil capsules providing n-3 PUFAs in the form of eicosapentaenoic (EPA) and DHA acids with combined doses ranging from 0.6 to 5.56 g/day in the intervention groups. Two studies administered high fat enteral supplements in form of medium-chain FAs (MCFAs) or long chain triglyceride (LCT) fat emulsion. The EF-C interventions consisted of dietary fish, including n-3 PUFAs-rich fish, or enriched plant oils, spreads, dairy products and cereal-based matrices, mainly as n-6 PUFAs. Oily fish interventions included servings ranging from 160 to 400 g per week, providing from 1.0 to 1.6 g/day of EPA+DHA. One study used an n-3 PUFAs enriched milk, providing 0.37 g/day EPA+DHA. The studies including enriched plant oils differed in their FAs content and doses. The ED interventions included diets with controlled FAs content, primarily based on the Mediterranean dietary pattern reinforcing the n-3 PUFAs intake. ED provided daily doses of PUFAs ranging from 0.4 to 6.8% of TEI.

Further details of studied bioactive lipid classes, FA-based dietary interventions and doses are presented in Table 2 of the ***Article 4*** (page 239 of the present thesis).

Results of the effects of dietary FAs on circulating bioactive lipids profile in subjects with CVD and CVD risk factors

A total of 18 RCTs and 1 RT that contained data on dietary FAs intake, including SFAs, MUFAS, PUFAs and TFAs, and circulating bioactive lipids, including PLs, Lyso-PLs or SLs classes and subclasses, and Lp-PLA2 enzymatic precursor, were systematically reviewed in subjects with CVD or with CVD risk factors. Additionally, a set of fixed-effects model meta-analyses were performed in 7 RCTs.

The results for **overweight and obesity** were reported in 5 studies (detailed in Table 3 of the ***Article 4***, page 242 of the present thesis). In the systematic review, significant increases in PC subclasses containing very long chain (VLC)-PUFAs were showed after TFA interventions at daily doses of 15.7 g (26 g hydrogenated soybean oil). Also, SFA interventions at daily doses of 21.4 g (40 g palm oil) showed significant increases in MUFAs- and SFAs-containing ceramides (Cer) and sphingomyelins (SM), whereas n-6 PUFA-rich oils at daily doses of 26.1 g (40 g sunflower oil) showed opposed effects. Moreover, an overall decrease in serum lyso-PC, lyso-PE and lyso-PI was reported after n-3 PUFAs ED interventions. In the meta-analysis, the effects of n-3 PUFA supplements were analyzed for 2 studies. It was found a significant increase in saturated lyso-PC(16:0 and 18:0) [SMD (95 % CI); 0.52 (0.02, 1.01) and 0.58 (0.09, 1.08) μM, respectively], after n-3 PUFAs supplemented at daily doses ranging from 0.37 to 1.9 g/day EPA+DHA, with low heterogeneity between studies.

The results for **dyslipidemia** were assessed in 7 studies (detailed in Table 4 of the ***Article 4***, page 246 of the present thesis). In line with obesity results, in the systematic review, significant increases in the LDL surface molar concentrations of saturated lyso-PC(18:0) were showed after 0.375 g/day EPA+DHA. In addition, oily fish ED showed significant increases in the

plasma percentage (%) of EPA- and DHA-containing lyso-PC(20:5 and 22:6) after 80 g of oily fish 5 days a week (1.6 g/day EPA+DHA). Also, the molar % of EPA-containing lyso-PC(20:5) was significantly increased after the intake of 20 g/day of a plant stanol ester-enriched rapeseed oil spread (3.3 g/day n-6 LA and 1.3 g/day n-3 ALA). The effects of n-3 PUFAs were more pronounced for plasma Lp-PLA2 mass in 4 studies, therefore, a meta-analysis was performed. The meta-analysis found a significant decrease in plasma Lp-PLA2 mass [SMD (95 % CI); -0.36 (-0.47, -0.25) ng/mL], after n-3 PUFAs provided as highly bioavailable capsules at daily doses ranging from 1 to 5.56 g/day EPA+DHA, with low heterogeneity between studies.

The results for **metabolic syndrome** were revealed in 4 studies (detailed in Table 5 of the ***Article 4***, page 250 of the present thesis). In the systematic review, a short-term significant increase in plasma long chain- and VLC-PUFAs-containing PC, PE and lyso-PC, were evidenced after 2-month of n-3 PUFAs Mediterranean-based ED providing from 4.2 to 6.2% TEI as n-3. However, in the long term (6 months), the levels of most VLC-PUFAs-containing PC, PE, lyso-PC and lyso-PE were drastically reduced. Exceptionally, lyso-PC(16:0, 18:1, 20:5 and 22:6) subclasses remained significantly increased at long term.

The results for **type 2 diabetes** were revealed in 2 studies (detailed in Table 6 of the ***Article 4***, page 255 of the present thesis). In the systematic review, a significant decrease was reported in MUFAs- and SFAs-containing SM and Cer after the administration of a MCFAs-rich diet, providing 33-34% of SFAs and only 1-2% PUFAs. Moreover, the decreases in SM subclasses were positively correlated with lower fasting plasma insulin and HOMA-IR. In

addition, a significant decrease in plasma Lp-PLA2 mass was reported after a daily dose of 1200 mg alpha lipoic acid.

The results for **hypertension** were revealed in 2 studies (detailed in Table 7 of the ***Article 4***, page 256 of the present thesis). In the systematic review, a broad array of serum bioactive lipids, including MUFAs- and long chain-PUFAs-containing Cer, SM, PC, PE, lyso-PC and lyso-PE, showed lower concentrations after a DASH ED, relative to control diet. Conversely, VLC-PUFAs-containing PC(40:6) and PE(40:6) showed higher concentrations. No significant results were found for Lp-PLA2 after n-3 PUFAs supplementation at daily doses of 3.36 g/day EPA+DHA.

The results for **stable CAD** were revealed in 2 studies (detailed in Table 8 of the ***Article 4***, page 258 of the present thesis). Because the effects of n-3 and n-6 PUFAs on plasma Lp-PLA2 mass were notable for 2 studies, a meta-analysis was performed. The meta-analysis found a significant decrease in plasma Lp-PLA2 mass [SMD (95% CI); -0.52 (-0.91, -0.12) ng/mL] after 4 to 6 weeks of n-3 and n-6 PUFAs supplementation at daily doses of 1 g EPA+DHA and 25 mL of canola oil (7.3 g n-6 PUFAs), respectively, with moderate heterogeneity between studies.

Results of the effects of dietary FAs on circulating bioactive lipids profile in healthy subjects

A total of 5 RCTs and 4 RTs that contained data on dietary FAs intake, circulating bioactive lipids, and Lp-PLA2 enzymatic precursor, were systematically reviewed in healthy subjects (Table 9 of the ***Article 4***, page 259 of the present thesis). Additionally, a set of fixed-effects models meta-analyses were performed in 4 RCTs.

The results for **ED** were reported in 2 short-term and postprandial crossover studies. In line with metabolic syndrome individuals, healthy individuals manifested a significant enrichment of HDL surface PLs, mainly long chain-PUFAs-containing PC and PE subclasses, after high-saturated fat (HSF) meals providing from 24 to 44.7 g SFAs. However, VLC-PUFAs-containing PC(40:6 and 40:7) only increased after low saturated fat meals.

The results for n-3 and n-6 PUFA **EF-C** were reported in 2 studies. Significant selective increases of plasma VLC-PUFAs-containing PC, PE and lyso-PE subclasses were showed after 8-week oily fish intervention, providing 180 g salmon per week (6.15 g/day EPA+DHA). Remarkably, the increases were produced in a dose-dependent manner. Compared to dyslipidemic individuals, results in healthy subjects did not found significant changes in VLC-PUFAs-containing lyso-PC(20:5 and 22:6) after inclusion of dietary salmon. In addition, a significant increase in Lp-PLA2 activity was evidenced after the intake of a high LA (n-6) enriched-soy oil provided at daily doses of 9.9 g (54.2% LA).

The results for **dietary supplements** were reported in 5 studies. In one postprandial RT, a significant increase (fold change > 1.5 at 2-hour) was observed in unsaturated PE, lyso-PE and Cer for the timecourse of a 5-hour oral lipid tolerance test, providing 96% of TEI as fat (31 g MUFAs and 16 g PUFAs). Contrary, saturated lyso-PC(16:0 and 18:0) were diminished (fold change < 1.5 at 5-hour). In two RCTs, a meta-analysis was performed to assess the effects of n-3 PUFA marine oil supplements on saturated lyso-PC. The meta-analysis found a marked but not significant decrease in saturated lyso-PC(16:0 and 18:0) [SMD (95% CI); -0.19 (-0.53, 0.16) and -0.17 (-0.51, 0.18) µM, respectively], after daily doses of 1.9 to 4 g EPA+DHA, which was

opposed to the changes found in obese individuals. Other meta-analysis included two RCTs which assessed the effects of n-3 PUFA supplements on plasma Lp-PLA2 mass. In concordance with dyslipidemic subjects, this meta-analysis showed a significant decrease in Lp-PLA2 mass [SMD (95% CI); -0.35 (-0.59, -0.10) ng/mL] after daily doses of 0.6 to 2 g EPA+DHA, or 0.57 g ALA from flaxseed oil, for a period of 1.5 to 2 months. The heterogeneity between studies was low.

Generally, the results from this systematic review and meta-analysis offer for the first time an overview on the effects of dietary FAs on particular circulating bioactive lipids. It is evidenced great versatility in bioactive lipid biochemical structures according to the type of FAs intervention, which reflects a highly dynamic lipidome. The most consistent effects were attributed to the relatively high doses of dietary marine n-3 PUFAs, provided as supplements or ED, although questions remain regarding the optimal doses and duration period. Notably, marine n-3 PUFAs, provided as supplements or as oily fish, at doses from 0.30 to 3.4 g/day EPA+DHA, showed the most marked changes in circulating bioactive lipids profile in subjects with CVD risk factors. Also, doses from 0.6 to 5.56 g/day EPA+DHA, lead to decreased plasma Lp-PLA2 mass in healthy subjects, with CVD and with CVD risk factors. These EPA+DHA doses are above the last recommendation of the European Food Safety Authority (EFSA) based on cardiovascular risk considerations for adults, which range from 250 to 500 mg/day of combined EPA+DHA.

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

The work presented in this thesis represents a breakthrough in the discovery of novel gut microbiota- and metabolite-based biomarkers with promising applicability for the early diagnosis of the main causal and modifiable CVD risk factors. This work provides a better understanding of diet-gut microbe and metabolite-host interactions that manifest in the preliminary stages of hypertension and hypercholesterolemia pathogenesis. The research leading to these results was framed in the cross-sectional *Cardiogut* and *Bioclaims* studies, which generated a large amount of data that was comprehensively analysed in the course of this thesis. The integration of multiomics approaches, mainly targeted metabolomics, including lipidomics, and gut metagenomics performed in blood and faeces, together with multivariate predictive model analyses allowed for the identification of particular bacterial signatures and faecal or circulating metabolites as candidate early biomarkers for hypertension and hypercholesterolemia. From the *Cardiogut* study, a set of bacterial taxa, mainly *Bacteroides* spp., were enriched in HT subjects, whereas *Faecalibacterium* spp., *Roseburia* spp., and other genera of the Ruminococcaceae and Christensenellaceae families were depleted. These findings suggest a strong association with hypertension. Moreover, HT subjects also showed a depletion of SCFAs in plasma and higher levels in faeces. These findings are indicative of less efficient absorption in hypertension mediated by imbalanced host-microbiome crosstalk. From the *Bioclaims* study, particular circulating lyso-PLs, mainly serum lyso-PE 18:2 and lyso-PC 15:0, were identified as suitable susceptibility/risk biomarkers for hypercholesterolemia progression. Furthermore, the inclusion of experimental animal models allows for the investigation into the hepatic

mechanisms by which human lyso-PE 18:2 counteracts hypercholesterolemia progression. Thus, these results confirm our primary hypothesis that particular gut microbes and faecal or circulating metabolites derived from gut microbiota and lipid metabolites act as novel biomarkers that assist in the early detection of the main causal and modifiable CVD risk factors prior to drug treatment. Additionally, our reported relationships in both studies between bioactive dietary compounds, including PCs and FAs, and faecal or circulating metabolites, including SCFAs, lyso-PLs and TMAO, provide further insights into the complex pathways involved in the gut microbiome and metabolome, as well as in bioactive lipid dynamics. In turn, these interactions highlight the role of particular dietary components as modulators of the gut and circulating metabolome either in the pathogenesis or prevention of hypertension and hypercholesterolemia, thus verifying our secondary hypothesis. Despite the evident associations found between diet, microbiome and metabolome in these two cross-sectional studies, we could not conclusively determine whether our reported associated pathways were causal, correlational, or consequential of CVD or CVD risk factors. Thus, our systematic review and meta-analysis of human randomized clinical trials offer, from a lipidomic angle, an overview of the effects of dietary FAs on circulating bioactive lipids, including some of the lyso-PL classes previously identified in the *Bioclaims* study, as lipidomic biomarkers involved in health, CVD or CVD risk factors. The present discussion assembles the main results of the articles constituting this thesis.

In ***Article 1*** (the *Cardiogut* study), through a comprehensive taxonomic and functional analysis of faecal samples from HT and NT subjects, we

identified a higher abundance of *Bacteroides coprocola*, *Bacteroides plebeius*, *Intestimonas* and genera of the Lachnospiraceae family in HT subjects. However, *Faecalibacterium prausnitzii, Roseburia hominis,* Ruminococcaceae NK4A214, Ruminococcaceae_UCG-010 and Christensenellaceae_R-7, were less abundant in HT subjects and increased in NT subjects. Moreover, *B. coprocola* (ASV s41 and s16) and *F. prausnitzii* (ASV s372) presented the highest discriminant power in the HT and NT groups, respectively. Remarkably, *F. prausnitzii,* which is a well-known butyrate producer (M. Zhang et al., 2019), was negatively associated with SBP and DBP, suggesting a favourable effect on BP homeostasis mediated by the anti-inflammatory action of butyrate. Additionally, the *Roseburia hominis* and Ruminococcaceae genera, enriched in NT, have been described as potent SCFA producers in healthy individuals (de la Cuesta-Zuluaga et al., 2018; Shoaie et al., 2015). Thus, it is to be expected that our observed depletion of SCFAs-producing bacteria in HT subjects could affect both the production and absorption of SCFAs, which is a mechanism of high BP. To test this theory, by appying metabolomics, we further analysed these fermentation metabolites in faeces and plasma. We found antagonistic results, reporting that HT subjects had significantly lower levels of plasma acetate, isobutyrate, butyrate and isovalerate, and higher faecal levels of acetate, propionate, butyrate and valerate than NT subjects. These results were corroborated by the significant positive correlations found between SBP and faecal SCFAs that were increased in HT. Similar to our findings, *de la Cuesta-Zuluaga et al.* reported that higher faecal SCFA concentrations were associated with a hallmark of hypertension and gut permeability (de la Cuesta-Zuluaga et al., 2018). Additionally, previous cross-sectional studies have reported higher

faecal SCFA levels in overweight and obese individuals than in lean individuals (Fernandes et al., 2014; Rahat-Rozenbloom et al., 2014; Schwiertz et al., 2010).

Nonetheless, evidence regarding the causative link between decreased plasma SCFAs and human hypertension is scarce. Perhaps, our observed diminished plasma SCFA levels in HT subjects could result from a downregulation of absorption mechanisms due to reduced expression of SCFA transporters in the colon or SCFA-sensing receptors in the hypothalamus, as recently shown in spontaneously hypertensive rats (T. Yang et al., 2019). This mechanism may explain the reduced central responsiveness to SCFAs, thus dismissing their beneficial effects on BP homeostasis. Despite this, we cannot disregard other factors, such as the dysfunction of the intestinal epithelial barrier or increased proinflammatory cells, which have been related to high BP (S. Kim et al., 2018). In this sense, gram-negative bacteria, such as *B. coprocola*, which is enriched in HT subjects, are a source of lypopolysaccharides (LPS). LPS are also known as endotoxins and have proinflammatory action on intestinal epithelial cells (Nighot et al., 2017). In contrast, SCFAs-producing bacteria through SCFAs, primarily butyrate, have shown anti-inflammatory effects via modulation of the function of intestinal macrophages by reduction of LPS-induced pro-inflammatory mediators and inhibition of histone deacetylase, as reported *in vitro* and *in vivo* models (Chang et al., 2014). Hence, depletion of SCFAs-producing bacteria in the gut may difficult the host's ability to repair the epithelium and regulate inflammation, a risk factor for hypertension (Yan et al., 2017). Other pathways that link circulating SCFAs with BP involve the activation of endothelial GPRs, primarily GPR41, which lowers baseline BP in

mice (Natarajan et al., 2016). Currently, there are no human intervention studies using SCFAs to target BP. However, recent RCTs with butyrate supplementation have demonstrated a potential adjuvant effect in the reduction of BP in subjects with type 2 diabetes (Roshanravan et al., 2017) and metabolic syndrome (Bouter et al., 2018).

From our results, it is noteworthy that no significant differences were found regarding microbial richness and alpha diversity between HT and NT subjects. In contrast to our results, as recently reviewed, several cross-sectional studies have reported a dysbiotic gut microbiome in HT individuals that is mediated by a decrease in microbial richness and alpha diversity (Y. Guo et al., 2021). Moreover, higher BP has been associated with lower gut microbiota alpha diversity. This reduction in dysbiosis markers could be explained by the fact that the majority of these studies did not adjust the analyses for important confounders such as age, BMI, or lifestyle and dietary factors, which could impair the gut microbial community. Likewise, in these studies, the use of antihypertensive drugs could have induced the reported compositional changes in gut microbiota. Thus, instead of a clear dysbiosis in HT subjects, our results suggest that prior to drug treatment, HT subjects could have specific alterations in particular bacterial signatures and metabolites. These findings indicate a new disease classifier to discriminate hypertension from a healthy status.

Despite that our baseline office BP record could be biased by white-coat or masked hypertension (Pioli et al., 2018), we ensured proper BP monitoring procedure according to the recommendations of the ESC/ECH Hypertension Guidelines (Williams et al., 2018). In fact, in line with our results, a cohort study monitoring 24-hour ambulatory BP also showed an association

between gut microbiota and BP homeostasis, as well as higher faecal SCFAs in HT than NT individuals (Huart et al., 2019), thus excluding a white-coat effect.

Diet has a substantial impact on gut microbiota composition. Carbohydrates are the most studied dietary component for their capacity to modulate the gut microbiome. In particular, nondigestible carbohydrates, such as dietary fibre and resistant starch, are good sources of "microbiota accessible carbohydrates" (MACs) which can be fermented by colonic gut microbes to provide the host with energy and a carbon source (Singh et al., 2017). Diets low in prebiotic fibres, such as fermentable oligosaccharides, disaccharides, monosaccharides and polyols, have been shown to reduce total bacterial abundance, mainly *Bifidobacteria* and *Lactobacilli*, and SCFAs faecal concentrations (Halmos et al., 2015). From our ***Article 1*** results, we revealed an overall inverse association between faecal propionate, valeric and isobutyric acids and total dietary fibre intake, which was assessed by 3-day dietary records. However, NT subjects reported significantly higher fibre intake. This inconsistent relationship could be related to the lower levels of faecal SCFAs detected in NT subjects compared to HT subjects. Future studies should establish correlations with particular types of fibre, such as soluble and insoluble, measured by objective intake methods. However, fibre-rich foods are usually good dietary sources of PCs and well-known bioactive dietary compounds with prebiotic properties on gut microbiota (Kawabata et al., 2019). Thus, in ***Article 2***, we focused on the multiple-way relationships between PCs and gut microbiota- and metabolite-based biomarkers that were identified in the nontreated HT and NT subjects in ***Article 1***.

In ***Article 2***, we hypothesized that specific dietary PCs from a habitual diet could be precursors for the occurrence of particular bacterial signatures that differ between HT and NT subjects. We performed a comprehensive quantification of regular PC intake from the *Phenol-Explorer* database and used multivariate regression to correlate the obtained data with discriminant bacterial taxa (ASVs) and SCFAs previously identified in HT and NT subjects.

According to our results, in NT subjects, positive associations were observed between olive fruit PCs, mainly anthocyanins, with NT-enriched Ruminococcaceae UCG-010 and Christensenellaceae R-7 taxa and with plasma butyrate and valerate. It should be noted that the average intake of olive fruits in NT subjects (25 g/day) was approximately three times the median consumption in the Spanish population (7 g/capita/day) (Ministerio de Agricultura, Pesca y Alimentación, 2018), providing 24.79 mg/day of PCs, mainly phenolic acids. These interrelationships could reflect favoured SCFA production and absorption in the colon of healthy subjects through an increase in SCFA-producers by olive fruit PCs. Moreover, the negative correlations previously found for Christensenellaceae R-7 with SBP and DBP (Calderón-Pérez et al., 2020) reinforce the role of these PCs in the prevention of hypertension. However, more studies are needed to decipher whether this observed link is due to olive fruit PCs or due to the imminent probiotic action of *Lactobacillus* strains used as fermentation starters (Perpetuini et al., 2020).

On the other hand, in HT subjects, we found that coffee PCs, mainly hydroxycinnamic acids and alkylmethoxyphenols, were positively associated with HT-enriched *B. coprocola* and *B. plebeius* and with faecal propionate, acetate and valerate. The mean coffee intake reported for the HT subjects

was 60.11 mL/day. In agreement with our results, a recent cross-sectional study in healthy subjects observed a positive correlation between methoxyphenols and alkylmethoxyphenols and the *Bacteroides-Prevotella-Porphyromonas* group in high coffee consumers (> 45 mL/day) (González et al., 2020). Furthermore, they also reported a direct association between *Bacteroides* spp. and caffeine, which suggests that caffeine could be a confounder that weakens the strength of the associations among coffee PCs and gut microbiota. Despite this, our additional observed relationship for coffee PCs with faecal SCFAs in HT supports the hypothesis that, in the first grade of hypertension, coffee PCs could modulate gut microbiota by favouring the growth of *Bacteroides* spp. This may contribute to less efficient SCFA absorption due to the disruption of the epithelial barrier. This theory was reinforced by the negative associations demonstrated in the NT subjects between coffee PCs and the NT-enriched *F. prausnitzii* and Christensenellaceae R-7, which could impair SCFA production preceding the alteration of BP. In fact, an overall positive association was found between main coffee PCs and SBP and DBP. Thus, given the negative impact of coffee PCs on the gut microbiome in both HT and NT subjects, our results indicate a new pattern in which coffee PCs could precede the rise in BP.

Despite our proposed theory linking coffee PCs, the gut microbiome, and hypertension, during the last decade, several prospective cohort studies have supported the association between long-term coffee consumption and the decreased risk of hypertension, as reported in a recent dose-response meta-analysis (Grosso et al., 2017). However, in this meta-analysis, a reported significant decrease in risk of 9% was observed for seven cups of coffee/day, whereas for up to 6 cups/day, the decrease in risk was not

significant. Taking into account that a cup of coffee equals 150-300 mL, it is evident that their observed decrease in hypertension risk only occurs in very-high coffee consumers, which is far from our estimated average intake in HT subjects (approximately 60 mL/day).

To date, the main mechanisms of action of coffee PCs on BP regulation rely on the antioxidant activity of chlorogenic acids, which are predominantly phenolic acids. They act through the inhibition of NAD(P)H oxidase expression and by directly scavenging for free radicals. They also have an anti-inflammatory action that acts through the suppression of proinflammatory cytokines, such as IL-8 and IL-6, via the modulation of key transcription factors (Liang & Kitts, 2015). Additionally, in spontaneously hypertensive rats, chlorogenic acids have been shown to stimulate NO bioavailability by inhibiting excessive production of ROS in the vasculature, therefore attenuating endothelial dysfunction, vascular hypertrophy and hypertension (Suzuki et al., 2006). Contrary to these previous findings, our microbial composition and metabolomics analysis provides for the first time evidence on the modulatory capacity of coffee PCs, primarily hydroxycinnamic acids and alkylmethoxyphenols, on the gut microbiota and its derived SCFAs as alternative pathways involved in the progression of hypertension.

Overall, the results from ***Article 1*** and ***Article 2*** in the *Cardiogut* study highlight the potential usefulness of particular faecal microbes and SCFAs as indicators of the gut microbiota ecosystem and metabolite dynamics in a first disease grade of hypertension prior to drug treatment. This discovery, along with the multiple interplays found between specific dietary PCs, targeted microbes, SCFAs and BP, provide knowledge on new pathways involved in

disease susceptibility or prevention (***Figure 26***). Nevertheless, the cross-sectional nature of this study failed to track changes within each individual, and limited the establishment of causal relationships. Thus, future studies should focus on collecting stool and blood samples overtime to assess longitudinal changes and better understanding the extent of these interactions on BP.

A determining limitation in the *Cardiogut* study was the reduced sample size, as it may have led to diminished statistical power for some biomarkers due to increased intraindividual variability. For instance, this limitation could explain the lack of significant associations found for total polyphenol intake or urinary total polyphenol excretion, as objective markers of exposure to dietary PCs, with BP values. Another limitation regarding procedures was the method used for faecal SCFA quantification. This method involved the use of a system consisting of gas chromatography coupled to a flame ionization detector (GC/FID), which could occasionally cause potential loss of SCFAs given the strong volatility of the derivatives; therefore, future studies should use more advanced techniques (H. Kim et al., 2019). Finally, although our analyses were adjusted for various known confounders, mainly clinical and dietary parameters, residual confounders remain possible. The existence of unmeasured human factors, such as interindividual variability in gut microbiota composition, may have influenced the results. Indeed, interindividual variations in the gut bacterial community could affect the intrinsic activity, metabolization and absorption rate through the gut barrier of PCs (Tomas-Barberan et al., 2016). Other uncontrolled factors are those affecting digestive efficiency, such as the transit time, which has been shown

to increase the richness and alter the composition of faecal microbial communities (Roager et al., 2016).

One distinctive point was the integration of 16S rRNA gene sequencing, metagenomics and targeted metabolomics that provided a complete picture of the gut microbial compositions and functions, and also of the metabolite dynamic shifts. Moreover, the application of suitable tools to report dietary PCs allowed collected accurate information on the regular intake.

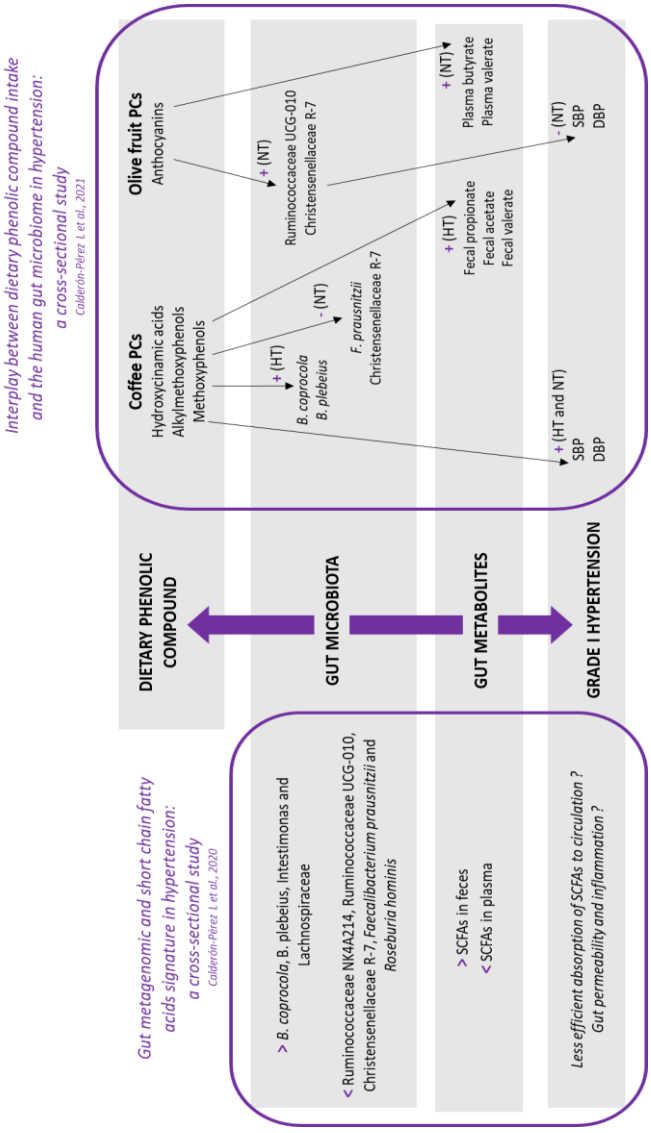


Figure 26 | Overview of the multiple-way relationships for gut microbiome and metabolome biomarkers, dietary phenolic compounds and blood pressure in the susceptibility or prevention of grade 1 hypertension. Abbreviations: DBP, diastolic blood pressure; HT, hypertensive; NT, normotensive; PCs,

Apart from hypertension, hypercholesterolemia (or high LDL cholesterol) constitutes a major modifiable CVD risk factor and key component of traditional risk prediction algorithms (Mach et al., 2020). Beyond conventional lipid-related markers (i.e., total cholesterol, LDL cholesterol, and HDL cholesterol), bioactive lipids (i.e., lyso-PLs) have emerged as molecules of special interest due to their independent predictive value for negative CV outcomes (Tan et al., 2020). Thus, in addition to the gut microbiome, the human lipidome hosts a niche of circulating metabolites with particular relevance as biomarkers for the early detection and progression of CVDs.

In ***Article 3*** (the *Bioclaims* study), through targeted lipidomic and metabolomic analyses, we determined the serum lyso-PLs and plasma TMAO concentrations in MH-LDL-c and L-LDL-c individuals. Our results confirmed the hypothesis that particular serum lyso-PLs could be precursors of hypercholesterolemia progression, as their rise occurs prior to LDL cholesterol increase. Notably, after performing predictive models, lyso-PC 15:0 and lyso-PE 18:2 were identified as the most discriminant lyso forms between the MH-LDL-c and L-LDL-c groups with potential as susceptibility/risk biomarkers of human hypercholesterolemia. Lyso-PC 15:0 was highly concentrated in MH-LDL-c subjects, and lyso-PE 18:2 was increased in L-LDL-c subjects but significantly decreased in MH-LDL-c subjects. Additionally, the relationships among the classical lipid-related markers in MH-LDL-c subjects demonstrated a strong positive association for lyso-PC 15:0 with total cholesterol, LDL cholesterol and the atherogenic index of LDL cholesterol/HDL cholesterol. These result are in accordance with previous human studies reporting a link between lyso-PC 15:0 and the

incidence of type 2 diabetes and increased DBP in diabetic individuals (Kröger et al., 2011; Petersen et al., 2017). Moreover, lyso-PC 15:0 has been recently reported to be among the main differential metabolites in coronary heart diseases, including stable angina and myocardial infarction (Lu et al., 2017). Nonetheless, our findings provide an additional association between lyso-PC 15:0 and lipid disorders and atherosclerosis. In contrast, the negative associations found in L-LDL-c between lyso-PE 18:2, total cholesterol and LDL cholesterol suggest a possible protective role of lyso-PE 18:2 against the onset of human hypercholesterolemia.

The fact that circulating levels of lyso-PE 18:2 were lower in MH-LDL-c subjects may be indicative of increased LDL cholesterol in the preliminary stage of hypercholesterolemia. To support this hypothesis and inquire into investigate the mechanisms involved, lyso-PL biomarkers were further evaluated from a secondary exploratory study with hamsters fed either a moderate HFD to induce isolated hypercholesterolemia or a LFD. Overall, we found an overall intrahepatic accumulation of polyunsaturated lyso-PE, including lyso-PE 18:2, in HFD-fed hamsters, which could explain the protective effect against the benign progression of diet-induced hepatic steatosis. This statement is in accordance with previous studies in rodents in which the chronic administration of diets rich in fats led to intrahepatic accumulation of lipids (Nam et al., 2015; Sampey et al., 2011; Zou et al., 2006). Moreover, similar to our results, in a recent study with HFD-fed hamsters lyso-PE 18:2 was among lyso-PLs that exhibited the earliest alterations in response to induced-dyslipidaemia (Suárez-García et al., 2017). However, in our model, the histological examination did not reveal hepatocyte balloon degeneration or fibrosis, and circulating transaminases

were unaltered. These results suggest that the degree of steatosis that was induced by diet was still incipient and that the liver may not have yet experienced major injuries that could affect its functionality. Thus, the increased hepatic lyso-PE 18:2 seems to reflect an adaptation mechanism of the liver in response to excess fat in the diet to protect itself from the detrimental effects of hepatic steatosis in animals and to counteract hypercholesterolemia progression in humans. Furthermore, the positive correlation between lyso-PE 18:2, liver GGT and dietary omega (n)-PUFAs, such as LA (C18:2n-6), found in MH-LDL-c subjects indicates that the proposed mechanism to counteract hypercholesterolemia progression could be comparable in both human and animal models.

In parallel to our observed intrahepatic modification of the lysophospholipidome in favour of polyunsaturated acyl lyso forms, from our *in vivo* study we also revealed an intrahepatic replacement of saturated and monounsaturated acyl lyso-PLs in HFD-fed hamsters. Even though the HFD was primarily enriched in SFAs and MUFAs, this event could be explained by sequential processes of desaturation and elongation of unsaturated lyso forms in rodent liver, as reported in several studies on free FAs (Legrand & Rioux, 2010; Rioux et al., 2005). Through this processes, dietary SFAs, such as myristic acid (14:0) or pentadecanoic acid (15:0), would contribute to the metabolism of LA and ALA in hepatocytes and therefore regulate the bioavailability of long-chain n-3 PUFAs, as previously described in rats (Rioux et al., 2005) and human (Dabadie et al., 2005). Thus, our strong association reported in MH-LDL-c subjects for SFAs intake, including whole dairy, with lyso-PC 15:0 and 17:0 could support the intermediate role of SFAs on the hepatic levels of polyunsaturated lyso-PLs.

It is generally accepted that PUFA-derived metabolites, such as arachidonic acid and LA, that are esterified to PLs in LDL are primary targets for free radical-induced lipid peroxidation (LPO) (Yin et al., 2011). When LPO is produced, oxidized PLs and their hydrolysed FAs may be a predominant feature and contributing factors to hypercholesterolemia, as recently reported in patients with coronary heart disease (Lu et al., 2017). Thus, rather than lyso-PL action, the pro-hypercholesterolaemic state in the MH-LDL-c group could be caused by the direct action of LPO. Despite this, our comprehensive analysis of dietary components allowed us to demonstrate interesting relationships among dietary FAs and particular lyso-PLs, which indicates a more complex clinical presentation of hypercholesterolemia than that represented by classical lipid markers.

From our results, TMAO was left out the main susceptibility/risk biomarkers for hypercholesterolemia or hypertension in the case of the *Cardiogut* study. Mechanistic preclinical studies have identified TMAO as a promising gut-derived metabolite involved in disease progression, and it has been associated with CV events (Bennett et al., 2013; Tang et al., 2013; Z. Wang et al., 2011). However, our results support the notion that in preliminary stages of human hypercholesterolemia and in nontreated grade 1 hypertension, certain circulating gut metabolites, such as Lyso-PLs and SCFAs, are better indicators of disease risk than TMAO. It is conceivable that the presence of CVDs might affect gut microbiota composition and lead, in turn, to an increased production of TMAO, which would refute its role as an early biomarker (Nowiński & Ufnal, 2018; Schiattarella et al., 2018).

In a recent meta-analysis involving 6176 hypertensive cases (Ge et al., 2020), it has been revealed a significant positive dose-dependent association

between high circulating TMAO concentrations and hypertension prevalence. The RR for hypertension prevalence increased by 9% and 20% per 5 µmol/L and 10 µmol/L increment of TMAO concentration, respectively. However, in this meta-analysis, all enrolled participants presented a high CVD risk and severe grade of hypertension, hindering to determine whether high TMAO is a triggering factor for hypertension. Thus, the association of TMAO with the hypertension risk still remain a classic chicken-and-egg mystery. Furthermore, our observed interindividual variability in circulating TMAO levels could be highly influenced by several factors, including gut microbiota composition and activity, liver function, permeability of the gut-blood barrier, and diet (Nowiński & Ufnal, 2018). In this sense, our observed positive associations between TMAO and fish intake in MH-LDL-c subjects in the *Bioclaims* study indicate fish consumption as a plausible dietary source of TMAO. Therefore, given the questionable usefulness of TMAO as a novel biomarker of CVD risk in primary prevention, further prospective studies will be needed to clarify its associations with the main causal and modifiable CVD risk factors. In addition, given that both TMAO and lyso-PLs originate from the enzymatic hydrolysis of primary molecules, such as TMA and PLs, in the gut (Hui, 2016) and that hydrolysed lyso-PLs are a substrate for gut bacteria to synthesize TMA, it would be of particular interest to study the intestinal synergies of these metabolites as possible mechanistic pathways involved in CVD development.

Finally, the *Bioclaims* study suggested that different FA-rich sources of foods could modulate the FA-acyl chain of circulating lyso-PLs. As shown by the strong associations between dietary PUFAs and lyso-PE 18:2 in both the MH-LDL-c and L-LDL-c groups, the intake of essential omega (n)-PUFAs, such

as LA (C18:2n-6), could modify the circulating lysophospholipidome. This reinforce the protective role of lyso-PE 18:2 against the onset of human hypercholesterolemia and hepatic steatosis that was demonstrated in hamsters (***Figure 27***). However, the existing body of literature regarding the dietary origins of lyso-PLs in humans is scarce. Given the lack of causality of our observed associations, future interventional studies are needed to better understand the extent to which dietary FAs modify the plasma lysophospholipidome in CVDs. In this context, ***Article 4*** focused on a systematic review and meta-analysis to further investigate this topic.

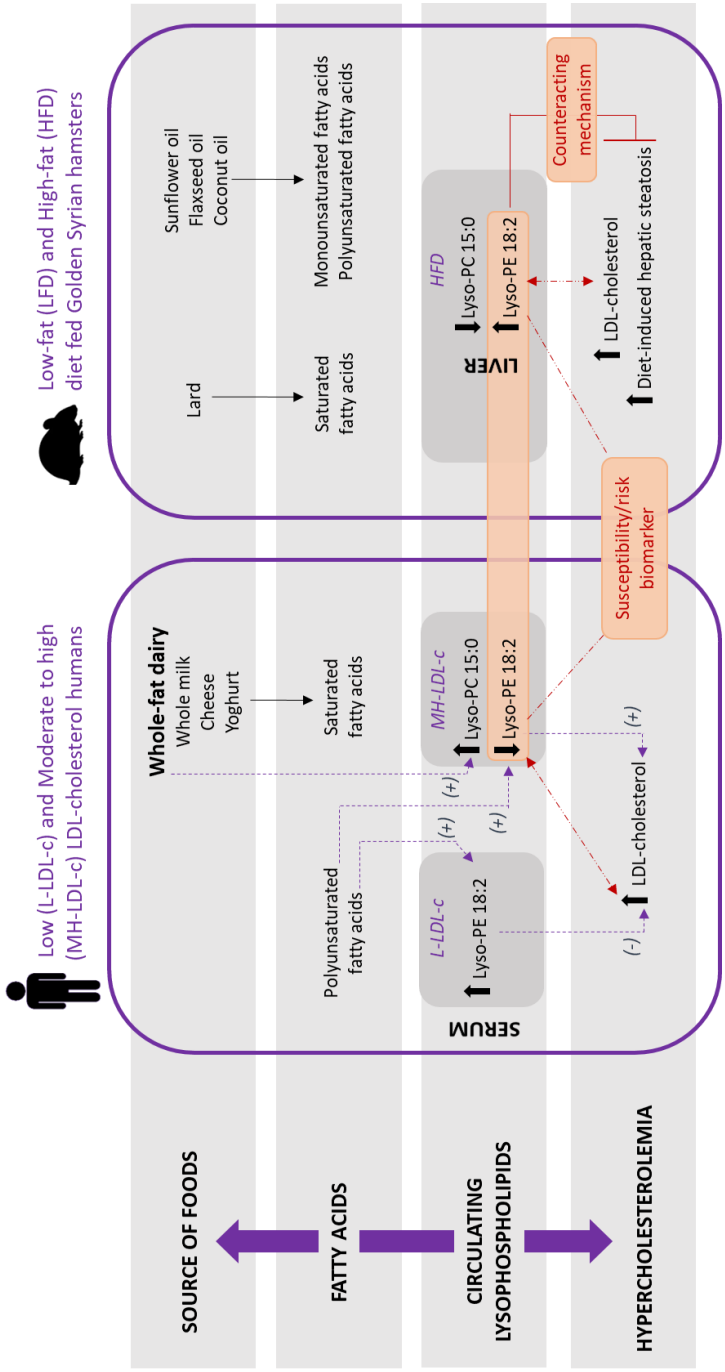


Figure 27 | Modulatory capacity of dietary fatty acids on the circulating lysophospholipidome in relation to human hypercholesterolemia and diet-induced hepatic steatosis in hamsters. Abbreviations: HFD, high-fat diet; LDL, low-density lipoprotein; LFD, low-fat diet; Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine. Own source.

In ***Article 4***, we systematically reviewed a total of 27 randomized clinical trials which provided available scientific evidence on the effects of dietary FAs, including SFAs, MUFAs, PUFAs and TFAs, on the circulating bioactive lipid profile of healthy subjects, with CVD or CVD risk factors. A set of meta-analyses were performed by including 10 RCTs assessing the effects of PUFA-supplemented interventions, mainly n-3 PUFAs, on bioactive lipid or enzymatic precursors.

We identified 11 bioactive lipid classes that represented more than 150 lipid subclasses differing in their fatty acyl chains. Remarkably, the results showed great versatility in the biochemical structures of circulating bioactive lipids according to the type of FA-based interventions, reflecting a highly dynamic lipidome. Primarily, our set of meta-analyses evidenced the direct effects of an increased intake of n-3 PUFAs on lyso-PC concentrations. In subjects at risk for CVD, including those with the major risk factors of obesity, dyslipidaemia and metabolic syndrome, a net increase (approximately +0.50 μM in those with obesity) in saturated-chain lyso-PC(16:0 and 18:0) was observed after n-3 PUFAs supplementation (daily doses from 0.37 to 1.9 g EPA+DHA). In contrast, in healthy subjects, the meta-analysis results for these saturated lyso-PC showed a decreasing trend. These opposing results could reflect a decrease in saturated lyso-PC catabolism under altered cardiometabolic conditions. Although the increase in lyso-PC(16:0 and 18:0) levels could be mediated by a chronic proinflammatory state in obesity (Ellulu et al., 2017), our results are in accordance with previous evidence suggesting an impairment in the response of the metabolism of lyso-PLs to n-3 PUFAs in an obese population (Del Bas et al., 2016). Furthermore, under altered cardiometabolic conditions the modulatory capacity of n-3 PUFAs, in

the form of EPA and DHA, on the fatty acyl moieties of saturated lyso-PC might be diminished (Browning et al., 2012).

Apart from supplemented n-3 PUFAs, we found that the intake of oily fish and ALA-rich ED and EF-C increased long-term LDL-surface concentrations of EPA- and DHA-containing lyso-PC(20:5 and 22:6), especially in subjects with dyslipidaemia. The consumption of oily fish, such as salmon at weekly doses of at least 400 g (1.6 g EPA+DHA), showed greater effects by increasing lyso-PC(20:5 and 22:6) and lowering other lipid-related markers, including triglyceride levels and LDL aggregation susceptibility. In agreement with these results, previous studies suggest that fish oil supplementation can alter the lipid metabolism of lyso-PLs, increasing the proportion of VLC-PUFA-containing lyso-PLs (Ottestad et al., 2012). Polyunsaturated lyso-PCs are able to promote anti-inflammatory and anti-atherogenic actions against the negative effects of saturated lyso-PC (Hung et al., 2012). Indeed, in the *Bioclaims* study (***Article 3***), lyso-PC 20:5, which was highly concentrated in the MH-LDL-c group, showed positive associations with fatty fish intake, indicating a beneficial role against the progression of hypercholesterolemia. Thus, EPA- and DHA-containing lyso-PC constitute promising bioactive lipids in the management of subjects at risk for dyslipidaemia, which is defined by elevated serum LDL cholesterol, or low levels of HDL cholesterol, and/or elevated triglyceride levels.

The discordant effects found for dietary n-3 PUFAs and n-6 PUFAs on unsaturated lyso-PE(20:4 and 22:6) among CVD risk and healthy individuals, provided further insights into the versatile response of these bioactive lipid to dietary FAs according to the cardiometabolic state. For instance, lyso-PE(22:6), which was significantly increased after n-6 PUFAs intake in obese

subjects, was decreased in healthy subjects following the consumption of n-6 and n-3 PUFA-enriched diets. Accordingly, in the *Bioclaims* study (***Article 3***), lyso-PE(22:6), which was among the most discriminant biomarkers in the MH-LDL-c group, showed a positive association with fatty fish intake, which is rich in n-3 PUFAs.

From our qualitative synthesis, we found that PLs and SLs, particularly PC, SM and Cer, were the most representative classes. These circulating bioactive lipids showed major changes in the postprandial period after HSF test meals. We revealed a wide range of net effects in circulating long chain- and VLC-PUFAs containing PC subclasses, depending on the dietary source of FAs. Interestingly, opposite results for the postprandial and sustained responses after exposure to SFAs and n-3 PUFAs were observed for long chain- and VLC-PUFA-containing PCs. While SFA- and TFA-rich food sources significantly increased unsaturated PC levels in the short term, a generalized decrease was reported after high n-3 PUFA ED. Nonetheless, specific PC(38:4, 38:6, 40:4 and 40:6) remained increased after sustained n-3 PUFA interventions. Our results were in accordance with a previous dose-response study where the PL-FA content exhibited increased unsaturation following SFAs and TFAs interventions compared to n-3 PUFA interventions (Browning et al., 2012). These effects could be explained by a general up-regulation in the formation of unsaturated PC through a preferential integration of SFAs and TFAs into de sn-1 position of the fatty acyl chains, all containing PUFAs in the sn-2 position (Gürdeniz et al., 2013). This reconfiguration process yield a more condensed shape of the PC molecule which may influence the distribution of n-3 PUFAs in lipid rafts (Wassall et al., 2018), thus impairing LDL cholesterol homeostasis. Despite this, the sustained increase of DHA-

containing PC(38:6 and 40:6) after n-3 PUFA ED may support a protective role in CVDs as have shown the negative associations in large prospective studies (Stegemann et al., 2014). From these results, we can suppose different detrimental or protective effects of long chain- and VLC-PUFAs-containing PC against CVD risk factors. However, whether these effects could be influenced by the duration and type of intervention need to be further investigated.

The Lp-PLA2 enzyme, also known as platelet activating Factor 2 acetyl hydrolase (PAF2AH), is responsible for the hydrolysis of lipoproteins and two acyl ester bonds of cell membrane PLs to form nonesterified fatty acids and Lyso-PLs (Madjid et al., 2010). In human plasma, it is frequently linked to LDL cholesterol particles, where it develops strong proinflammatory and proatherosclerotic effects. Thus, it has become a prominent inflammatory biomarker and independent risk predictor for CVDs (Diaconu et al., 2021). The intermediate role of Lp-PLA2 is as an enzymatic precursor in the synthesis of lyso-PLs and its concentration can be highly related to diet, as recently reported with Western dietary patterns (Seyedi et al., 2020). Thus, we also systematically reviewed the effects of dietary FAs on the plasma levels of this enzyme. From our meta-analysis, concordant decreases (approximetely -0.35 ng/mL) were found in plasma Lp-PLA2 mass in healthy subjects, with CVD and CVD risk factors after n-3 PUFA supplementation (daily doses from 1 to 5.56 g EPA+DHA). These effects were more pronounced in stable CAD subjects (-0.52 ng/mL). However, the meta-analysis provides additional evidence on the effects of n-3 PUFAs on plasma Lp-PLA2 mass.

To the best of our knowledge, the present study is the first to systematically review the effects of dietary FAs on particular bioactive lipids

in human randomized clinical trials. The application of targeted and untargeted high-throughput lipidomic analysis through MS quantitative approaches allowed us to characterize a wide set of bioactive lipid moieties with promising applications for the management and prevention of the main causal and modifiable CVD risk factors. Remarkably, the supplementation with EPA+DHA doses from 0.3 to 3.4 g/day showed the most marked changes in circulating bioactive lipid profile in CVD risk subjects. Conversely, EPA+DHA doses from 0.6 to 5.56 g/day lead to decreased plasma Lp-PLA2 mass in healthy subjects, with CVD and CVD risk factors. These reported doses are above the last recommendation of the European Food Safety Authority (EFSA) based on cardiovascular risk considerations for European adults, which range from 250 to 500 mg/day of combined EPA+DHA (EFSA Panel on Dietetic Products, 2012).

However, our research had some determining limitations related to the included studies, such as small sample sizes, varied designs and durations, and different forms of administration and dosages, which limited pooling all results in a single meta-analysis. In addition, the smaller number of studies for certain CVD risk factors, mainly type 2 diabetes and hypertension, limited the establishment of definitive conclusions. Additionally, potential confounders, such as sex, ethnicity, statin therapy and dietary compounds, could have led to an overestimation of the observed effects on bioactive lipids. Therefore, future lipidomic research is needed to clarify the relationships between dietary FAs and circulating bioactive lipids and to determine the extent of their prognostic and therapeutic utility as health or CVD risk biomarkers.

OVERALL DISCUSSION

Considering all the results in the framework of the *Cardiogut* and *Bioclaims* studies, we identified a range of gut microbiota- and metabolite-based biomarkers with promising applicability in the initial or preliminary stages of progressive diseases such as hypertension and hypercholesterolemia.

With the main data on discriminant bacterial signatures, the target faecal and circulating metabolites, and their relationships with bioactive dietary compounds, we propose a reliable health and disease classifier that is composed of novel biomarkers to discern hypertension, hypercholesterolemia and other causal and modifiable CVD risk factors from the healthy status (***Figure 28***).

On the one hand, higher intestinal abundance of *B. coprocola*, *B. plebeius*, *Intestimonas* and genera of Lachnospiraceae, together with lower abundance of *F. prausnitzii, Roseburia hominis,* Ruminococcaceae NK4A214, Ruminococcaceae_UCG-010 and Christensenellaceae_R-7, and higher faecal excretion of SCFAs, and lower plasmatic levels, could suggest a first grade of hypertension in humans (***Article 1***). Moreover, the dietary inclusion of coffee PCs could exasperate this clinical scenario by promoting the growth of *Bacteroides* spp. and inhibiting SCFA-producers in the gut (***Article 2***). On the other hand, lower serum levels of lyso-PE 18:2, together with its increased intrahepatic accumulation, could designate hypercholesterolemia in humans.

Additionally, increased serum levels of lyso-PC 15:0 could be indicative of hypercholesterolemia progression, and the dietary intake of SFAs from whole dairy may promote its formation (***Article 3***).

Nonetheless, in the healthy status, the dietary intake of olive fruit PCs favour the growth of SCFA-producers, such as Ruminococcaceae_UCG-010 and Christensenellaceae_R-7, and encourages SCFA colonic absorption, protecting against the onset of hypertension (***Article 2***). In addition, dietary omega (n)-PUFAs, mainly LA (C18:2n-6), promote the synthesis of lyso-PE 18:2 in the liver, thus reinforcing the protective role suggested for hepatic lyso-PE 18:2 against the onset of human hypercholesterolemia and hepatic steatosis in hamsters (***Article 3***). Therefore, both olive fruit PCs and omega (n)-PUFAs emerge as promising dietary components for future nutrition-based therapies to prevent the onset of the main causal and modifiable CVD risk factors and to maintain CV health.

It is noteworthy that the cross-sectional nature of our reported diet-gut microbe and metabolite-host interactions might limit the inferences on causality. Despite this, our results have proven helpful in gaining insights into the complex pathways involved in the pathogenesis or prevention of the main causal and modifiable CVD risk factors and have allowed us to formulate new hypotheses. Moreover, the integration of metabolomics, lipidomics and 16S rRNA metagenomics approaches, together with the application of multivariate predictive models, such as LEfSe analysis, PCA, PLS-DA and ROC curves, have provided robustness and consistency to our identified biomarkers. Likewise, the inclusion of an experimental animal model in ***Article 3*** verified the human results and delved into the mechanisms.

Finally, concerning our systematic review and meta-analysis of randomized clinical trials (***Article 4***), the results increase the scientific evidence of this field. These results offer for the first time an overview of the

effects of dietary FAs on circulating bioactive lipids and Lp-PLA2 enzymatic precursor in health, CVD and CVD risk factors. Notably, under altered cardiometabolic conditions, such as obesity, the relatively high doses of dietary n-3 PUFAs, provided as supplements, increase circulating proinflammatory lyso-PC(16:0 and 18:0) in obese subjects, which suggests a decreased response in the catabolism of saturated lyso-PC to n-3 PUFAs in obesity. Additionally, the increases in EPA- and DHA-containing lyso-PC(20:5 and 22:6) on the LDL surface of healthy and CVD risk subjects, and the decreases in Lp-PLA2 mass in subjects at risk for CVD, suggest a beneficial effect of dietary n-3 PUFAs, provided as oily fish ED or supplements, in healthy subjects, with CVD or CVD risk factors.

Hence, our approach presents an essential scientific basis for candidate biomarkers that could be useful for the development of future therapeutic interventions to prevent or counteract the progression of the main causal and modifiable CVD risk factors (***Figure 28***).

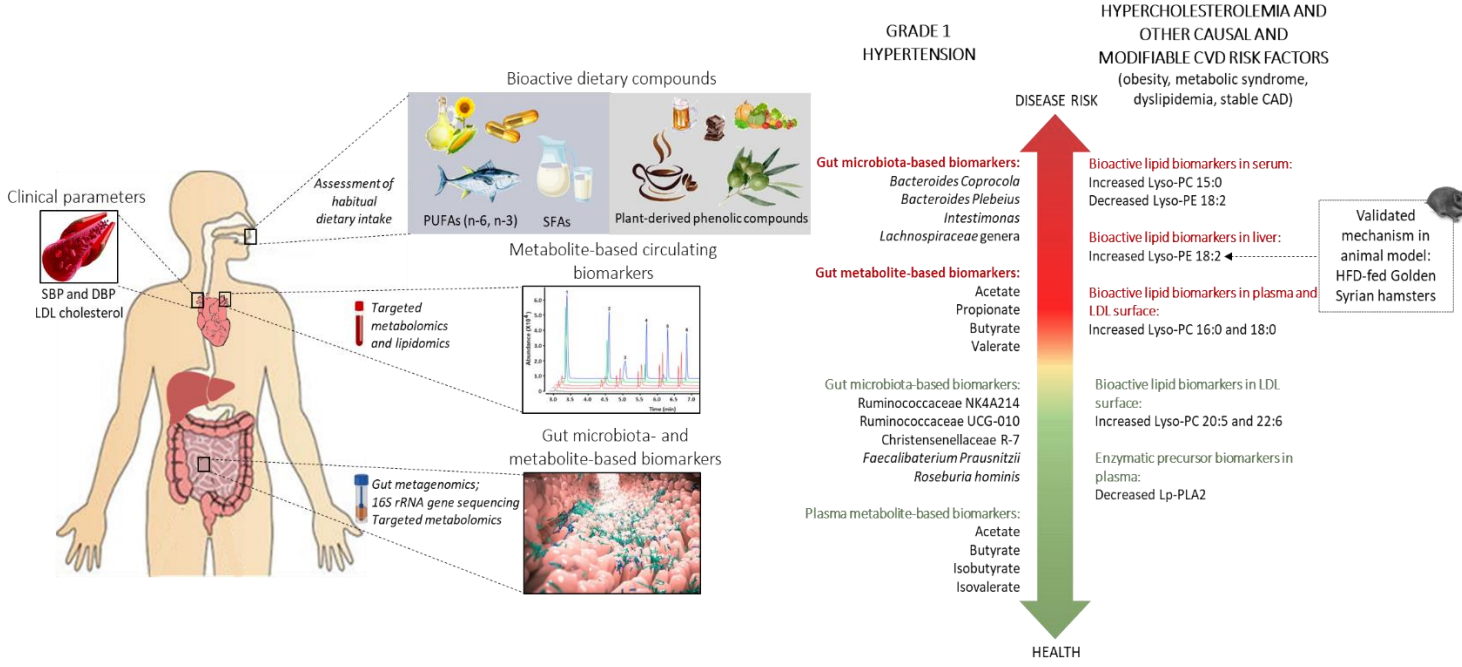
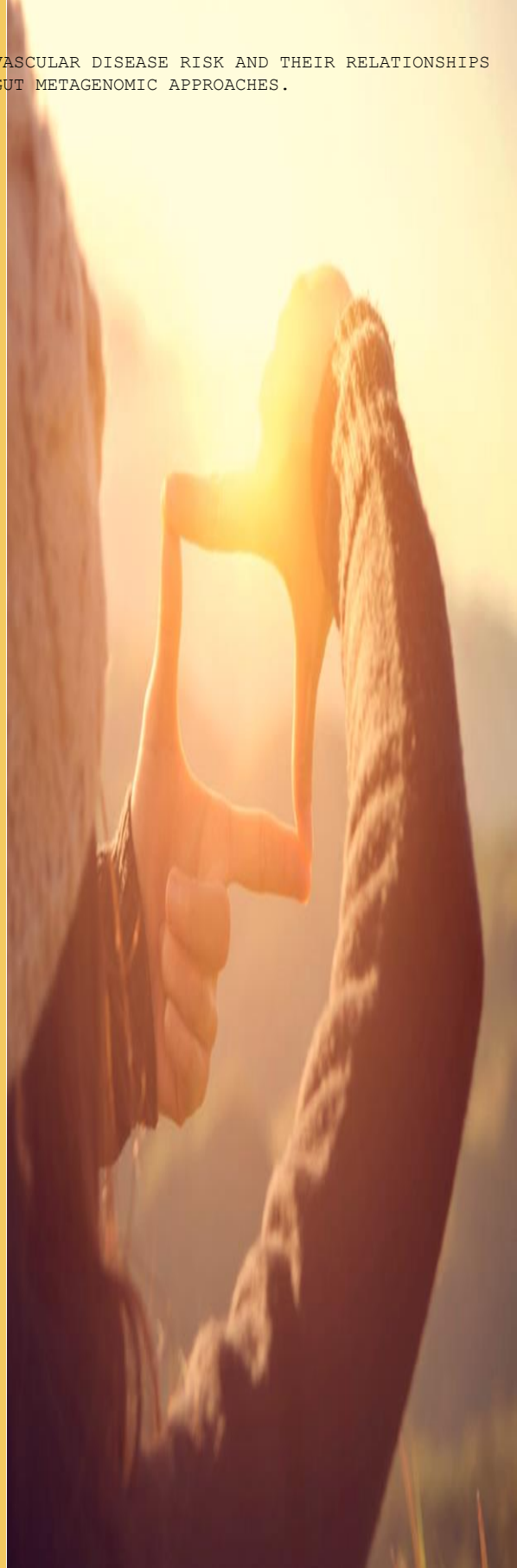


Figure 28 | Health and disease classifier consisting of novel gut microbiota- and metabolite-based biomarkers to discern between the healthy state and the main causal and modifiable CVD risk factors. Abbreviations: CAD, coronary artery disease; DBP, diastolic blood pressure; Lp-PLA2, lipoprotein-associated phospholipase A2; Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine; PUFAs, polyunsaturated fatty acids; SBP, systolic blood pressure; SFAs, saturated fatty acids. Own source.

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

FUTURE PESRPECTIVES



UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

From our work, we have identified promising gut microbiota- and metabolite- based biomarkers useful for the prevention or prediction of main causal and modifiable CVD risk factors. Their multiple relationships with dietary bioactive compounds and the host variables, such as BP and LDL cholesterol, provide a complete picture of the complex pathways involved in the onset or progression of CVDs. Despite this, there are still some aspects that merit future research:

First, given the associative nature of our work, a major challenge is elucidating the underlying biological mechanisms involved in the associations and testing whether the associations are due to a causal relationship. Large prospective studies are needed to determine the directionality and the extent of the associations between imbalances in the gut microbiota and CVDs, and between diet and gut microbiome/metabolome. In the frame of a prospective study, it would be interesting to collect and analyze faecal samples at baseline and overtime in order to enable additional statistical analyses to account for variation and longitudinal changes in gut microbiota and metabolites. In this context, RCTs would be optimal to assess the impact of dietary compounds, administered as single ingredient, on the bacterial community and its derived metabolites. The use of crossover designs with washout periods may be useful to account for the inter-individual variability in gut microbiota composition. *In vivo* animal models must also be utilized to investigate mechanisms.

Second, we are aware that several uncontrolled external or pysicochemical factors can influence PCs bioavailability, including food processing, polarity, molecular mass and structure, plant matrix, transit time, or interactions with other nutrients or ingredients in food matrices, such as

their associations with dietary fiber (Ozdal et al., 2016). Moreover, PCs bioaccesibility is determinant for their release and solubility during digestion. Despite that our multiple linear regression models were adjusted for dietary fiber, age and energy intake, we cannot discern whether the observed relationships for PCs, mainly coffee and olive fruit PCs, with bacterial taxa, SCFAs and BP may be influenced by other unmeasured factors. Therefore, in order to confirm these relationships, it would be of especial interest in the future the application of *in vitro* digestion-fermentation assays. As recently reported (Pérez-Burillo et al., 2019), these *in vitro* models are able to simulate lower-gastrointestinal-tract conditions to better understanding the changes, interactions and bioaccesibility of PCs, as well as their metabolite-producing capacity. In this way, the digestion of different coffee and olive fruit varieties might provide additional information to our observed associations.

Third, future studies focusing on diet-gut microbe and metabolite-host interactions may unravel how certain metabolites function to modulate the disease process, as well as deepen into the microbial pathways involved into the biosynthesis of CVD related metabolites. Despite metagenomics is increasing our knowledge of gene content in the microbiome, it has a very limited function in revealing their activity or gene expression. Thus, future studies should integrate other complementary 'omics', such as metatranscriptomics or metaproteomics. Metatranscriptomics would allow to discriminate the active members of the gut microbiome and their functionality by using 16s rRNA transcripts as a marker of the phylogenetic structure of active bacterial community (Gosalbes et al., 2011), while metaproteomics would identify various proteins able to reveal the presence

of particular bacterial functional profiles (Kolmeder et al., 2012). Both approaches could reveal the mechanisms by which our targeted bacterial taxa impact on the intestinal ecosystem of healthy and CVD risk individuals.

Forth, it is evident from animal studies that certain anaerobic bacteria, such as *Akkermansia muciniphila*, reside primarily in the mucosal layers of the gut and are not readily detected in analyses of only faeces. Indeed, the microbial composition throughout the gut can vary considerably with respect to the anatomic location along the intestinal tract (Ghazalpour et al., 2016). Thus, beyond faecal samples, the collection of samples from different anatomical regions within the intestines poses significant challenges to get a more complete picture of the gut microbiota in the future.

Fifth, there exist consolidated evidence suggesting that variations in gut bacteria are associated with blood lipid levels, as firstly reported by Fu et al. in a large population-based cohort of 893 subjects (Fu et al., 2015). In this study, several bacterial taxa were associated with classical lipid markers, such as triglyceride or HDL cholesterol. However, so far no study has linked changes in the composition of the gut microbiota with circulating bioactive lipids. In this sense, an extension of the *Bioclaims* study by obtaining faecal samples would be of considerable interest to know if the circulating lysophospholipidome could interact with the gut microbiota.

Sixth, another interesting opportunity arising around the gut microbiome and metabolome is personalized nutrition. Today, many guidelines on CV health offer advices to try to decrease the prevalence of CVDs. However, to date they have not been efficiently controlled suggesting the one-size-fits-all dietary advices might not be sufficient. As our work provide a large amount of data related to diet, intestinal microbiota (i.e. biomarker ASVs), as well as

faecal and circulating metabolites on the particular phenotypes of hypertension and hypercholesterolemia, it could offer the basis for personalized advice. For this purpose, our identified biomarkers should be further validated in large nutritional studies to better know the inter-individual variabilities in dietary responses, as well as complemented with genomic analyses to deep into gene-diet interactions.

Finally, despite the potential of our identified biomarkers for CVD risk detection, further studies are needed to elucidate their clear clinical utility since useful biomarkers must have high accuracy, reliability, and therapeutic impact with early intervention (J. Wang et al., 2017). In order to better identify high-risk individuals, the implementation of multi-marker strategies from novel susceptibility/risk biomarkers could be a feasible approach for better risk stratification. It is also important to note that the implementation of such strategies should be done in a cost-effective manner. Thus, once warranted the multi-marker usefulness and accuracy, a cost-utility study would be necessary.

CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

According to the results obtained from the present thesis, it is reasonable to conclude the following:

1. **After the *Cardiogut* study (Article 1):**
   - A lower intestinal abundance of the bacterial taxa *Faecalibacterium prausnitzii, Roseburia hominis,* Ruminococcaceae NK4A214, Ruminococcaceae_UCG-010 and Christensenellaceae_R-7, together with a higher abundance of *B. coprocola*, *B. plebeius*, *Intestimonas* and genera of Lachnospiraceae, represent promising biomarkers of the gut microbiota ecosystem to discriminate HT individuals from NT individuals.
   - Higher faecal excretion of the main SCFAs, including acetate, propionate, butyrate and valerate, together with lower plasmatic levels of these SCFAs, is associated with grade 1 hypertension.
   - The antagonistic results of the faecal and plasma SCFAs could indicate less efficient SCFA absorption in HT individuals, which may reflect imbalanced host-microbiome crosstalk.
   - SCFA faecal levels do not reflect SCFA levels in circulation, highlighting the importance of analysing SCFAs in plasma, where they can interact with host proteins to influence host physiology.
2. **After the *Cardiogut* study (Article 2):**
   - Coffee PCs, including hydroxycinnamic acids and alkylmethoxyphenols, could contribute to grade 1 hypertension pathogenesis because of their multiple-way positive relationships with SBP and DBP, faecal SCFAs, and the discriminant bacterial taxa *Bacteroides coprocola* and *Bacteroides plebeius* in HT individuals. This

is reinforced by the negative relationships between coffee PCs and the beneficial SCFA-producing bacteria in NT individuals.

- Olive fruit PCs, mainly anthocyanins, could have a protective role against grade 1 hypertension, given their positive relationships with Ruminococcaceae UCG-010, Christensenellaceae R-7 and plasma SCFAs in NT individuals.

3. After the *Bioclaims* study (Article 3):

- Targeted lipidomic analysis of the lysophospholipidome identified low serum levels of Lyso-PE 18:2 as an indicator of hypercholesterolemia progression in humans, owing to the lower concentrations in MH-LDL-c individuals than in L-LDL-c individuals.
- Increased serum levels of lyso-PC 15:0 could predict human hypercholesterolemia, given its positive relationship with LDL cholesterol. In turn, dietary intake of SFAs from whole dairy may promote lyso-PC 15:0 synthesis.
- The intrahepatic accumulation of lyso-PE 18:2 in HFD-fed hamsters reveals an adaptation mechanism of the liver to protect itself from diet-induced hepatic steatosis. These results may be generalized to MH-LDC-c individuals, explaining a response of the human liver to counteract hypercholesterolemia progression.
- The dietary intake of essential omega (n)-PUFAs, such as LA, may promote the intrahepatic synthesis of lyso-PE 18:2, and this is supported by the positive interactions among lyso-PE 18:2, PUFAs and liver GGT.

4. After both the *Cardiogut* and the *Bioclaims* studies:

- In preliminary stages of hypertension and hypercholesterolemia, lyso-PLs and SCFAs are better indicators of the onset of these CVD risk factors than TMAO.
- Different gut microbiota compositions in subjects at risk for CVD might promote TMAO production, hindering its identification as an early-stage biomarker.

5. Concerning the systematic review and meta-analysis (Article 4):

- The daily marine n-3 PUFA supplementation, provided as EPA+DHA at doses from 1 to 5.56 g, and consumed from 1 to 6 months, exhibit positive effects reducing plasma Lp-PLA2 mass in healthy, dyslipidemic and stable CAD subjects, suggesting an anti-inflammatory effect by lowering pro-inflammatory lyso-PCs.
- The daily supplementation with 0.37 to 1.9 g EPA+DHA increases plasma pro-inflammatory lyso-PC(16:0 and 18:0) in obese subjects. Thus, an impaired saturated lyso-PC response to n-3 PUFAs is suggested.
- The dietary intake of n-3 PUFA-rich oily fish at weekly doses of at least 400 g increases plasma polyunsaturated lyso-PC(20:5 and 22:6) in dyslipidemic subjects, which may positively contribute to the management of subjects at risk for dyslipidemia.
- Larger meta-analysis with high-quality RCTs are required to reinforce the results and further inquire into the optimal dose of n-3 PUFAs and the treatment duration period.

OVERALL CONCLUSION

It is of much interest to understand the mutual interactions between the CV system, the gut microbiome and metabolome, and bioactive dietary

compounds. In this context, the identification of particular gut bacterial signatures, faecal and plasma SCFAs, and circulating bioactive lipids by using noninvasive techniques and multiomics approaches provides useful biomarkers for the onset of hypertension and hypercholesterolemia. Additionally, knowledge of these biomarker relationships with dietary compounds, such as PCs and FAs, adds relevant information about the complex molecular-restructuring or microbial modulation pathways involved in the pathogenesis or prevention of the main causal and modifiable CVD risk factors.

Overall, our results offer novel promising health/disease biomarkers that may help to more accurately detect the main causal and modifiable CVD risk factors in their preliminary stages, prior to drug treatment. Nevertheless, further prospective studies are required for successful predictions of CVD risk. In addition, targeting diet-microbe/metabolite-host interactions represents a step towards the development of potent nutritional therapeutic strategies to protect against CVD progression.

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

REFERENCES


Gut Reboot
VASSILIOS FANOS
METABOLOMICS AND MICROBIOMICS
PERSONALIZED MEDICINE FROM THE FETUS TO THE ADULT
CLASSIC REPRINT SERIES
ANTONY VAN LEEUWENHOEK AND HIS "LITTLE ANIMALS"
by Antoni van Leeuwenhoek
Gut Microbiome-

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., & Versalovic, J. (2014). The placenta harbors a unique microbiome. *Science Translational Medicine*, *6*(237), 237ra65. https://doi.org/10.1126/scitranslmed.3008599

Agus, A., Clément, K., & Sokol, H. (2021). Gut microbiota-derived metabolites as central regulators in metabolic disorders. *Gut*, *70*(6), 1174 LP – 1182. https://doi.org/10.1136/gutjnl-2020-323071

Akerele, O. A., & Cheema, S. K. (2015). Fatty acyl composition of lysophosphatidylcholine is important in atherosclerosis. *Medical Hypotheses*, *85*(6), 754–760. https://doi.org/10.1016/j.mehy.2015.10.013

Aleixandre, A., & Miguel, M. (2016). Dietary fiber and blood pressure control. *Food & Function*, *7*(4), 1864–1871. https://doi.org/10.1039/c5fo00950b

Aleksandrova, K., Jenab, M., Bueno-de-Mesquita, H. B., Fedirko, V., Kaaks, R., Lukanova, A., van Duijnhoven, F. J. B., Jansen, E., Rinaldi, S., Romieu, I., Ferrari, P., Murphy, N., Gunter, M. J., Riboli, E., Westhpal, S., Overvad, K., Tjønneland, A., Halkjær, J., Boutron-Ruault, M.-C., … Boeing, H. (2014). Biomarker patterns of inflammatory and metabolic pathways are associated with risk of colorectal cancer: results from the European Prospective Investigation into Cancer and Nutrition (EPIC). *European Journal of Epidemiology*, *29*(4), 261–275. https://doi.org/10.1007/s10654-014-9901-8

Ali, S. E., Farag, M. A., Holvoet, P., Hanafi, R. S., & Gad, M. Z. (2016). A Comparative Metabolomics Approach Reveals Early Biomarkers for Metabolic Response to Acute Myocardial Infarction. *Scientific Reports*, *6*(1), 36359. https://doi.org/10.1038/srep36359

Allen, J. M., Mailing, L. J., Niemiro, G. M., Moore, R., Cook, M. D., White, B. A., Holscher, H. D., & Woods, J. A. (2018). Exercise Alters Gut Microbiota Composition and Function in Lean and Obese Humans. *Medicine and Science in Sports and Exercise*, *50*(4), 747–757. https://doi.org/10.1249/MSS.0000000000001495

Amini, M., Zayeri, F., & Salehi, M. (2021). Trend analysis of cardiovascular disease mortality, incidence, and mortality-to-incidence ratio: results from global burden of disease study 2017. *BMC Public Health*, *21*(1), 401. https://doi.org/10.1186/s12889-021-10429-0

Antman, E. M., & Loscalzo, J. (2018). Ischemic Heart Disease. In J. L. Jameson, A. S. Fauci, D. L. Kasper, S. L. Hauser, D. L. Longo, & J. Loscalzo (Eds.), *Harrison's Principles of Internal Medicine, 20e*. McGraw-Hill Education. http://accessmedicine.mhmedical.com/content.aspx?aid=1160046856

Aprikian, O., Duclos, V., Guyot, S., Besson, C., Manach, C., Bernalier, A., Morand, C., Rémésy, C., & Demigné, C. (2003). Apple pectin and a polyphenol-rich apple

concentrate are more effective together than separately on cecal fermentations and plasma lipids in rats. *The Journal of Nutrition*, *133*(6), 1860–1865. https://doi.org/10.1093/jn/133.6.1860

Bahadoran, Z., Mirmiran, P., Kabir, A., Azizi, F., & Ghasemi, A. (2017). The Nitrate-Independent Blood Pressure-Lowering Effect of Beetroot Juice: A Systematic Review and Meta-Analysis. *Advances in Nutrition (Bethesda, Md.)*, *8*(6), 830–838. https://doi.org/10.3945/an.117.016717

Baigent, C., Blackwell, L., Emberson, J., Holland, L. E., Reith, C., Bhala, N., Peto, R., Barnes, E. H., Keech, A., Simes, J., & Collins, R. (2010). Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. *Lancet (London, England)*, *376*(9753), 1670–1681. https://doi.org/10.1016/S0140-6736(10)61350-5

Bajinka, O., Tan, Y., Abdelhalim, K. A., Özdemir, G., & Qiu, X. (2020). Extrinsic factors influencing gut microbes, the immediate consequences and restoring eubiosis. *AMB Express*, *10*(1), 130. https://doi.org/10.1186/s13568-020-01066-8

Battson, M. L., Lee, D. M., Jarrell, D. K., Hou, S., Ecton, K. E., Weir, T. L., & Gentile, C. L. (2018). Suppression of gut dysbiosis reverses Western diet-induced vascular dysfunction. *American Journal of Physiology. Endocrinology and Metabolism*, *314*(5), E468–E477. https://doi.org/10.1152/ajpendo.00187.2017

Battson, M. L., Lee, D. M., Weir, T. L., & Gentile, C. L. (2018). The gut microbiota as a novel regulator of cardiovascular function and disease. *The Journal of Nutritional Biochemistry*, *56*, 1–15. https://doi.org/https://doi.org/10.1016/j.jnutbio.2017.12.010

Bennett, B. J., de Aguiar Vallim, T. Q., Wang, Z., Shih, D. M., Meng, Y., Gregory, J., Allayee, H., Lee, R., Graham, M., Crooke, R., Edwards, P. A., Hazen, S. L., & Lusis, A. J. (2013). Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metabolism*, *17*(1), 49–60. https://doi.org/10.1016/j.cmet.2012.12.011

Bialonska, D., Ramnani, P., Kasimsetty, S. G., Muntha, K. R., Gibson, G. R., & Ferreira, D. (2010). The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. *International Journal of Food Microbiology*, *140*(2–3), 175–182. https://doi.org/10.1016/j.ijfoodmicro.2010.03.038

Bibbins-Domingo, K., Grossman, D. C., Curry, S. J., Davidson, K. W., Epling, J. W. J., García, F. A. R., Gillman, M. W., Kemper, A. R., Krist, A. H., Kurth, A. E., Landefeld, C. S., LeFevre, M. L., Mangione, C. M., Phillips, W. R., Owens, D. K.,

Phipps, M. G., & Pignone, M. P. (2016). Statin Use for the Primary Prevention of Cardiovascular Disease in Adults: US Preventive Services Task Force Recommendation Statement. *JAMA*, *316*(19), 1997–2007. https://doi.org/10.1001/jama.2016.15450

Bioclaims Group. (2015). *BIOCLAIMS project*. BIOCLAIMS Project [Internet]. https://bioclaims.uib.eu/

Biomarkers Definitions Working Group. (2001). Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clinical Pharmacology and Therapeutics*, *69*(3), 89–95. https://doi.org/10.1067/mcp.2001.113989

Bohn, T. (2014). Dietary factors affecting polyphenol bioavailability. *Nutrition Reviews*, *72*(7), 429–452. https://doi.org/10.1111/nure.12114

Bouter, K., Bakker, G. J., Levin, E., Hartstra, A. V, Kootte, R. S., Udayappan, S. D., Katiraei, S., Bahler, L., Gilijamse, P. W., Tremaroli, V., Stahlman, M., Holleman, F., van Riel, N. A. W., Verberne, H. J., Romijn, J. A., Dallinga-Thie, G. M., Serlie, M. J., Ackermans, M. T., Kemper, E. M., … Nieuwdorp, M. (2018). Differential metabolic effects of oral butyrate treatment in lean versus metabolic syndrome subjects. *Clinical and Translational Gastroenterology*, *9*(5), 155. https://doi.org/10.1038/s41424-018-0025-4

Brennan, L., & Hu, F. B. (2019). Metabolomics-Based Dietary Biomarkers in Nutritional Epidemiology-Current Status and Future Opportunities. *Molecular Nutrition & Food Research*, *63*(1), e1701064. https://doi.org/10.1002/mnfr.201701064

Brglez Mojzer, E., Knez Hrnčič, M., Škerget, M., Knez, Ž., & Bren, U. (2016). Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. In *Molecules* (Vol. 21, Issue 7). https://doi.org/10.3390/molecules21070901

Brial, F., Le Lay, A., Dumas, M. E., & Gauguier, D. (2018). Implication of gut microbiota metabolites in cardiovascular and metabolic diseases. *Cellular and Molecular Life Sciences*, *75*(21), 3977–3990. https://doi.org/10.1007/s00018-018-2901-1

Brown, J. M., & Hazen, S. L. (2018). Microbial modulation of cardiovascular disease. *Nature Reviews. Microbiology*, *16*(3), 171–181. https://doi.org/10.1038/nrmicro.2017.149

Browning, L. M., Walker, C. G., Mander, A. P., West, A. L., Madden, J., Gambell, J. M., Young, S., Wang, L., Jebb, S. A., & Calder, P. C. (2012). Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. *The*

American Journal of Clinical Nutrition, *96*(4), 748–758. https://doi.org/10.3945/ajcn.112.041343

Buysse, D. J., Reynolds, C. F., Monk, T. H., Berman, S. R., & Kupfer, D. J. (1989). The Pittsburgh sleep quality index: A new instrument for psychiatric practice and research. *Psychiatry Research*, *28*(2), 193–213. https://doi.org/https://doi.org/10.1016/0165-1781(89)90047-4

Calderón-Pérez, L., Gosalbes, M. J., Yuste, S., Valls, R. M., Pedret, A., Llauradó, E., Jimenez-Hernandez, N., Artacho, A., Pla-Pagà, L., Companys, J., Ludwig, I., Romero, M.-P., Rubió, L., & Solà, R. (2020). Gut metagenomic and short chain fatty acids signature in hypertension: a cross-sectional study. *Scientific Reports*, *10*(1), 6436. https://doi.org/10.1038/s41598-020-63475-w

Caliceti, C., Franco, P., Spinozzi, S., Roda, A., & Cicero, A. F. G. (2016). Berberine: New Insights from Pharmacological Aspects to Clinical Evidences in the Management of Metabolic Disorders. *Current Medicinal Chemistry*, *23*(14), 1460–1476. https://doi.org/10.2174/0929867323666160411143314

Califf, R. M. (2018). Biomarker definitions and their applications. *Experimental Biology and Medicine (Maywood, N.J.)*, *243*(3), 213–221. https://doi.org/10.1177/1535370217750088

Canfora, E. E., Jocken, J. W., & Blaak, E. E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature Reviews. Endocrinology*, *11*(10), 577–591. https://doi.org/10.1038/nrendo.2015.128

Cao, Y., Liu, H., Qin, N., Ren, X., Zhu, B., & Xia, X. (2020). Impact of food additives on the composition and function of gut microbiota: A review. *Trends in Food Science & Technology*, *99*, 295–310. https://doi.org/https://doi.org/10.1016/j.tifs.2020.03.006

Carretero, O. A., & Oparil, S. (2000). Essential hypertension. Part I: definition and etiology. *Circulation*, *101*(3), 329–335. https://doi.org/10.1161/01.cir.101.3.329

Chang, P. V, Hao, L., Offermanns, S., & Medzhitov, R. (2014). The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(6), 2247–2252. https://doi.org/10.1073/pnas.1322269111

Chen, M., Li, Y., Sun, Q., Pan, A., Manson, J. E., Rexrode, K. M., Willett, W. C., Rimm, E. B., & Hu, F. B. (2016). Dairy fat and risk of cardiovascular disease in 3 cohorts of US adults. *The American Journal of Clinical Nutrition*, *104*(5), 1209–1217. https://doi.org/10.3945/ajcn.116.134460

Chiu, S., Williams, P. T., & Krauss, R. M. (2017). Effects of a very high saturated fat diet on LDL particles in adults with atherogenic dyslipidemia: A randomized controlled trial. *PloS One*, *12*(2), e0170664. https://doi.org/10.1371/journal.pone.0170664

Cicero, A. F. G., Fogacci, F., & Colletti, A. (2017). Food and plant bioactives for reducing cardiometabolic disease risk: an evidence based approach. *Food & Function*, *8*(6), 2076–2088. https://doi.org/10.1039/C7FO00178A

Cicero, A. F. G., Grassi, D., Tocci, G., Galletti, F., Borghi, C., & Ferri, C. (2019). Nutrients and Nutraceuticals for the Management of High Normal Blood Pressure: An Evidence-Based Consensus Document. *High Blood Pressure & Cardiovascular Prevention*, *26*(1), 9–25. https://doi.org/10.1007/s40292-018-0296-6

Claesson, M. J., Wang, Q., O'Sullivan, O., Greene-Diniz, R., Cole, J. R., Ross, R. P., & O'Toole, P. W. (2010). Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Research*, *38*(22), e200–e200. https://doi.org/10.1093/nar/gkq873

Consortium, H. M. P. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234

Corella, D., & Ordovás, J. M. (2014). How does the Mediterranean diet promote cardiovascular health? Current progress toward molecular mechanisms: gene-diet interactions at the genomic, transcriptomic, and epigenomic levels provide novel insights into new mechanisms. *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology*, *36*(5), 526–537. https://doi.org/10.1002/bies.201300180

Costea, P. I., Zeller, G., Sunagawa, S., Pelletier, E., Alberti, A., Levenez, F., Tramontano, M., Driessen, M., Hercog, R., Jung, F. E., Kultima, J. R., Hayward, M. R., Coelho, L. P., Allen-Vercoe, E., Bertrand, L., Blaut, M., Brown, J. R. M., Carton, T., Cools-Portier, S., … Bork, P. (2017). Towards standards for human fecal sample processing in metagenomic studies. *Nature Biotechnology*, *35*(11), 1069–1076. https://doi.org/10.1038/nbt.3960

Dabadie, H., Peuchant, E., Bernard, M., LeRuyet, P., & Mendy, F. (2005). Moderate intake of myristic acid in sn-2 position has beneficial lipidic effects and enhances DHA of cholesteryl esters in an interventional study. *The Journal of Nutritional Biochemistry*, *16*(6), 375–382. https://doi.org/10.1016/j.jnutbio.2005.01.010

Damjanov, I. (2005). ROBBINS AND COTRAN PATHOLOGIC BASIS OF DISEASE, 7TH

EDITION. *Shock*, *23*(5). https://journals.lww.com/shockjournal/Fulltext/2005/05000/ROBBINS_AND_COTRAN_PATHOLOGIC_BASIS_OF_DISEASE,.16.aspx

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V, Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563. https://doi.org/10.1038/nature12820

De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I. B., La Storia, A., Laghi, L., Serrazanetti, D. I., Di Cagno, R., Ferrocino, I., Lazzi, C., Turroni, S., Cocolin, L., Brigidi, P., Neviani, E., Gobbetti, M., O'Toole, P. W., & Ercolini, D. (2016). High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut*, *65*(11), 1812–1821. https://doi.org/10.1136/gutjnl-2015-309957

de la Cuesta-Zuluaga, J., Mueller, N. T., Álvarez-Quintero, R., Velásquez-Mejía, E. P., Sierra, J. A., Corrales-Agudelo, V., Carmona, J. A., Abad, J. M., & Escobar, J. S. (2018). Higher Fecal Short-Chain Fatty Acid Levels Are Associated with Gut Microbiome Dysbiosis, Obesity, Hypertension and Cardiometabolic Disease Risk Factors. *Nutrients*, *11*(1), 51. https://doi.org/10.3390/nu11010051

Del Bas, J. M., Caimari, A., Rodriguez-Naranjo, M. I., Childs, C. E., Paras Chavez, C., West, A. L., Miles, E. A., Arola, L., & Calder, P. C. (2016). Impairment of lysophospholipid metabolism in obesity: altered plasma profile and desensitization to the modulatory properties of n-3 polyunsaturated fatty acids in a randomized controlled trial. *The American Journal of Clinical Nutrition*, *104*(2), 266–279. https://doi.org/10.3945/ajcn.116.130872

Diaconu, A., Coculescu, B.-I., Manole, G., Vultur, H., Coculescu, E. C., Stocheci, C. M., Tudorache, I.-S., Dincă, A.-L., & Dincă, V. G. (2021). Lipoprotein-associated phospholipase A2 (Lp-PLA2) – possible diagnostic and risk biomarker in chronic ischaemic heart disease. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *36*(1), 68–73. https://doi.org/10.1080/14756366.2020.1839447

Donaldson, G. P., Lee, S. M., & Mazmanian, S. K. (2016). Gut biogeography of the bacterial microbiota. *Nature Reviews. Microbiology*, *14*(1), 20–32. https://doi.org/10.1038/nrmicro3552

Durgan, D. J., Ganesh, B. P., Cope, J. L., Ajami, N. J., Phillips, S. C., Petrosino, J. F., Hollister, E. B., & Bryan Jr, R. M. (2016). Role of the Gut Microbiome in Obstructive Sleep Apnea-Induced Hypertension. *Hypertension (Dallas, Tex. : 1979)*, *67*(2), 469–474. https://doi.org/10.1161/HYPERTENSIONAHA.115.06672

EFSA Panel on Dietetic Products, N. and A. (NDA). (2012). Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). *EFSA Journal*, *10*(7), 2815. https://doi.org/https://doi.org/10.2903/j.efsa.2012.2815

Ellulu, M. S., Patimah, I., Khaza'ai, H., Rahmat, A., & Abed, Y. (2017). Obesity and inflammation: the linking mechanism and the complications. *Archives of Medical Science : AMS*, *13*(4), 851–863. https://doi.org/10.5114/aoms.2016.58928

Emmett, P. M., Jones, L. R., Northstone, K., Pounis, G., & Taylor, C. M. (2019). *Chapter 3 - Collection and Management of Dietary Data* (G. B. T.-A. in N. R. Pounis (ed.); pp. 43–73). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-814556-2.00003-8

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M.-I., Corella, D., Arós, F., Gómez-Gracia, E., Ruiz-Gutiérrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R. M., Serra-Majem, L., Pintó, X., Basora, J., Muñoz, M. A., Sorlí, J. V, Martínez, J. A., Fitó, M., Gea, A., … Martínez-González, M. A. (2018). Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts. *The New England Journal of Medicine*, *378*(25), e34. https://doi.org/10.1056/NEJMoa1800389

Ettehad, D., Emdin, C. A., Kiran, A., Anderson, S. G., Callender, T., Emberson, J., Chalmers, J., Rodgers, A., & Rahimi, K. (2016). Blood pressure lowering for prevention of cardiovascular disease and death: a systematic review and meta-analysis. *The Lancet*, *387*(10022), 957–967. https://doi.org/10.1016/S0140-6736(15)01225-8

Fahmy, H., Hegazi, N., El-Shamy, S., & Farag, M. A. (2020). Pomegranate juice as a functional food: a comprehensive review of its polyphenols, therapeutic merits, and recent patents. *Food & Function*, *11*(7), 5768–5781. https://doi.org/10.1039/d0fo01251c

Fahy, E., Subramaniam, S., Murphy, R. C., Nishijima, M., Raetz, C. R. H., Shimizu, T., Spener, F., van Meer, G., Wakelam, M. J. O., & Dennis, E. A. (2009). Update of the LIPID MAPS comprehensive classification system for lipids. *Journal of Lipid Research*, *50 Suppl*(Suppl), S9–S14. https://doi.org/10.1194/jlr.R800095-JLR200

Ference, B. A., Ginsberg, H. N., Graham, I., Ray, K. K., Packard, C. J., Bruckert, E., Hegele, R. A., Krauss, R. M., Raal, F. J., Schunkert, H., Watts, G. F., Borén, J., Fazio, S., Horton, J. D., Masana, L., Nicholls, S. J., Nordestgaard, B. G., van de Sluis, B., Taskinen, M.-R., … Catapano, A. L. (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the

European Atherosclerosis Society Consensus Panel. *European Heart Journal*, *38*(32), 2459–2472. https://doi.org/10.1093/eurheartj/ehx144

Fernandes, J., Su, W., Rahat-Rozenbloom, S., Wolever, T. M. S., & Comelli, E. M. (2014). Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. *Nutrition & Diabetes*, *4*(6), e121–e121. https://doi.org/10.1038/nutd.2014.23

Fernández-Ballart, J. D., Piñol, J. L., Zazpe, I., Corella, D., Carrasco, P., Toledo, E., Perez-Bauer, M., Martínez-González, M. Á., Salas-Salvadó, J., & Martn-Moreno, J. M. (2010). Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *British Journal of Nutrition*, *103*(12), 1808–1816. https://doi.org/10.1017/S0007114509993837

Fernandez-Navarro, T., Salazar, N., Gutierrez-Diaz, I., Sanchez, B., Ruas-Madiedo, P., de Los Reyes-Gavilan, C. G., Margolles, A., Gueimonde, M., & Gonzalez, S. (2018). Bioactive compounds from regular diet and faecal microbial metabolites. *European Journal of Nutrition*, *57*(2), 487–497. https://doi.org/10.1007/s00394-016-1332-8

Fischbach, M. A., & Segre, J. A. (2016). Signaling in Host-Associated Microbial Communities. *Cell*, *164*(6), 1288–1300. https://doi.org/10.1016/j.cell.2016.02.037

Fitó, M., Melander, O., Martínez, J. A., Toledo, E., Carpéné, C., & Corella, D. (2016). Advances in Integrating Traditional and Omic Biomarkers When Analyzing the Effects of the Mediterranean Diet Intervention in Cardiovascular Prevention. *International Journal of Molecular Sciences*, *17*(9). https://doi.org/10.3390/ijms17091469

Fitzstevens, J. L., Smith, K. C., Hagadorn, J. I., Caimano, M. J., Matson, A. P., & Brownell, E. A. (2017). Systematic Review of the Human Milk Microbiota. *Nutrition in Clinical Practice : Official Publication of the American Society for Parenteral and Enteral Nutrition*, *32*(3), 354–364. https://doi.org/10.1177/0884533616670150

Flack, J. M., & Adekola, B. (2020). Blood pressure and the new ACC/AHA hypertension guidelines. *Trends in Cardiovascular Medicine*, *30*(3), 160–164. https://doi.org/https://doi.org/10.1016/j.tcm.2019.05.003

Fotschki, B., Juśkiewicz, J., Jurgoński, A., Kołodziejczyk, K., Milala, J., Kosmala, M., & Zduńczyk, Z. (2016). Anthocyanins in Strawberry Polyphenolic Extract Enhance the Beneficial Effects of Diets with Fructooligosaccharides in the Rat Cecal Environment. *PloS One*, *11*(2), e0149081. https://doi.org/10.1371/journal.pone.0149081

Fu, J., Bonder, M. J., Cenit, M. C., Tigchelaar, E. F., Maatman, A., Dekens, J. A. M., Brandsma, E., Marczynska, J., Imhann, F., Weersma, R. K., Franke, L., Poon, T. W., Xavier, R. J., Gevers, D., Hofker, M. H., Wijmenga, C., & Zhernakova, A. (2015). The Gut Microbiome Contributes to a Substantial Proportion of the Variation in Blood Lipids. *Circulation Research*, *117*(9), 817–824. https://doi.org/10.1161/CIRCRESAHA.115.306807

G., A. E., Hendrik, B., Andras, M., Lajos, M., Helge, W., Nicola, W., P., R. S., K., F. S., & N., M. D. (2021). The Gut Microbiome in Hypertension. *Circulation Research*, *128*(7), 934–950. https://doi.org/10.1161/CIRCRESAHA.121.318065

Gao, Q., Praticò, G., Scalbert, A., Vergères, G., Kolehmainen, M., Manach, C., Brennan, L., Afman, L. A., Wishart, D. S., Andres-Lacueva, C., Garcia-Aloy, M., Verhagen, H., Feskens, E. J. M., & Dragsted, L. O. (2017). A scheme for a flexible classification of dietary and health biomarkers. *Genes & Nutrition*, *12*, 34. https://doi.org/10.1186/s12263-017-0587-x

García-Closas, M., Vermeulen, R., Cox, D., Lan, Q., Caporaso, N. E., & Rothman, N. (2011). Population-based study designs in molecular epidemiology. *IARC Scientific Publications*, *163*, 241–259.

Gaulke, C. A., & Sharpton, T. J. (2018). The influence of ethnicity and geography on human gut microbiome composition. *Nature Medicine*, *24*(10), 1495–1496. https://doi.org/10.1038/s41591-018-0210-8

Gay, H. C., Rao, S. G., Vaccarino, V., & Ali, M. K. (2016). Effects of Different Dietary Interventions on Blood Pressure: Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Hypertension (Dallas, Tex. : 1979)*, *67*(4), 733–739. https://doi.org/10.1161/HYPERTENSIONAHA.115.06853

Ge, X., Zheng, L., Zhuang, R., Yu, P., Xu, Z., Liu, G., Xi, X., Zhou, X., & Fan, H. (2020). The Gut Microbial Metabolite Trimethylamine N-Oxide and Hypertension Risk: A Systematic Review and Dose–Response Meta-analysis. *Advances in Nutrition*, *11*(1), 66–76. https://doi.org/10.1093/advances/nmz064

Gerards, M. C., Terlou, R. J., Yu, H., Koks, C. H. W., & Gerdes, V. E. A. (2015). Traditional Chinese lipid-lowering agent red yeast rice results in significant LDL reduction but safety is uncertain - a systematic review and meta-analysis. *Atherosclerosis*, *240*(2), 415–423. https://doi.org/10.1016/j.atherosclerosis.2015.04.004

Ghazalpour, A., Cespedes, I., Bennett, B. J., & Allayee, H. (2016). Expanding role of gut microbiota in lipid metabolism. *Current Opinion in Lipidology*, *27*(2), 141–147. https://doi.org/10.1097/MOL.0000000000000278

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., & Reid, G.

(2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology*, *14*(8), 491–502. https://doi.org/10.1038/nrgastro.2017.75

Gomez-Arango, L. F., Barrett, H. L., McIntyre, H. D., Callaway, L. K., Morrison, M., & Dekker Nitert, M. (2016). Increased Systolic and Diastolic Blood Pressure Is Associated With Altered Gut Microbiota Composition and Butyrate Production in Early Pregnancy. *Hypertension (Dallas, Tex. : 1979)*, *68*(4), 974–981. https://doi.org/10.1161/HYPERTENSIONAHA.116.07910

Gómez-Guzmán, M., Toral, M., Romero, M., Jiménez, R., Galindo, P., Sánchez, M., Zarzuelo, M. J., Olivares, M., Gálvez, J., & Duarte, J. (2015). Antihypertensive effects of probiotics Lactobacillus strains in spontaneously hypertensive rats. *Molecular Nutrition & Food Research*, *59*(11), 2326–2336. https://doi.org/10.1002/mnfr.201500290

González, S., Salazar, N., Ruiz-Saavedra, S., Gómez-Martín, M., de Los Reyes-Gavilán, C. G., & Gueimonde, M. (2020). Long-Term Coffee Consumption is Associated with Fecal Microbial Composition in Humans. *Nutrients*, *12*(5), 1287. https://doi.org/10.3390/nu12051287

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J. T., Spector, T. D., Clark, A. G., & Ley, R. E. (2014). Human genetics shape the gut microbiome. *Cell*, *159*(4), 789–799. https://doi.org/10.1016/j.cell.2014.09.053

Gosalbes, M. J., Durbán, A., Pignatelli, M., Abellan, J. J., Jiménez-Hernández, N., Pérez-Cobas, A. E., Latorre, A., & Moya, A. (2011). Metatranscriptomic approach to analyze the functional human gut microbiota. *PloS One*, *6*(3), e17447. https://doi.org/10.1371/journal.pone.0017447

Granger, J. P., Alexander, B. T., & Llinas, M. (2002). Mechanisms of pressure natriuresis. *Current Hypertension Reports*, *4*(2), 152–159. https://doi.org/10.1007/s11906-002-0040-3

Gregory, J. C., Buffa, J. A., Org, E., Wang, Z., Levison, B. S., Zhu, W., Wagner, M. A., Bennett, B. J., Li, L., DiDonato, J. A., Lusis, A. J., & Hazen, S. L. (2015). Transmission of atherosclerosis susceptibility with gut microbial transplantation. *The Journal of Biological Chemistry*, *290*(9), 5647–5660. https://doi.org/10.1074/jbc.M114.618249

Grosso, G. (2018). Effects of Polyphenol-Rich Foods on Human Health. In *Nutrients* (Vol. 10, Issue 8). https://doi.org/10.3390/nu10081089

Grosso, G., Micek, A., Godos, J., Pajak, A., Sciacca, S., Bes-Rastrollo, M., Galvano, F., & Martinez-Gonzalez, M. A. (2017). Long-Term Coffee Consumption Is

Associated with Decreased Incidence of New-Onset Hypertension: A Dose-Response Meta-Analysis. *Nutrients*, *9*(8), 890. https://doi.org/10.3390/nu9080890

Grosso, G., Mistretta, A., Frigiola, A., Gruttadauria, S., Biondi, A., Basile, F., Vitaglione, P., D'Orazio, N., & Galvano, F. (2014). Mediterranean diet and cardiovascular risk factors: a systematic review. *Critical Reviews in Food Science and Nutrition*, *54*(5), 593–610. https://doi.org/10.1080/10408398.2011.596955

Guasch-Ferré, M., Hu, F. B., Ruiz-Canela, M., Bulló, M., Toledo, E., Wang, D. D., Corella, D., Gómez-Gracia, E., Fiol, M., Estruch, R., Lapetra, J., Fitó, M., Arós, F., Serra-Majem, L., Ros, E., Dennis, C., Liang, L., Clish, C. B., Martínez-González, M. A., & Salas-Salvadó, J. (2017). Plasma Metabolites From Choline Pathway and Risk of Cardiovascular Disease in the PREDIMED (Prevention With Mediterranean Diet) Study. *Journal of the American Heart Association*, *6*(11). https://doi.org/10.1161/JAHA.117.006524

Guo, Y., Li, X., Wang, Z., & Yu, B. (2021). Gut Microbiota Dysbiosis in Human Hypertension: A Systematic Review of Observational Studies. *Frontiers in Cardiovascular Medicine*, *8*, 650227. https://doi.org/10.3389/fcvm.2021.650227

Guo, Z., Liu, X. M., Zhang, Q. X., Shen, Z., Tian, F. W., Zhang, H., Sun, Z. H., Zhang, H. P., & Chen, W. (2011). Influence of consumption of probiotics on the plasma lipid profile: a meta-analysis of randomised controlled trials. *Nutrition, Metabolism, and Cardiovascular Diseases : NMCD*, *21*(11), 844–850. https://doi.org/10.1016/j.numecd.2011.04.008

Gürdeniz, G., Rago, D., NT, B., Savorani, F., Astrup, A., & LO, D. (2013). Effect of trans fatty acid intake on LC-MS and NMR plasma profiles. *PLoS One*, *8*(7 PG-e69589), e69589. https://doi.org/10.1371/journal.pone.0069589

Halmos, E. P., Christophersen, C. T., Bird, A. R., Shepherd, S. J., Gibson, P. R., & Muir, J. G. (2015). Diets that differ in their FODMAP content alter the colonic luminal microenvironment. *Gut*, *64*(1), 93–100. https://doi.org/10.1136/gutjnl-2014-307264

Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J., & Goodman, R. M. (1998). Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chemistry & Biology*, *5*(10), R245-9. https://doi.org/10.1016/s1074-5521(98)90108-9

Hinterwirth, H., Stegemann, C., & Mayr, M. (2014). Lipidomics. *Circulation: Cardiovascular Genetics*, *7*(6), 941–954. https://doi.org/10.1161/CIRCGENETICS.114.000550

Hishikawa, D., Hashidate, T., Shimizu, T., & Shindou, H. (2014). Diversity and function of membrane glycerophospholipids generated by the remodeling pathway in mammalian cells. *Journal of Lipid Research*, *55*(5), 799–807. https://doi.org/https://doi.org/10.1194/jlr.R046094

Holčapek, M., Liebisch, G., & Ekroos, K. (2018). Lipidomic Analysis. *Analytical Chemistry*, *90*(7), 4249–4257. https://doi.org/10.1021/acs.analchem.7b05395

Holscher, H. D. (2017). Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut Microbes*, *8*(2), 172–184. https://doi.org/10.1080/19490976.2017.1290756

Honour, J. W. (2015). Historical perspective: gut dysbiosis and hypertension. *Physiological Genomics*, *47*(10), 443–446. https://doi.org/10.1152/physiolgenomics.00063.2015

Hooper, L. V, & Macpherson, A. J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nature Reviews. Immunology*, *10*(3), 159–169. https://doi.org/10.1038/nri2710

Huart, J., Leenders, J., Taminiau, B., Descy, J., Saint-Remy, A., Daube, G., Krzesinski, J.-M., Melin, P., de Tullio, P., & Jouret, F. (2019). Gut Microbiota and Fecal Levels of Short-Chain Fatty Acids Differ Upon 24-Hour Blood Pressure Levels in Men. *Hypertension (Dallas, Tex. : 1979)*, *74*(4), 1005–1013. https://doi.org/10.1161/HYPERTENSIONAHA.118.12588

Huff, T., Boyd, B., & Jialal, I. (2021). *Physiology, Cholesterol.*

Hui, D. Y. (2016). Intestinal phospholipid and lysophospholipid metabolism in cardiometabolic disease. *Current Opinion in Lipidology*, *27*(5), 507–512. https://doi.org/10.1097/MOL.0000000000000334

Humphreys, C. (2020). *19 - Intestinal Permeability* (J. E. Pizzorno & M. T. B. T.-T. of N. M. (Fifth E. Murray (eds.); pp. 166-177.e4). Churchill Livingstone. https://doi.org/https://doi.org/10.1016/B978-0-323-43044-9.00019-4

Hung, N. D., Sok, D.-E., & Kim, M. R. (2012). Prevention of 1-palmitoyl lysophosphatidylcholine-induced inflammation by polyunsaturated acyl lysophosphatidylcholine. *Inflammation Research : Official Journal of the European Histamine Research Society ... [et Al.]*, *61*(5), 473–483. https://doi.org/10.1007/s00011-012-0434-x

Ianiro, G., Tilg, H., & Gasbarrini, A. (2019). Antibiotics as deep modulators of gut microbiota: between good and evil. *Gut*, *65*(11), 1906 LP – 1915. https://doi.org/10.1136/gutjnl-2016-312297

IOM. (2010). *Evaluation of Biomarkers and Surrogate Endpoints in Chronic Disease*

(C. M. Micheel & J. R. Ball (eds.)). The National Academies Press. https://doi.org/10.17226/12869

Isabella, V. M., Ha, B. N., Castillo, M. J., Lubkowicz, D. J., Rowe, S. E., Millet, Y. A., Anderson, C. L., Li, N., Fisher, A. B., West, K. A., Reeder, P. J., Momin, M. M., Bergeron, C. G., Guilmain, S. E., Miller, P. F., Kurtz, C. B., & Falb, D. (2018). Development of a synthetic live bacterial therapeutic for the human metabolic disease phenylketonuria. *Nature Biotechnology*, *36*(9), 857–864. https://doi.org/10.1038/nbt.4222

Ivanova, E. A., Myasoedova, V. A., Melnichenko, A. A., Grechko, A. V, & Orekhov, A. N. (2017). Small Dense Low-Density Lipoprotein as Biomarker for Atherosclerotic Diseases. *Oxidative Medicine and Cellular Longevity*, *2017*, 1273042. https://doi.org/10.1155/2017/1273042

J., S. M., M, R. P., & J., D. V. (2004). Risk Factor Criteria. *Circulation*, *109*(25_suppl_1), IV-3-IV–5. https://doi.org/10.1161/01.CIR.0000133446.69171.7d

Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Nageshwar Reddy, D. (2015). Role of the normal gut microbiota. *World Journal of Gastroenterology*, *21*(29), 8787–8803. https://doi.org/10.3748/wjg.v21.i29.8787

Jin, L., Shi, X., Yang, J., Zhao, Y., Xue, L., Xu, L., & Cai, J. (2021). Gut microbes in cardiovascular diseases and their potential therapeutic applications. *Protein & Cell*, *12*(5), 346–359. https://doi.org/10.1007/s13238-020-00785-9

Jin, Q., Black, A., Kales, S. N., Vattem, D., Ruiz-Canela, M., & Sotos-Prieto, M. (2019). Metabolomics and Microbiomes as Potential Tools to Evaluate the Effects of the Mediterranean Diet. *Nutrients*, *11*(1). https://doi.org/10.3390/nu11010207

Johnson, J. S., Spakowicz, D. J., Hong, B.-Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications*, *10*(1), 5029. https://doi.org/10.1038/s41467-019-13036-1

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S., Okuda, S., Tokimatsu, T., & Yamanishi, Y. (2008). KEGG for linking genomes to life and the environment. *Nucleic Acids Research*, *36*(Database issue), D480-4. https://doi.org/10.1093/nar/gkm882

Kannel, W. (1961). Factors of risk in the development of coronary heart disease--six year follow-up experience. The Framingham Study. *Annals of Internal Medicine*, *55*, 33–50. https://doi.org/10.7326/0003-4819-55-1-33

Kawabata, K., Yoshioka, Y., & Terao, J. (2019). Role of Intestinal Microbiota in the Bioavailability and Physiological Functions of Dietary Polyphenols. *Molecules (Basel, Switzerland)*, *24*(2). https://doi.org/10.3390/molecules24020370

Kim, H., Kwon, J., Choi, S. Y., & Ahn, Y. G. (2019). Method development for the quantitative determination of short chain fatty acids in microbial samples by solid phase extraction and gas chromatography with flame ionization detection. *Journal of Analytical Science and Technology*, *10*(1), 28. https://doi.org/10.1186/s40543-019-0184-2

Kim, S., Goel, R., Kumar, A., Qi, Y., Lobaton, G., Hosaka, K., Mohammed, M., Handberg, E. M., Richards, E. M., Pepine, C. J., & Raizada, M. K. (2018). Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure. *Clinical Science (London, England : 1979)*, *132*(6), 701–718. https://doi.org/10.1042/CS20180087

Kleger, A., Liebau, S., Lin, Q., von Wichert, G., & Seufferlein, T. (2011). The Impact of Bioactive Lipids on Cardiovascular Development. *Stem Cells International*, *2011*, 916180. https://doi.org/10.4061/2011/916180

Knuplez, E., & Marsche, G. (2020). An Updated Review of Pro- and Anti-Inflammatory Properties of Plasma Lysophosphatidylcholines in the Vascular System. *International Journal of Molecular Sciences*, *21*(12), 4501. https://doi.org/10.3390/ijms21124501

Koeth, R. A., Levison, B. S., Culley, M. K., Buffa, J. A., Wang, Z., Gregory, J. C., Org, E., Wu, Y., Li, L., Smith, J. D., Tang, W. H. W., DiDonato, J. A., Lusis, A. J., & Hazen, S. L. (2014). γ-Butyrobetaine is a proatherogenic intermediate in gut microbial metabolism of L-carnitine to TMAO. *Cell Metabolism*, *20*(5), 799–812. https://doi.org/10.1016/j.cmet.2014.10.006

Koeth, R. A., Wang, Z., Levison, B. S., Buffa, J. A., Org, E., Sheehy, B. T., Britt, E. B., Fu, X., Wu, Y., Li, L., Smith, J. D., DiDonato, J. A., Chen, J., Li, H., Wu, G. D., Lewis, J. D., Warrier, M., Brown, J. M., Krauss, R. M., … Hazen, S. L. (2013). Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nature Medicine*, *19*(5), 576–585. https://doi.org/10.1038/nm.3145

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., & Bäckhed, F. (2016). From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell*, *165*(6), 1332–1345. https://doi.org/10.1016/j.cell.2016.05.041

Kolmeder, C. A., de Been, M., Nikkilä, J., Ritamo, I., Mättö, J., Valmu, L., Salojärvi, J., Palva, A., Salonen, A., & de Vos, W. M. (2012). Comparative metaproteomics and diversity analysis of human intestinal microbiota testifies for its

temporal stability and expression of core functions. *PloS One*, *7*(1), e29913. https://doi.org/10.1371/journal.pone.0029913

Kopp, W. (2019). How Western Diet And Lifestyle Drive The Pandemic Of Obesity And Civilization Diseases. *Diabetes, Metabolic Syndrome and Obesity : Targets and Therapy*, *12*, 2221–2236. https://doi.org/10.2147/DMSO.S216791

Kotchen, T. A. (2018). Hypertensive Vascular Disease. In J. L. Jameson, A. S. Fauci, D. L. Kasper, S. L. Hauser, D. L. Longo, & J. Loscalzo (Eds.), *Harrison's Principles of Internal Medicine, 20e*. McGraw-Hill Education. http://accessmedicine.mhmedical.com/content.aspx?aid=1176804125

Kratz, M., Cullen, P., Kannenberg, F., Kassner, A., Fobker, M., Abuja, P. M., Assmann, G., & Wahrburg, U. (2002). Effects of dietary fatty acids on the composition and oxidizability of low-density lipoprotein. *European Journal of Clinical Nutrition*, *56*(1), 72–81. https://doi.org/10.1038/sj.ejcn.1601288

Kriaa, A., Bourgin, M., Potiron, A., Mkaouar, H., Jablaoui, A., Gérard, P., Maguin, E., & Rhimi, M. (2019). Microbial impact on cholesterol and bile acid metabolism: current status and future prospects. *Journal of Lipid Research*, *60*(2), 323–332. https://doi.org/10.1194/jlr.R088989

Kröger, J., Zietemann, V., Enzenbach, C., Weikert, C., Jansen, E. H., Döring, F., Joost, H.-G., Boeing, H., & Schulze, M. B. (2011). Erythrocyte membrane phospholipid fatty acids, desaturase activity, and dietary fatty acids in relation to risk of type 2 diabetes in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *The American Journal of Clinical Nutrition*, *93*(1), 127–142. https://doi.org/10.3945/ajcn.110.005447

Kuang, A., Erlund, I., Herder, C., Westerhuis, J. A., Tuomilehto, J., & Cornelis, M. C. (2018). Lipidomic Response to Coffee Consumption. *Nutrients*, *10*(12). https://doi.org/10.3390/nu10121851

Kumar Singh, A., Cabral, C., Kumar, R., Ganguly, R., Kumar Rana, H., Gupta, A., Rosaria Lauro, M., Carbone, C., Reis, F., & Pandey, A. K. (2019). Beneficial Effects of Dietary Polyphenols on Gut Microbiota and Strategies to Improve Delivery Efficiency. *Nutrients*, *11*(9). https://doi.org/10.3390/nu11092216

Lam, K. N., Alexander, M., & Turnbaugh, P. J. (2019). Precision Medicine Goes Microscopic: Engineering the Microbiome to Improve Drug Outcomes. *Cell Host & Microbe*, *26*(1), 22–34. https://doi.org/10.1016/j.chom.2019.06.011

Lane, N. (2015). The unseen world: reflections on Leeuwenhoek (1677) "Concerning little animals." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *370*(1666), 20140344. https://doi.org/10.1098/rstb.2014.0344

REFERENCES

Lau, K., Srivatsav, V., Rizwan, A., Nashed, A., Liu, R., Shen, R., & Akhtar, M. (2017). Bridging the Gap between Gut Microbial Dysbiosis and Cardiovascular Diseases. *Nutrients*, *9*(8), 859. https://doi.org/10.3390/nu9080859

Lee, S. H., Yun, Y., Kim, S. J., Lee, E.-J., Chang, Y., Ryu, S., Shin, H., Kim, H.-L., Kim, H.-N., & Lee, J. H. (2018). Association between Cigarette Smoking Status and Composition of Gut Microbiota: Population-Based Cross-Sectional Study. *Journal of Clinical Medicine*, *7*(9), 282. https://doi.org/10.3390/jcm7090282

Legrand, P., & Rioux, V. (2010). The complex and important cellular and metabolic functions of saturated fatty acids. *Lipids*, *45*(10), 941–946. https://doi.org/10.1007/s11745-010-3444-x

Li, J., Zhao, F., Wang, Y., Chen, J., Tao, J., Tian, G., Wu, S., Liu, W., Cui, Q., Geng, B., Zhang, W., Weldon, R., Auguste, K., Yang, L., Liu, X., Chen, L., Yang, X., Zhu, B., & Cai, J. (2017). Gut microbiota dysbiosis contributes to the development of hypertension. *Microbiome*, *5*(1), 1–19. https://doi.org/10.1186/s40168-016-0222-x

Li, X., & Xu, J. (2013). Lycopene supplement and blood pressure: an updated meta-analysis of intervention trials. *Nutrients*, *5*(9), 3696–3712. https://doi.org/10.3390/nu5093696

Li, Y., Hao, Y., Fan, F., & Zhang, B. (2018). The Role of Microbiome in Insomnia, Circadian Disturbance and Depression . In *Frontiers in Psychiatry* (Vol. 9, p. 669). https://www.frontiersin.org/article/10.3389/fpsyt.2018.00669

Liang, N., & Kitts, D. D. (2015). Role of Chlorogenic Acids in Controlling Oxidative and Inflammatory Stress Conditions. *Nutrients*, *8*(1). https://doi.org/10.3390/nu8010016

Libby, P. (2018). Atlas of Atherosclerosis. In J. L. Jameson, A. S. Fauci, D. L. Kasper, S. L. Hauser, D. L. Longo, & J. Loscalzo (Eds.), *Harrison's Principles of Internal Medicine, 20e*. McGraw-Hill Education. http://accessmedicine.mhmedical.com/content.aspx?aid=1164034657

Lu, J., Chen, B., Chen, T., Guo, S., Xue, X., Chen, Q., Zhao, M., Xia, L., Zhu, Z., Zheng, L., & Yin, H. (2017). Comprehensive metabolomics identified lipid peroxidation as a prominent feature in human plasma of patients with coronary heart diseases. *Redox Biology*, *12*, 899–907. https://doi.org/https://doi.org/10.1016/j.redox.2017.04.032

Lüdicke, F., Magnette, J., Baker, G., & Weitkunat, R. (2015). A Japanese cross-sectional multicentre study of biomarkers associated with cardiovascular disease in smokers and non-smokers. *Biomarkers : Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals*, *20*(6–7), 411–421. https://doi.org/10.3109/1354750X.2015.1096303

Lyngbakken, M. N., Myhre, P. L., Røsjø, H., & Omland, T. (2019). Novel biomarkers of cardiovascular disease: Applications in clinical practice. *Critical Reviews in Clinical Laboratory Sciences*, *56*(1), 33–60. https://doi.org/10.1080/10408363.2018.1525335

Ma, J., & Li, H. (2018). The Role of Gut Microbiota in Atherosclerosis and Hypertension . In *Frontiers in Pharmacology* (Vol. 9, p. 1082). https://www.frontiersin.org/article/10.3389/fphar.2018.01082

Mach, F., Baigent, C., Catapano, A. L., Koskinas, K. C., Casula, M., Badimon, L., Chapman, M. J., De Backer, G. G., Delgado, V., Ference, B. A., Graham, I. M., Halliday, A., Landmesser, U., Mihaylova, B., Pedersen, T. R., Riccardi, G., Richter, D. J., Sabatine, M. S., Taskinen, M.-R., … Wiklund, O. (2020). 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk: The Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS). *European Heart Journal*, *41*(1), 111–188. https://doi.org/10.1093/eurheartj/ehz455

Madison, A., & Kiecolt-Glaser, J. K. (2019). Stress, depression, diet, and the gut microbiota: human-bacteria interactions at the core of psychoneuroimmunology and nutrition. *Current Opinion in Behavioral Sciences*, *28*, 105–110. https://doi.org/10.1016/j.cobeha.2019.01.011

Madjid, M., Ali, M., & Willerson, J. T. (2010). Lipoprotein-associated phospholipase A2 as a novel risk marker for cardiovascular disease: a systematic review of the literature. *Texas Heart Institute Journal*, *37*(1), 25–39. https://pubmed.ncbi.nlm.nih.gov/20200624

Mansoori, S., Kushner, N., Suminski, R. R., Farquhar, W. B., & Chai, S. C. (2019). Added Sugar Intake is Associated with Blood Pressure in Older Females. In *Nutrients* (Vol. 11, Issue 9). https://doi.org/10.3390/nu11092060

Marcobal, A., Kashyap, P. C., Nelson, T. A., Aronov, P. A., Donia, M. S., Spormann, A., Fischbach, M. A., & Sonnenburg, J. L. (2013). A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. *The ISME Journal*, *7*(10), 1933–1943. https://doi.org/10.1038/ismej.2013.89

Marhuenda-Muñoz, M., Laveriano-Santos, E. P., Tresserra-Rimbau, A., Lamuela-Raventós, R. M., Martínez-Huélamo, M., & Vallverdú-Queralt, A. (2019). Microbial Phenolic Metabolites: Which Molecules Actually Have an Effect on Human Health? *Nutrients*, *11*(11). https://doi.org/10.3390/nu11112725

Marín, L., Miguélez, E. M., Villar, C. J., & Lombó, F. (2015). Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties.

BioMed Research International, *2015*, 905215. https://doi.org/10.1155/2015/905215

Markowiak, P., & Śliżewska, K. (2017). Effects of Probiotics, Prebiotics, and Synbiotics on Human Health. *Nutrients*, *9*(9). https://doi.org/10.3390/nu9091021

Marques, F. Z., Nelson, E., Chu, P. Y., Horlock, D., Fiedler, A., Ziemann, M., Tan, J. K., Kuruppu, S., Rajapakse, N. W., El-Osta, A., Mackay, C. R., & Kaye, D. M. (2017). High-fiber diet and acetate supplementation change the gut microbiota and prevent the development of hypertension and heart failure in hypertensive mice. *Circulation*, *135*(10), 964–977. https://doi.org/10.1161/CIRCULATIONAHA.116.024545

Martin, R.-P., O., L. L., Joerg, H., Tatsuya, S., Claudio, N., & Amir, L. (2003). Hypercholesterolemia and Hypertension Have Synergistic Deleterious Effects on Coronary Endothelial Function. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *23*(5), 885–891. https://doi.org/10.1161/01.ATV.0000069209.26507.BF

Martinez-Hervas, S., & Ascaso, J. F. (2019). *Hypercholesterolemia* (I. Huhtaniemi & L. B. T.-E. of E. D. (Second E. Martini (eds.); pp. 320–326). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-801238-3.65340-0

Mell, B., Jala, V. R., Mathew, A. V., Byun, J., Waghulde, H., Zhang, Y., Haribabu, B., Vijay-Kumar, M., Pennathur, S., & Joe, B. (2015). Evidence for a link between gut microbiota and hypertension in the Dahl rat. *Physiological Genomics*, *47*(6), 187–197. https://doi.org/10.1152/physiolgenomics.00136.2014

Méndez-del Villar, M., González-Ortiz, M., Martínez-Abundis, E., Pérez-Rubio, K. G., & Lizárraga-Valdez, R. (2014). Effect of resveratrol administration on metabolic syndrome, insulin sensitivity, and insulin secretion. *Metabolic Syndrome and Related Disorders*, *12*(10), 497–501. https://doi.org/10.1089/met.2014.0082

Meyer, K. A., Benton, T. Z., Bennett, B. J., Jacobs, D. R. J., Lloyd-Jones, D. M., Gross, M. D., Carr, J. J., Gordon-Larsen, P., & Zeisel, S. H. (2016). Microbiota-Dependent Metabolite Trimethylamine N-Oxide and Coronary Artery Calcium in the Coronary Artery Risk Development in Young Adults Study (CARDIA). *Journal of the American Heart Association*, *5*(10). https://doi.org/10.1161/JAHA.116.003970

Micha, R., Peñalvo, J. L., Cudhea, F., Imamura, F., Rehm, C. D., & Mozaffarian, D. (2017). Association Between Dietary Factors and Mortality From Heart Disease, Stroke, and Type 2 Diabetes in the United States. *JAMA*, *317*(9), 912–924. https://doi.org/10.1001/jama.2017.0947

Ministerio de Agricultura, Pesca y Alimentación, (Organización). (2018). Informe del Consumo Alimentario en España 2018. *Gobierno de España*, 242. https://doi.org/10.1002/chin.200343156

Ministry of Health. (2018). *2018 Annual Report on the National Health System - Executive Summary*. https://cpage.mpr.gob.es/

Misra, B. B., Langefeld, C. D., Olivier, M., & Cox, L. A. (2018). Integrated Omics: Tools, Advances, and Future Approaches. *Journal of Molecular Endocrinology*. https://doi.org/10.1530/JME-18-0055

Modi, S. R., Collins, J. J., & Relman, D. A. (2014). Antibiotics and the gut microbiota. *The Journal of Clinical Investigation*, *124*(10), 4212–4218. https://doi.org/10.1172/JCI72333

Morrison, D. J., & Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes*, *7*(3), 189–200. https://doi.org/10.1080/19490976.2015.1134082

Mosele, J. I., Macià, A., & Motilva, M.-J. (2015). Metabolic and Microbial Modulation of the Large Intestine Ecosystem by Non-Absorbed Diet Phenolic Compounds: A Review. *Molecules (Basel, Switzerland)*, *20*(9), 17429–17468. https://doi.org/10.3390/molecules200917429

Murray, C. J. L., Aravkin, A. Y., Zheng, P., Abbafati, C., Abbas, K. M., Abbasi-Kangevari, M., Abd-Allah, F., Abdelalim, A., Abdollahi, M., Abdollahpour, I., Abegaz, K. H., Abolhassani, H., Aboyans, V., Abreu, L. G., Abrigo, M. R. M., Abualhasan, A., Abu-Raddad, L. J., Abushouk, A. I., Adabi, M., … Lim, S. S. (2020). Global burden of 87 risk factors in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. *The Lancet*, *396*(10258), 1223–1249. https://doi.org/https://doi.org/10.1016/S0140-6736(20)30752-2

Mushenkova, N. V, Bezsonov, E. E., Orekhova, V. A., Popkova, T. V, Starodubova, A. V, & Orekhov, A. N. (2021). Recognition of Oxidized Lipids by Macrophages and Its Role in Atherosclerosis Development. *Biomedicines*, *9*(8). https://doi.org/10.3390/biomedicines9080915

Nam, M., Choi, M.-S., Jung, S., Jung, Y., Choi, J.-Y., Ryu, D. H., & Hwang, G.-S. (2015). Lipidomic Profiling of Liver Tissue from Obesity-Prone and Obesity-Resistant Mice Fed a High Fat Diet. *Scientific Reports*, *5*, 16984. https://doi.org/10.1038/srep16984

Natarajan, N., Hori, D., Flavahan, S., Steppan, J., Flavahan, N. A., Berkowitz, D. E., & Pluznick, J. L. (2016). Microbial short chain fatty acid metabolites lower blood pressure via endothelial G protein-coupled receptor 41. *Physiological Genomics*, *48*(11), 826–834.

https://doi.org/10.1152/physiolgenomics.00089.2016

Naz, S., Vallejo, M., García, A., & Barbas, C. (2014). Method validation strategies involved in non-targeted metabolomics. *Journal of Chromatography. A*, *1353*, 99–105. https://doi.org/10.1016/j.chroma.2014.04.071

NCEPIII. (2002). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation*, *106*(25), 3143–3421.

Nelson, J. R., Wani, O., May, H. T., & Budoff, M. (2017). Potential benefits of eicosapentaenoic acid on atherosclerotic plaques. *Vascular Pharmacology*, *91*, 1–9. https://doi.org/https://doi.org/10.1016/j.vph.2017.02.004

Newgard, C. B. (2017). Metabolomics and Metabolic Diseases: Where Do We Stand? *Cell Metabolism*, *25*(1), 43–56. https://doi.org/10.1016/j.cmet.2016.09.018

Nighot, M., Al-Sadi, R., Guo, S., Rawat, M., Nighot, P., Watterson, M. D., & Ma, T. Y. (2017). Lipopolysaccharide-Induced Increase in Intestinal Epithelial Tight Permeability Is Mediated by Toll-Like Receptor 4/Myeloid Differentiation Primary Response 88 (MyD88) Activation of Myosin Light Chain Kinase Expression. *The American Journal of Pathology*, *187*(12), 2698–2710. https://doi.org/10.1016/j.ajpath.2017.08.005

Nowiński, A., & Ufnal, M. (2018). Trimethylamine N-oxide: A harmful, protective or diagnostic marker in lifestyle diseases? *Nutrition (Burbank, Los Angeles County, Calif.)*, *46*, 7–12. https://doi.org/10.1016/j.nut.2017.08.001

O'Gorman, A., Gibbons, H., Ryan, M. F., Gibney, E. R., Gibney, M. J., Frost, G. S., Roche, H. M., & Brennan, L. (2017). Exploring the Links between Diet and Health in an Irish Cohort: A Lipidomic Approach. *Journal of Proteome Research*, *16*(3), 1280–1287. https://doi.org/10.1021/acs.jproteome.6b00912

Ohira, H., Tsutsui, W., & Fujioka, Y. (2017). Are Short Chain Fatty Acids in Gut Microbiota Defensive Players for Inflammation and Atherosclerosis? *Journal of Atherosclerosis and Thrombosis*, *24*(7), 660–672. https://doi.org/10.5551/jat.RV17006

Oliphant, K., & Allen-Vercoe, E. (2019). Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome*, *7*(1), 91. https://doi.org/10.1186/s40168-019-0704-8

Oliveros, E., Patel, H., Kyung, S., Fugar, S., Goldberg, A., Madan, N., & Williams, K. A. (2020). Hypertension in older adults: Assessment, management, and challenges. *Clinical Cardiology*, *43*(2), 99–107.

https://doi.org/10.1002/clc.23303

Olivier, M., Asmis, R., Hawkins, G. A., Howard, T. D., & Cox, L. A. (2019). The Need for Multi-Omics Biomarker Signatures in Precision Medicine. *International Journal of Molecular Sciences*, *20*(19), 4781. https://doi.org/10.3390/ijms20194781

Othman, R. A., Moghadasian, M. H., & Jones, P. J. (2011). Cholesterol-lowering effects of oat β-glucan. *Nutrition Reviews*, *69*(6), 299–309. https://doi.org/10.1111/j.1753-4887.2011.00401.x

Ottestad, I., Hassani, S., Borge, G. I., Kohler, A., Vogt, G., Hyötyläinen, T., Orešič, M., Brønner, K. W., Holven, K. B., Ulven, S. M., & Myhrstad, M. C. W. (2012). Fish oil supplementation alters the plasma lipidomic profile and increases long-chain PUFAs of phospholipids and triglycerides in healthy subjects. *PloS One*, *7*(8), e42550. https://doi.org/10.1371/journal.pone.0042550

Ozdal, T., Sela, D. A., Xiao, J., Boyacioglu, D., Chen, F., & Capanoglu, E. (2016). The Reciprocal Interactions between Polyphenols and Gut Microbiota and Effects on Bioaccessibility. *Nutrients*, *8*(2), 78. https://doi.org/10.3390/nu8020078

Pandey, K. R., Naik, S. R., & Vakil, B. V. (2015). Probiotics, prebiotics and synbiotics-a review. *Journal of Food Science and Technology*, *52*(12), 7577–7587. https://doi.org/10.1007/s13197-015-1921-1

Patti, G. J., Yanes, O., & Siuzdak, G. (2012). Innovation: Metabolomics: the apogee of the omics trilogy. *Nature Reviews. Molecular Cell Biology*, *13*(4), 263–269. https://doi.org/10.1038/nrm3314

Pedret, A., Valls, R. M., Calderón-Pérez, L., Llauradó, E., Companys, J., Pla-Pagà, L., Moragas, A., Martín-Luján, F., Ortega, Y., Giralt, M., Caimari, A., Chenoll, E., Genovés, S., Martorell, P., Codoñer, F. M., Ramón, D., Arola, L., & Solà, R. (2019). Effects of daily consumption of the probiotic Bifidobacterium animalis subsp. lactis CECT 8145 on anthropometric adiposity biomarkers in abdominally obese subjects: a randomized controlled trial. *International Journal of Obesity (2005)*, *43*(9), 1863–1868. https://doi.org/10.1038/s41366-018-0220-0

Pérez-Burillo, S., Mehta, T., Esteban-Muñoz, A., Pastoriza, S., Paliy, O., & Ángel Rufián-Henares, J. (2019). Effect of in vitro digestion-fermentation on green and roasted coffee bioactivity: The role of the gut microbiota. *Food Chemistry*, *279*, 252–259. https://doi.org/10.1016/j.foodchem.2018.11.137

Pérez-Jiménez, J., Neveu, V., Vos, F., & Scalbert, A. (2010). Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database. *European Journal of Clinical Nutrition*, *64 Suppl 3*, S112-20. https://doi.org/10.1038/ejcn.2010.221

Perpetuini, G., Prete, R., Garcia-Gonzalez, N., Khairul Alam, M., & Corsetti, A. (2020). Table Olives More than a Fermented Food. *Foods (Basel, Switzerland)*, *9*(2), 178. https://doi.org/10.3390/foods9020178

Petersen, K. S., Keogh, J. B., Lister, N., Weir, J. M., Meikle, P. J., & Clifton, P. M. (2017). Association between dairy intake, lipids and vascular structure and function in diabetes. *World Journal of Diabetes*, *8*(5), 202–212. https://doi.org/10.4239/wjd.v8.i5.202

Piano, M. R. (2017). Alcohol's Effects on the Cardiovascular System. *Alcohol Research : Current Reviews*, *38*(2), 219–241. https://pubmed.ncbi.nlm.nih.gov/28988575

Pichler, G., Amigo, N., Tellez-Plaza, M., Pardo-Cea, M. A., Dominguez-Lucas, A., Marrachelli, V. G., Monleon, D., Martin-Escudero, J. C., Ascaso, J. F., Chaves, F. J., Carmena, R., & Redon, J. (2018). LDL particle size and composition and incident cardiovascular disease in a South-European population: The Hortega-Liposcale Follow-up Study. *International Journal of Cardiology*, *264*, 172–178. https://doi.org/https://doi.org/10.1016/j.ijcard.2018.03.128

Picó, C., Serra, F., Rodríguez, A. M., Keijer, J., & Palou, A. (2019). Biomarkers of Nutrition and Health: New Tools for New Approaches. *Nutrients*, *11*(5), 1092. https://doi.org/10.3390/nu11051092

Pioli, M. R., Ritter, A. M., de Faria, A. P., & Modolo, R. (2018). White coat syndrome and its variations: differences and clinical impact. *Integrated Blood Pressure Control*, *11*, 73–79. https://doi.org/10.2147/IBPC.S152761

Pluznick, J. L. (2013). Renal and cardiovascular sensory receptors and blood pressure regulation. *American Journal of Physiology. Renal Physiology*, *305*(4), F439-44. https://doi.org/10.1152/ajprenal.00252.2013

Pluznick, J. L. (2017). Microbial Short-Chain Fatty Acids and Blood Pressure Regulation. *Current Hypertension Reports*, *19*(4), 1–9. https://doi.org/10.1007/s11906-017-0722-5

Potischman, N., & Freudenheim, J. L. (2003). Biomarkers of nutritional exposure and nutritional status: an overview. *The Journal of Nutrition*, *133*(3), 873S-874S. https://doi.org/10.1093/jn/133.3.873S

Raatz, S. K., Rosenberger, T. A., Johnson, L. K., Wolters, W. W., Burr, G. S., & Picklo, M. J. S. (2013). Dose-dependent consumption of farmed Atlantic salmon (Salmo salar) increases plasma phospholipid n-3 fatty acids differentially. *Journal of the Academy of Nutrition and Dietetics*, *113*(2), 282–287. https://doi.org/10.1016/j.jand.2012.09.022

Rahat-Rozenbloom, S., Fernandes, J., Gloor, G. B., & Wolever, T. M. S. (2014).

Evidence for greater production of colonic short-chain fatty acids in overweight than lean humans. *International Journal of Obesity (2005)*, *38*(12), 1525–1531. https://doi.org/10.1038/ijo.2014.46

Randrianarisoa, E., Lehn-Stefan, A., Wang, X., Hoene, M., Peter, A., Heinzmann, S. S., Zhao, X., Königsrainer, I., Königsrainer, A., Balletshofer, B., Machann, J., Schick, F., Fritsche, A., Häring, H.-U., Xu, G., Lehmann, R., & Stefan, N. (2016). Relationship of Serum Trimethylamine N-Oxide (TMAO) Levels with early Atherosclerosis in Humans. *Scientific Reports*, *6*, 26745. https://doi.org/10.1038/srep26745

Rastmanesh, R. (2011). High polyphenol, low probiotic diet for weight loss because of intestinal microbiota interaction. *Chemico-Biological Interactions*, *189*(1–2), 1–8. https://doi.org/10.1016/j.cbi.2010.10.002

Rath, S., Heidrich, B., Pieper, D. H., & Vital, M. (2017). Uncovering the trimethylamine-producing bacteria of the human gut microbiota. *Microbiome*, *5*(1), 54. https://doi.org/10.1186/s40168-017-0271-9

Reynolds, I., Page, R. L., & Boxer, R. S. (2019). *Cardiovascular Health and Healthy Aging BT - Healthy Aging: A Complete Guide to Clinical Management* (P. P. Coll (ed.); pp. 31–51). Springer International Publishing. https://doi.org/10.1007/978-3-030-06200-2_5

Ried, K., Fakler, P., & Stocks, N. P. (2017). Effect of cocoa on blood pressure. *Cochrane Database of Systematic Reviews*, *4*. https://doi.org/10.1002/14651858.CD008893.pub3

Rioux, V., Catheline, D., Bouriel, M., & Legrand, P. (2005). Dietary myristic acid at physiologically relevant levels increases the tissue content of C20:5 n-3 and C20:3 n-6 in the rat. *Reproduction, Nutrition, Development*, *45*(5), 599–612. https://doi.org/10.1051/rnd:2005048

Roager, H. M., Hansen, L. B. S., Bahl, M. I., Frandsen, H. L., Carvalho, V., Gøbel, R. J., Dalgaard, M. D., Plichta, D. R., Sparholt, M. H., Vestergaard, H., Hansen, T., Sicheritz-Pontén, T., Nielsen, H. B., Pedersen, O., Lauritzen, L., Kristensen, M., Gupta, R., & Licht, T. R. (2016). Colonic transit time is related to bacterial metabolism and mucosal turnover in the gut. *Nature Microbiology*, *1*(9), 16093. https://doi.org/10.1038/nmicrobiol.2016.93

Rohner, A., Ried, K., Sobenin, I. A., Bucher, H. C., & Nordmann, A. J. (2015). A systematic review and metaanalysis on the effects of garlic preparations on blood pressure in individuals with hypertension. *American Journal of Hypertension*, *28*(3), 414–423. https://doi.org/10.1093/ajh/hpu165

Rombaldova, M., Janovska, P., Kopecky, J., & Kuda, O. (2017). Omega-3 fatty acids promote fatty acid utilization and production of pro-resolving lipid mediators

in alternatively activated adipose tissue macrophages. *Biochemical and Biophysical Research Communications*, *490*(3), 1080–1085. https://doi.org/10.1016/j.bbrc.2017.06.170

Roshanravan, N., Mahdavi, R., Alizadeh, E., Jafarabadi, M. A., Hedayati, M., Ghavami, A., Alipour, S., Alamdari, N. M., Barati, M., & Ostadrahimi, A. (2017). Effect of Butyrate and Inulin Supplementation on Glycemic Status, Lipid Profile and Glucagon-Like Peptide 1 Level in Patients with Type 2 Diabetes: A Randomized Double-Blind, Placebo-Controlled Trial. *Hormone and Metabolic Research = Hormon- Und Stoffwechselforschung = Hormones et Metabolisme*, *49*(11), 886–891. https://doi.org/10.1055/s-0043-119089

Roth, G. A., Mensah, G. A., Johnson, C. O., Addolorato, G., Ammirati, E., Baddour, L. M., Barengo, N. C., Beaton, A. Z., Benjamin, E. J., Benziger, C. P., Bonny, A., Brauer, M., Brodmann, M., Cahill, T. J., Carapetis, J., Catapano, A. L., Chugh, S. S., Cooper, L. T., Coresh, J., … Fuster, V. (2020). Global Burden of Cardiovascular Diseases and Risk Factors, 1990-2019: Update From the GBD 2019 Study. *Journal of the American College of Cardiology*, *76*(25), 2982–3021. https://doi.org/10.1016/j.jacc.2020.11.010

Rutayisire, E., Huang, K., Liu, Y., & Tao, F. (2016). The mode of delivery affects the diversity and colonization pattern of the gut microbiota during the first year of infants' life: a systematic review. *BMC Gastroenterology*, *16*(1), 86. https://doi.org/10.1186/s12876-016-0498-0

Sacks, F. M., Svetkey, L. P., Vollmer, W. M., Appel, L. J., Bray, G. A., Harsha, D., Obarzanek, E., Conlin, P. R., Miller, E. R. 3rd, Simons-Morton, D. G., Karanja, N., & Lin, P. H. (2001). Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group. *The New England Journal of Medicine*, *344*(1), 3–10. https://doi.org/10.1056/NEJM200101043440101

Sahebkar, A., Ferri, C., Giorgini, P., Bo, S., Nachtigal, P., & Grassi, D. (2017). Effects of pomegranate juice on blood pressure: A systematic review and meta-analysis of randomized controlled trials. *Pharmacological Research*, *115*, 149–161. https://doi.org/10.1016/j.phrs.2016.11.018

Sampey, B. P., Vanhoose, A. M., Winfield, H. M., Freemerman, A. J., Muehlbauer, M. J., Fueger, P. T., Newgard, C. B., & Makowski, L. (2011). Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. *Obesity (Silver Spring, Md.)*, *19*(6), 1109–1117. https://doi.org/10.1038/oby.2011.18

Sanders, M. E. (2008). Probiotics: definition, sources, selection, and uses. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *46 Suppl 2*, S58-61; discussion S144-51.

https://doi.org/10.1086/523341

Santini, A., & Novellino, E. (2017). Nutraceuticals in hypercholesterolaemia: an overview. *British Journal of Pharmacology*, *174*(11), 1450–1463. https://doi.org/10.1111/bph.13636

Sayin, S. I., Wahlström, A., Felin, J., Jäntti, S., Marschall, H.-U., Bamberg, K., Angelin, B., Hyötyläinen, T., Orešič, M., & Bäckhed, F. (2013). Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metabolism*, *17*(2), 225–235. https://doi.org/10.1016/j.cmet.2013.01.003

Schiattarella, G. G., Sannino, A., Toscano, E., Giugliano, G., Gargiulo, G., Franzone, A., Trimarco, B., Esposito, G., & Perrino, C. (2018). *Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker : a systematic review and dose-response meta-analysis*. *June*, 2948–2956. https://doi.org/10.1093/eurheartj/ehx342

Schwiertz, A., Taras, D., Schäfer, K., Beijer, S., Bos, N. A., Donus, C., & Hardt, P. D. (2010). *Microbiota and SCFA in Lean and Overweight Healthy Subjects*. *18*(1). https://doi.org/10.1038/oby.2009.167

Seyedi, S. H. S., Mottaghi, A., Mirmiran, P., Hedayati, M., & Azizi, F. (2020). The relationship between dietary patterns and lipoprotein-associated phospholipase A2 levels in adults with cardiovascular risk factors: Tehran Lipid and Glucose Study. *Journal of Research in Medical Sciences : The Official Journal of Isfahan University of Medical Sciences*, *25*, 3. https://doi.org/10.4103/jrms.JRMS_256_19

Shaffer, M., Armstrong, A. J. S., Phelan, V. V, Reisdorph, N., & Lozupone, C. A. (2017). Microbiome and metabolome data integration provides insight into health and disease. *Translational Research : The Journal of Laboratory and Clinical Medicine*, *189*, 51–64. https://doi.org/10.1016/j.trsl.2017.07.001

Shah, S. H., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Crosslin, D. R., Haynes, C., Dungan, J., Newby, L. K., Hauser, E. R., Ginsburg, G. S., Newgard, C. B., & Kraus, W. E. (2010). Association of a Peripheral Blood Metabolic Profile With Coronary Artery Disease and Risk of Subsequent Cardiovascular Events. *Circulation: Cardiovascular Genetics*, *3*(2), 207–214. https://doi.org/10.1161/CIRCGENETICS.109.852814

Shere, A., Eletta, O., & Goyal, H. (2017). Circulating blood biomarkers in essential hypertension: a literature review. *Journal of Laboratory and Precision Medicine; Vol 2, No 12 (December 2017): Journal of Laboratory and Precision Medicine*. https://jlpm.amegroups.com/article/view/3942

Shoaie, S., Ghaffari, P., Kovatcheva-Datchary, P., Mardinoglu, A., Sen, P., Pujos-

Guillot, E., De Wouters, T., Juste, C., Rizkalla, S., Chilloux, J., Hoyles, L., Nicholson, J. K., Dore, J., Dumas, M. E., Clement, K., Bäckhed, F., & Nielsen, J. (2015). Quantifying Diet-Induced Metabolic Changes of the Human Gut Microbiome. *Cell Metabolism*, *22*(2), 320–331. https://doi.org/10.1016/j.cmet.2015.07.001

Singh, R. K., Chang, H. W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T. H., Bhutani, T., & Liao, W. (2017). Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine*, *15*(1), 1–17. https://doi.org/10.1186/s12967-017-1175-y

Smith, W. S., Johnston, S. C., & Hemphill J. Claude, I. I. I. (2018). Ischemic Stroke. In J. L. Jameson, A. S. Fauci, D. L. Kasper, S. L. Hauser, D. L. Longo, & J. Loscalzo (Eds.), *Harrison's Principles of Internal Medicine, 20e*. McGraw-Hill Education. http://accessmedicine.mhmedical.com/content.aspx?aid=1179962137

Snopek, L., Mlcek, J., Sochorova, L., Baron, M., Hlavacova, I., Jurikova, T., Kizek, R., Sedlackova, E., & Sochor, J. (2018). Contribution of Red Wine Consumption to Human Health Protection. *Molecules (Basel, Switzerland)*, *23*(7). https://doi.org/10.3390/molecules23071684

Stegemann, C., Pechlaner, R., Willeit, P., Langley, S. R., Mangino, M., Mayr, U., Menni, C., Moayyeri, A., Santer, P., Rungger, G., Spector, T. D., Willeit, J., Kiechl, S., & Mayr, M. (2014). Lipidomics Profiling and Risk of Cardiovascular Disease in the Prospective Population-Based Bruneck Study. *Circulation*, *129*(18), 1821–1831. https://doi.org/10.1161/CIRCULATIONAHA.113.002500

Sterpu, I., Fransson, E., Hugerth, L. W., Du, J., Pereira, M., Cheng, L., Radu, S. A., Calderón-Pérez, L., Zha, Y., Angelidou, P., Pennhag, A., Boulund, F., Scheynius, A., Engstrand, L., Wiberg-Itzel, E., & Schuppe-Koistinen, I. (2021). No evidence for a placental microbiome in human pregnancies at term. *American Journal of Obstetrics and Gynecology*, *224*(3), 296.e1-296.e23. https://doi.org/10.1016/j.ajog.2020.08.103

Strimbu, K., & Tavel, J. A. (2010). What are biomarkers? *Current Opinion in HIV and AIDS*, *5*(6), 463–466. https://doi.org/10.1097/COH.0b013e32833ed177

Suárez-García, S., Caimari, A., Del Bas, J. M., Suárez, M., & Arola, L. (2017). Serum lysophospholipid levels are altered in dyslipidemic hamsters. *Scientific Reports*, *7*(1), 10431. https://doi.org/10.1038/s41598-017-10651-0

Surma, S., Czober, T., Lepich, T., Sierka, O., & Bajor, G. (2020). Selected biomarkers of atherosclerosis - clinical aspects. *Acta Angiologica*, *26*(1), 28–39. https://doi.org/10.5603/AA.2020.0005

Sutters, M. (2021). Overview: Systemic Hypertension. In M. A. Papadakis, S. J.

McPhee, & M. W. Rabow (Eds.), *Current Medical Diagnosis & Treatment 2021*. McGraw-Hill Education. http://accessmedicine.mhmedical.com/content.aspx?aid=1175799283

Suzuki, A., Yamamoto, N., Jokura, H., Yamamoto, M., Fujii, A., Tokimitsu, I., & Saito, I. (2006). Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats. *Journal of Hypertension*, *24*(6), 1065–1073. https://doi.org/10.1097/01.hjh.0000226196.67052.c0

Syväranta, S., Alanne-Kinnunen, M., Oörni, K., Oksjoki, R., Kupari, M., Kovanen, P. T., & Helske-Suihko, S. (2014). Potential pathological roles for oxidized low-density lipoprotein and scavenger receptors SR-AI, CD36, and LOX-1 in aortic valve stenosis. *Atherosclerosis*, *235*(2), 398–407. https://doi.org/10.1016/j.atherosclerosis.2014.05.933

Tan, S. T., Ramesh, T., Toh, X. R., & Nguyen, L. N. (2020). Emerging roles of lysophospholipids in health and disease. *Progress in Lipid Research*, *80*, 101068. https://doi.org/https://doi.org/10.1016/j.plipres.2020.101068

Tang, W. H. W., & Hazen, S. L. (2014). The contributory role of gut microbiota in cardiovascular disease. *The Journal of Clinical Investigation*, *124*(10), 4204–4211. https://doi.org/10.1172/JCI72331

Tang, W. H. W., Kitai, T., & Hazen, S. L. (2017). Gut Microbiota in Cardiovascular Health and Disease. *Circulation Research*, *120*(7), 1183–1196. https://doi.org/10.1161/CIRCRESAHA.117.309715

Tang, W. H. W., Wang, Z., Kennedy, D. J., Wu, Y., Buffa, J. A., Agatisa-Boyle, B., Li, X. S., Levison, B. S., & Hazen, S. L. (2015). Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. *Circulation Research*, *116*(3), 448–455. https://doi.org/10.1161/CIRCRESAHA.116.305360

Tang, W. H. W., Wang, Z., Levison, B. S., Koeth, R. A., Britt, E. B., Fu, X., Wu, Y., & Hazen, S. L. (2013). Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *The New England Journal of Medicine*, *368*(17), 1575–1584. https://doi.org/10.1056/NEJMoa1109400

Tay, J., Luscombe-Marsh, N. D., Thompson, C. H., Noakes, M., Buckley, J. D., Wittert, G. A., Yancy, W. S. J., & Brinkworth, G. D. (2015). Comparison of low- and high-carbohydrate diets for type 2 diabetes management: a randomized trial. *The American Journal of Clinical Nutrition*, *102*(4), 780–790. https://doi.org/10.3945/ajcn.115.112581

Tertov, V. V, Kaplun, V. V, Sobenin, I. A., Boytsova, E. Y., Bovin, N. V, & Orekhov, A. N. (2001). Human plasma trans-sialidase causes atherogenic modification of low density lipoprotein. *Atherosclerosis*, *159*(1), 103–115.

https://doi.org/10.1016/s0021-9150(01)00498-1

Thursby, E., & Juge, N. (2017). Introduction to the human gut microbiota. *The Biochemical Journal*, *474*(11), 1823–1836. https://doi.org/10.1042/BCJ20160510

Tokede, O. A., Onabanjo, T. A., Yansane, A., Gaziano, J. M., & Djoussé, L. (2015). Soya products and serum lipids: a meta-analysis of randomised controlled trials. *The British Journal of Nutrition*, *114*(6), 831–843. https://doi.org/10.1017/S0007114515002603

Tomas-Barberan, F. A., Selma, M. V, & Espin, J. C. (2016). Interactions of gut microbiota with dietary polyphenols and consequences to human health. *Current Opinion in Clinical Nutrition and Metabolic Care*, *19*(6), 471–476. https://doi.org/10.1097/MCO.0000000000000314

Toral, M., Robles-Vera, I., de la Visitación, N., Romero, M., Yang, T., Sánchez, M., Gómez-Guzmán, M., Jiménez, R., Raizada, M. K., & Duarte, J. (2019). Critical Role of the Interaction Gut Microbiota - Sympathetic Nervous System in the Regulation of Blood Pressure. *Frontiers in Physiology*, *10*, 231. https://doi.org/10.3389/fphys.2019.00231

Touyz, R. M., & Burger, D. (2012). *Biomarkers in Hypertension BT - Special Issues in Hypertension* (A. E. Berbari & G. Mancia (eds.); pp. 237–246). Springer Milan. https://doi.org/10.1007/978-88-470-2601-8_19

Tuohy, K. M., Fava, F., & Viola, R. (2014). 'The way to a man's heart is through his gut microbiota'--dietary pro- and prebiotics for the management of cardiovascular risk. *The Proceedings of the Nutrition Society*, *73*(2), 172–185. https://doi.org/10.1017/S0029665113003911

Tzounis, X., Rodriguez-Mateos, A., Vulevic, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. E. (2011). Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *The American Journal of Clinical Nutrition*, *93*(1), 62–72. https://doi.org/10.3945/ajcn.110.000075

Tzounis, X., Vulevic, J., Kuhnle, G. G. C., George, T., Leonczak, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. E. (2018). Flavanol monomer-induced changes to the human faecal microflora. *The British Journal of Nutrition*, *99*(4), 782–792. https://doi.org/10.1017/S0007114507853384

Urpi-Sarda, M., Casas, R., Chiva-Blanch, G., Romero-Mamani, E. S., Valderas-Martínez, P., Arranz, S., Andres-Lacueva, C., Llorach, R., Medina-Remón, A., Lamuela-Raventos, R. M., & Estruch, R. (2012). Virgin olive oil and nuts as key foods of the Mediterranean diet effects on inflammatory biomakers related to atherosclerosis. *Pharmacological Research*, *65*(6), 577–583.

https://doi.org/10.1016/j.phrs.2012.03.006

Ursell, L. K., Haiser, H. J., Van Treuren, W., Garg, N., Reddivari, L., Vanamala, J., Dorrestein, P. C., Turnbaugh, P. J., & Knight, R. (2014). The intestinal metabolome: an intersection between microbiota and host. *Gastroenterology*, *146*(6), 1470–1476. https://doi.org/10.1053/j.gastro.2014.03.001

Vallbona Calbó, C., Roure Cuspinera, E., Violan Fors, M., & Alegre Martín, J. (2007). *Guia de prescripció d'exercici físic per a la salut (PEFS)*. https://doi.org/http://hdl.handle.net/11351/3541

van Bussel, E. F., Hoevenaar-Blom, M. P., Poortvliet, R. K. E., Gussekloo, J., van Dalen, J. W., van Gool, W. A., Richard, E., & Moll van Charante, E. P. (2020). Predictive value of traditional risk factors for cardiovascular disease in older people: A systematic review. *Preventive Medicine*, *132*, 105986. https://doi.org/https://doi.org/10.1016/j.ypmed.2020.105986

Vauzour, D., Rodriguez-Mateos, A., Corona, G., Oruna-Concha, M. J., & Spencer, J. P. E. (2010). Polyphenols and human health: prevention of disease and mechanisms of action. *Nutrients*, *2*(11), 1106–1131. https://doi.org/10.3390/nu2111106

Veugen, M. G. J., Henry, R. M. A., Brunner-La Rocca, H.-P., Dagnelie, P. C., Schram, M. T., van Agtmaal, M. J. M., van der Kallen, C. J. H., Sep, S. J. S., van Boxtel, M. P. J., Bekers, O., Meex, S. J. R., Jansen, J. F. A., Kroon, A. A., & Stehouwer, C. D. A. (2018). Cross-Sectional Associations Between Cardiac Biomarkers, Cognitive Performance, and Structural Brain Changes Are Modified by Age. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *38*(8), 1948–1958. https://doi.org/10.1161/ATVBAHA.118.311082

Visseren, F. L. J., Mach, F., Smulders, Y. M., Carballo, D., Koskinas, K. C., Bäck, M., Benetos, A., Biffi, A., Boavida, J.-M., Capodanno, D., Cosyns, B., Crawford, C., Davos, C. H., Desormais, I., Di Angelantonio, E., Franco, O. H., Halvorsen, S., Hobbs, F. D. R., Hollander, M., … Williams, B. (2021). 2021 ESC Guidelines on cardiovascular disease prevention in clinical practice. *European Heart Journal*, *42*(34), 3227–3337. https://doi.org/10.1093/eurheartj/ehab484

Wang, D., Özen, C., Abu-Reidah, I. M., Chigurupati, S., Patra, J. K., Horbanczuk, J. O., Jóźwik, A., Tzvetkov, N. T., Uhrin, P., & Atanasov, A. G. (2018). Vasculoprotective Effects of Pomegranate (Punica granatum L.). *Frontiers in Pharmacology*, *9*, 544. https://doi.org/10.3389/fphar.2018.00544

Wang, J., Tan, G.-J., Han, L.-N., Bai, Y.-Y., He, M., & Liu, H.-B. (2017). Novel biomarkers for cardiovascular risk prediction. *Journal of Geriatric Cardiology : JGC*, *14*(2), 135–150. https://doi.org/10.11909/j.issn.1671-5411.2017.02.008

Wang, L., Zeng, B., Liu, Z., Liao, Z., Zhong, Q., Gu, L., Wei, H., & Fang, X. (2018). Green Tea Polyphenols Modulate Colonic Microbiota Diversity and Lipid Metabolism in High-Fat Diet Treated HFA Mice. *Journal of Food Science*, *83*(3), 864–873. https://doi.org/10.1111/1750-3841.14058

Wang, W.-L., Xu, S.-Y., Ren, Z.-G., Tao, L., Jiang, J.-W., & Zheng, S.-S. (2015). Application of metagenomics in the human gut microbiome. *World Journal of Gastroenterology*, *21*(3), 803–814. https://doi.org/10.3748/wjg.v21.i3.803

Wang, Z., Klipfell, E., Bennett, B. J., Koeth, R., Levison, B. S., Dugar, B., Feldstein, A. E., Britt, E. B., Fu, X., Chung, Y.-M., Wu, Y., Schauer, P., Smith, J. D., Allayee, H., Tang, W. H. W., DiDonato, J. A., Lusis, A. J., & Hazen, S. L. (2011). Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*, *472*(7341), 57–63. https://doi.org/10.1038/nature09922

Wang, Z., Tang, W. H. W., Buffa, J. A., Fu, X., Britt, E. B., Koeth, R. A., Levison, B. S., Fan, Y., Wu, Y., & Hazen, S. L. (2014). Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. *European Heart Journal*, *35*(14), 904–910. https://doi.org/10.1093/eurheartj/ehu002

Wang, Z., & Zhao, Y. (2018). Gut microbiota derived metabolites in cardiovascular health and disease. *Protein & Cell*, *9*(5), 416–431. https://doi.org/10.1007/s13238-018-0549-0

Warrier, M., Shih, D. M., Burrows, A. C., Ferguson, D., Gromovsky, A. D., Brown, A. L., Marshall, S., McDaniel, A., Schugar, R. C., Wang, Z., Sacks, J., Rong, X., Vallim, T. de A., Chou, J., Ivanova, P. T., Myers, D. S., Brown, H. A., Lee, R. G., Crooke, R. M., … Brown, J. M. (2015). The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance. *Cell Reports*, *10*(3), 326–338. https://doi.org/10.1016/j.celrep.2014.12.036

Wassall, S. R., Leng, X., Canner, S. W., Pennington, E. R., Kinnun, J. J., Cavazos, A. T., Dadoo, S., Johnson, D., Heberle, F. A., Katsaras, J., & Shaikh, S. R. (2018). Docosahexaenoic acid regulates the formation of lipid rafts: A unified view from experiment and simulation. *Biochimica et Biophysica Acta. Biomembranes*, *1860*(10), 1985–1993. https://doi.org/10.1016/j.bbamem.2018.04.016

WHO. (2012). Guideline: Sodium intake for adults and children. *World Health Organization*, 1–56. http://apps.who.int/iris/handle/10665/77985%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed?term=Sodium%5BTitle%5D AND intake%5BTitle%5D AND adults%5BTitle%5D AND children%5BTitle%5D AND WHO%5BTitle%5D%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed?term=Sodium%255BTitle%255

WHO. (2016). Technical package for cardiovascular disease management in primary health care. In *Report* (pp. 1–76). https://www.who.int/cardiovascular_diseases/publications/en/

Wilkins, L. J., Monga, M., & Miller, A. W. (2019). Defining Dysbiosis for a Cluster of Chronic Diseases. *Scientific Reports*, *9*(1), 12918. https://doi.org/10.1038/s41598-019-49452-y

Williams, B., Mancia, G., Spiering, W., Rosei, E. A., Azizi, M., Burnier, M., Clement, D. L., Coca, A., De Simone, G., Dominiczak, A., Kahan, T., Mahfoud, F., Redon, J., Ruilope, L., Zanchetti, A., Kerins, M., Kjeldsen, S. E., Kreutz, R., Laurent, S., … Zamorano, J. L. (2018). 2018 ESC/ESH Guidelines for themanagement of arterial hypertension. In *European Heart Journal*. https://doi.org/10.1093/eurheartj/ehy339

Wirsching, J., Graßmann, S., Eichelmann, F., Harms, L. M., Schenk, M., Barth, E., Berndzen, A., Olalekan, M., Sarmini, L., Zuberer, H., & Aleksandrova, K. (2018). Development and reliability assessment of a new quality appraisal tool for cross-sectional studies using biomarker data (BIOCROSS). *BMC Medical Research Methodology*, *18*(1), 122. https://doi.org/10.1186/s12874-018-0583-x

Woodhouse, C., Singanayagam, A., & Patel, V. C. (2020). Modulating the gut–liver axis and the pivotal role of the faecal microbiome in cirrhosis. *Clinical Medicine*, *20*(5), 493 LP – 500. https://doi.org/10.7861/clinmed.2020-0676

Würtz, P., Havulinna, A. S., Soininen, P., Tynkkynen, T., Prieto-Merino, D., Tillin, T., Ghorbani, A., Artati, A., Wang, Q., Tiainen, M., Kangas, A. J., Kettunen, J., Kaikkonen, J., Mikkilä, V., Jula, A., Kähönen, M., Lehtimäki, T., Lawlor, D. A., Gaunt, T. R., … Salomaa, V. (2015). Metabolite Profiling and Cardiovascular Event Risk. *Circulation*, *131*(9), 774–785. https://doi.org/10.1161/CIRCULATIONAHA.114.013116

Xia, J., Broadhurst, D. I., Wilson, M., & Wishart, D. S. (2013). Translational biomarker discovery in clinical metabolomics: an introductory tutorial. *Metabolomics : Official Journal of the Metabolomic Society*, *9*(2), 280–299. https://doi.org/10.1007/s11306-012-0482-9

Xiao, S., Fei, N., Pang, X., Shen, J., Wang, L., Zhang, B., Zhang, M., Zhang, X., Zhang, C., Li, M., Sun, L., Xue, Z., Wang, J., Feng, J., Yan, F., Zhao, N., Liu, J., Long, W., & Zhao, L. (2014). A gut microbiota-targeted dietary intervention for amelioration of chronic inflammation underlying metabolic syndrome. *FEMS Microbiology Ecology*, *87*(2), 357–367. https://doi.org/10.1111/1574-6941.12228

Yan, Q., Gu, Y., Li, X., Yang, W., Jia, L., Chen, C., Han, X., Huang, Y., Zhao, L., Li, P.,

Fang, Z., Zhou, J., Guan, X., Ding, Y., Wang, S., Khan, M., Xin, Y., Li, S., & Ma, Y. (2017). Alterations of the Gut Microbiome in Hypertension. *Frontiers in Cellular and Infection Microbiology*, *7*, 381. https://doi.org/10.3389/fcimb.2017.00381

Yang, K., & Han, X. (2016). Lipidomics: Techniques, Applications, and Outcomes Related to Biomedical Sciences. *Trends in Biochemical Sciences*, *41*(11), 954–969. https://doi.org/10.1016/j.tibs.2016.08.010

Yang, T., Magee, K. L., Colon-Perez, L. M., Larkin, R., Liao, Y.-S., Balazic, E., Cowart, J. R., Arocha, R., Redler, T., Febo, M., Vickroy, T., Martyniuk, C. J., Reznikov, L. R., & Zubcevic, J. (2019). Impaired butyrate absorption in the proximal colon, low serum butyrate and diminished central effects of butyrate on blood pressure in spontaneously hypertensive rats. *Acta Physiologica*, *226*(2), e13256. https://doi.org/10.1111/apha.13256

Yang, T., Santisteban, M. M., Rodriguez, V., Li, E., Ahmari, N., Carvajal, J. M., Zadeh, M., Gong, M., Qi, Y., Zubcevic, J., Sahay, B., Pepine, C. J., Raizada, M. K., & Mohamadzadeh, M. (2015). Gut dysbiosis is linked to hypertension. *Hypertension (Dallas, Tex. : 1979)*, *65*(6), 1331–1340. https://doi.org/10.1161/HYPERTENSIONAHA.115.05315

Yin, H., Xu, L., & Porter, N. A. (2011). Free Radical Lipid Peroxidation: Mechanisms and Analysis. *Chemical Reviews*, *111*(10), 5944–5972. https://doi.org/10.1021/cr200084z

Younis, N., Sharma, R., Soran, H., Charlton-Menys, V., Elseweidy, M., & Durrington, P. N. (2008). Glycation as an atherogenic modification of LDL. *Current Opinion in Lipidology*, *19*(4), 378–384. https://doi.org/10.1097/MOL.0b013e328306a057

Žáček, P., Bukowski, M., Johnson, L., SK, R., & Picklo, M. (2018). Selective enrichment of n-3 fatty acids in human plasma lipid motifs following intake of marine fish. *J Nutr Biochem*, *54*(PG-57-65), 57–65. https://doi.org/10.1016/j.jnutbio.2017.11.002

Zampelas, A., & Magriplis, E. (2020). Dietary patterns and risk of cardiovascular diseases: a review of the evidence. *The Proceedings of the Nutrition Society*, *79*(1), 68–75. https://doi.org/10.1017/S0029665119000946

Zanzer, Y. C., Plaza, M., Dougkas, A., Turner, C., Björck, I., & Östman, E. (2017). Polyphenol-rich spice-based beverages modulated postprandial early glycaemia, appetite and PYY after breakfast challenge in healthy subjects: A randomized, single blind, crossover study. *Journal of Functional Foods*, *35*, 574–583. https://doi.org/https://doi.org/10.1016/j.jff.2017.06.016

Zhang, F., Zhang, T., Zhu, H., & Borody, T. J. (2019). Evolution of fecal microbiota

transplantation in methodology and ethical issues. *Current Opinion in Pharmacology*, *49*, 11–16. https://doi.org/10.1016/j.coph.2019.04.004

Zhang, M., Zhou, L., Wang, Y., Dorfman, R. G., Tang, D., Xu, L., Pan, Y., Zhou, Q., Li, Y., Yin, Y., Zhao, S., Wu, J., & Yu, C. (2019). Faecalibacterium prausnitzii produces butyrate to decrease c-Myc-related metabolism and Th17 differentiation by inhibiting histone deacetylase 3. *International Immunology*, *31*(8), 499–514. https://doi.org/10.1093/intimm/dxz022

Zhang, X., Li, Q., Xu, Z., & Dou, J. (2020). Mass spectrometry-based metabolomics in health and medical science: a systematic review. *RSC Advances*, *10*(6), 3092–3104. https://doi.org/10.1039/C9RA08985C

Zhong, H.-J., Zeng, H.-L., Cai, Y.-L., Zhuang, Y.-P., Liou, Y.-L., Wu, Q., & He, X.-X. (2021). Washed Microbiota Transplantation Lowers Blood Pressure in Patients With Hypertension. *Frontiers in Cellular and Infection Microbiology*, *11*, 679624. https://doi.org/10.3389/fcimb.2021.679624

Zhu, W., Gregory, J. C., Org, E., Buffa, J. A., Gupta, N., Wang, Z., Li, L., Fu, X., Wu, Y., Mehrabian, M., Sartor, R. B., McIntyre, T. M., Silverstein, R. L., Tang, W. H. W., DiDonato, J. A., Brown, J. M., Lusis, A. J., & Hazen, S. L. (2016). Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk. *Cell*, *165*(1), 111–124. https://doi.org/10.1016/j.cell.2016.02.011

Zhu, W., Wang, Z., Tang, W. H. W., & Hazen, S. L. (2017). Gut Microbe-Generated Trimethylamine N-Oxide From Dietary Choline Is Prothrombotic in Subjects. In *Circulation* (Vol. 135, Issue 17, pp. 1671–1673). https://doi.org/10.1161/CIRCULATIONAHA.116.025338

Zhu, Y., Bo, Y., & Liu, Y. (2019). Dietary total fat, fatty acids intake, and risk of cardiovascular disease: a dose-response meta-analysis of cohort studies. *Lipids in Health and Disease*, *18*(1), 91. https://doi.org/10.1186/s12944-019-1035-2

Zhuang, R., Ge, X., Han, L., Yu, P., Gong, X., Meng, Q., Zhang, Y., Fan, H., Zheng, L., Liu, Z., & Zhou, X. (2019). Gut microbe-generated metabolite trimethylamine N-oxide and the risk of diabetes: A systematic review and dose-response meta-analysis. *Obesity Reviews : An Official Journal of the International Association for the Study of Obesity*, *20*(6), 883–894. https://doi.org/10.1111/obr.12843

Zierer, J., Jackson, M. A., Kastenmüller, G., Mangino, M., Long, T., Telenti, A., Mohney, R. P., Small, K. S., Bell, J. T., Steves, C. J., Valdes, A. M., Spector, T. D., & Menni, C. (2018). The fecal metabolome as a functional readout of the gut microbiome. *Nature Genetics*, *50*(6), 790–795.

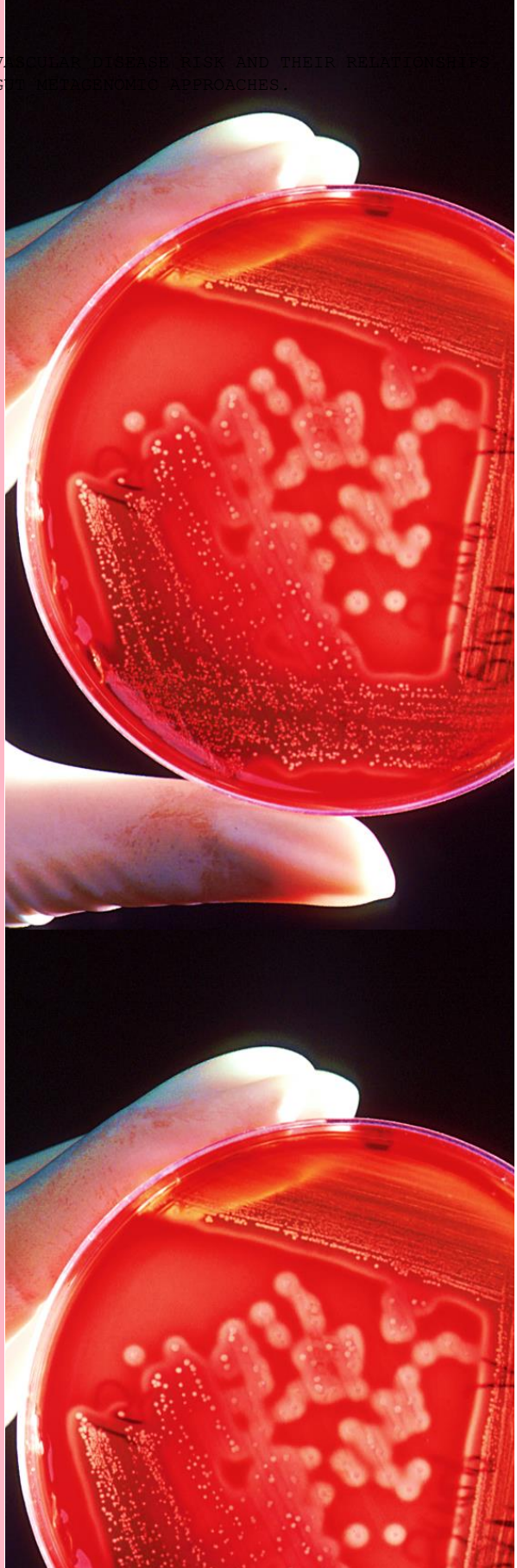
REFERENCES

https://doi.org/10.1038/s41588-018-0135-7

Żółkiewicz, J., Marzec, A., Ruszczyński, M., & Feleszko, W. (2020). Postbiotics-A Step Beyond Pre- and Probiotics. *Nutrients*, *12*(8). https://doi.org/10.3390/nu12082189

Zou, Y., Li, J., Lu, C., Wang, J., Ge, J., Huang, Y., Zhang, L., & Wang, Y. (2006). High-fat emulsion-induced rat model of nonalcoholic steatohepatitis. *Life Sciences*, *79*(11), 1100–1107. https://doi.org/10.1016/j.lfs.2006.03.021

ANNEXES



UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez

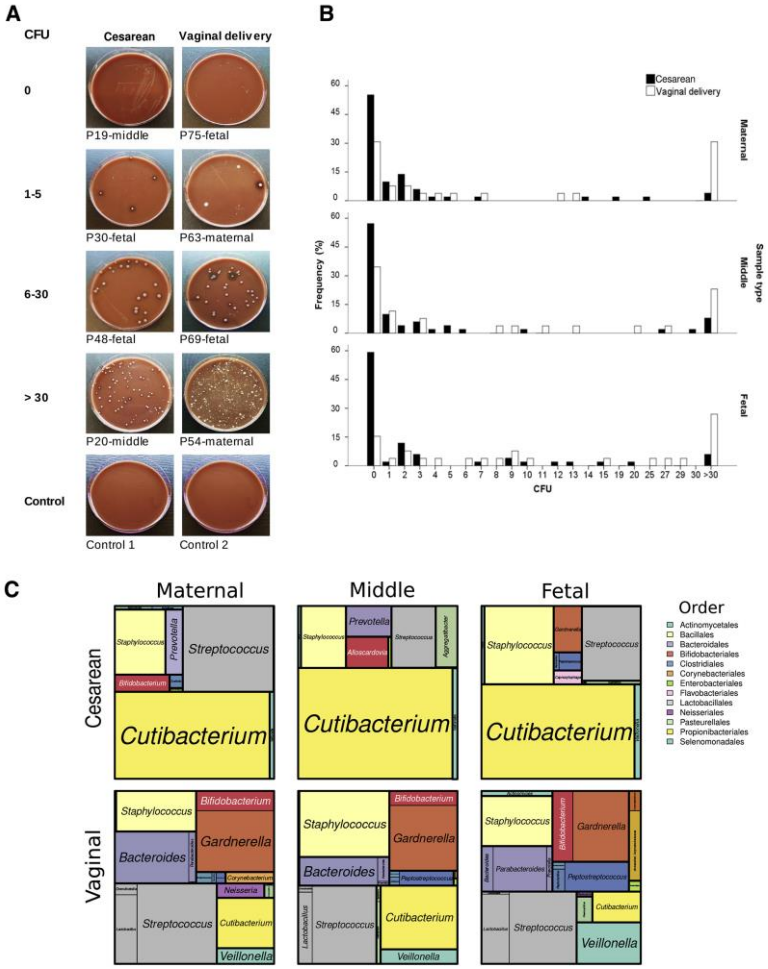
ANNEX I. International stay at the Center for Translational Microbiome Research, Karolinska Institutet, *Stockholm*

Visit to the Center for Translational Microbiome Research (CTMR), Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden. Stay period: September 16th - December 20th, 2019.

During the international stay at the CTMR, the PhD student had the chance to actively participate into large-scale projects focused on the study of the microbiota in reproductive health. In particular, she joined in the *PlaMi study* which was focused on answering the following question: *Does microbiome acquisition start in utero?,* which is a subject of much current debate in the scientific community relating to whether the placenta hosts microbiota.

The *PlaMi study* collected samples from 50 healthy women who had a planned Caesarian section and 26 with a normal vaginal delivery. After obtaining multiple samples from placenta, amniotic fluid, umbilical cord blood, vernix, vagina, saliva and faeces at delivery, a comprehensive analysis through DNA sequencing techniques combined with bacterial culture experiments was performed. The PhD student conducted over 200 bacterial cultures with different placenta tissues from Caesarean section and vaginal delivery, and extracted DNA from the cultured bacterial colonies for further 16S rRNA gene sequencing. She also performed the analysis of culturing and contributed writing part of the manuscript for this project (Sterpu et al., 2021). In the section of the manuscript *"Bacterial culture of placental specimens yielded typical vaginal and skin bacteria"* she actively participated in preparing the results, tables and figures, as summarized below:

Figure 5. in the published article. Bacteria grown from placentas are predominantly typical skin and vaginal taxa.



A, GC agar plates showing the bacterial growth from placental tissues after 48 hours. The placenta cultures are represented in ranges according to the number of CFUs. "P" stands for participant. **B**, Histogram showing frequency (percentage) distributions of CFUs according to the placental sample type and the delivery mode. Chi-squared tests (Fisher exact tests) were performed with significance level at P¼.05. The comparison was between vaginal delivery (white bars) and cesarean delivery (black bars) in each CFU range group. **C**, Treemaps showing the relative proportion of the taxa that grew in culture by location in the tissue and mode of delivery. Each area is colored according to the bacterial order, as shown in the legend, and the genus is overlaid on the boxes themselves. CFU, colony-forming unit; GC, guanine-cytosine.

Table 2. in the published article. Bacterial growth of placental tissues according to the delivery mode.

TABLE 2
Bacterial growth of placental tissues according to the delivery mode

	Delivery mode						
	Cesarean delivery (N=152)[a]			Vaginal delivery (N=78)			Cesarean delivery vs vaginal delivery
CFU range	Maternal (n=51)	Middle (n=50)	Fetal (n=51)	Maternal (n=26)	Middle (n=26)	Fetal (n=26)	*P* value
0	28 (54.9)	29 (58.0)	30 (58.8)	8 (30.8)	9 (34.6)	4 (15.4)	<.001
1–5	17 (33.4)	13 (26.0)	10 (19.6)	7 (26.9)	5 (19.2)	5 (19.2)	.787
6–30	4 (7.8)	3 (6.0)	8 (15.7)	3 (11.5)	6 (23.1)	10 (38.5)	.996
>30	2 (3.9)	5 (10.0)	3 (5.9)	8 (30.8)	6 (23.1)	7 (26.9)	<.001

Data are expressed as absolute and relative values for total plate count number (percentages). P value shows the differences between delivery groups at the level of the CFU range. The significance level was set at P<.05. The Fisher exact test was used for comparisons. CFU, colony-forming unit. a One mother delivered after a dichorionic diamniotic pregnancy.

Publication:

Sterpu I, Fransson E, Hugerth LW, Du J, Pereira M, Cheng L, Radu SA, Calderón-Pérez L, Zha Y, Angelidou P, Pennhag A, Boulund F, Scheynius A, Engstrand L, Wiberg-Itzel E, Schuppe-Koistinen I. No evidence for a placental microbiome in human pregnancies at term. Am J Obstet Gynecol. 2021 Mar;224(3):296.e1-296.e23. doi: 10.1016/j.ajog.2020.08.103.

Karolinska Institutet

European PhD stay

Student name: Lorena Calderón Pérez

Origin group:
NFOC-SALUT Research Group
Department of Medicine and Surgery
Rovira i Virgili University, Faculty of Medicine and Health Sciences
C/ Sant Llorenç 21, CP 43201, Reus, Spain

Hosting group;
Center for Translational Microbiome Research, CTMR
Department. of Microbiology, Tumor and Cell Biology
Karolinska Institutet, Biomedicum, A8
Solnavägen 9, 17165 Solna, Sweden

Group supervisor:
Juan Du | Assistant Professor
Centre for Translational Microbiome Research (CTMR)
Department of Microbiology, Tumor and Cell Biology (MTC)
Karolinska Institutet

Start date: 16th September 2019

End date: 20th December 2019

Projects:
Lorena has involved in the following two projects during her stay:

1. Placental Microbiome project (PlaMi) – Is there a placental microbiome in healthy women?

In this Project, Lorena cultured bacterial colonies from over 200 placenta tissues from both C-section and vaginal delivery participates. Lorena also prepared DNA from these colonies for further 16S rRNA sequencing. She finished all the analysis of culturing and prepared tables and manuscript for this project.

2. Anaerobically Cultivated Human Vaginal Microflora (ACHVaM study).

The aim of this Project is to develop different culture medium simulating vaginal flora conditions for future investigation of bacterial infection in the genital tract. Lorena tested vaginosis and healthy-live bacteria species by using different media and conditions. After culturing for one week, DNA extraction, qPCR and Real-Time PCR were used for identifing the growth of *Lactobacilli* and other pathogenic bacteria.

During her stay, she has attended to weekly seminars and meetings on Microbiome research. She has also attended the International Conference entitled "Emerging Roles of the Microbiome in Typical and Atypical Brain Development" held at the Nobel Forum on September 23-24. She also gave one presentation about her work in the center seminar.

Signature and date:

Supervisor

19-12-18

PhD student

19-12-18

ANNEX II. Scientific contributions published during the course of the doctoral thesis

2021 Publications

Valls RM, Pedret A, Calderón-Pérez L, Llauradó E, Pla-Pagà L, Companys J, Moragas A, Martín-Luján F, Ortega Y, Giralt M, Rubió L, Canela N, Puiggrós F, Caimari A, Del Bas JM, Arola L, Solà R. Hesperidin in orange juice improves human endothelial function in subjects with elevated blood pressure and stage 1 hypertension: A randomized, controlled trial (Citrus study). Journal of Functional Foods. 2021 Oct;85:104646. doi: 10.1016/j.jff.2021.104646.

Pla-Pagà L, Valls RM, Pedret A, Calderón-Pérez L, Llauradó E, Companys J, Domenech-Coca C, Canela N, Del Bas JM, Caimari A, Puiggròs F, Mi C, Arola L, Solà R. Effect of the consumption of hesperidin in orange juice on the transcriptomic profile of subjects with elevated blood pressure and stage 1 hypertension: A randomized controlled trial (CITRUS study). Clin Nutr. 2021 Oct;40(12):5812-5822. doi: 10.1016/j.clnu.2021.10.009.

Pla-Pagà L, Pedret A, Valls RM, Calderón-Pérez L, Llauradó E, Companys J, Martín-Luján F, Moragas A, Canela N, Puiggròs F, Caimari A, Del Bas JM, Arola L, Solà R, Mayneris-Perxachs J. Effects of Hesperidin Consumption on the Cardiovascular System in Pre- and Stage 1 Hypertensive Subjects: Targeted and Non-Targeted Metabolomic Approaches (CITRUS Study). Mol Nutr Food Res. 2021 Sep;65(17):e2001175. doi: 10.1002/mnfr.202001175.

Companys J, Gosalbes MJ, Pla-Pagà L, Calderón-Pérez L, Llauradó E, Pedret A, Valls RM, Jiménez-Hernández N, Sandoval-Ramirez BA, Del Bas JM, Caimari A, Rubió L, Solà R. Gut Microbiota Profile and Its Association with Clinical Variables and Dietary Intake in Overweight/Obese and Lean Subjects: A Cross-Sectional Study. Nutrients. 2021 Jun 13;13(6):2032. doi: 10.3390/nu13062032.

Calderón-Pérez L, Llauradó E, Companys J, Pla-Pagà L, Boqué N, Puiggrós F,

Valls RM, Pedret A, Llabrés JM, Arola L, Solà R. Acute Effects of Turmeric Extracts on Knee Joint Pain: A Pilot, Randomized Controlled Trial. J Med Food. 2021 Apr;24(4):436-440. doi: 10.1089/jmf.2020.0074.

Valls RM, Pedret A, Calderón-Pérez L, Llauradó E, Pla-Pagà L, Companys J, Moragas A, Martín-Luján F, Ortega Y, Giralt M, Romeu M, Rubió L, Mayneris-Perxachs J, Canela N, Puiggrós F, Caimari A, Del Bas JM, Arola L, Solà R. Effects of hesperidin in orange juice on blood and pulse pressures in mildly hypertensive individuals: a randomized controlled trial (Citrus study). Eur J Nutr. 2021 Apr;60(3):1277-1288. doi: 10.1007/s00394-020-02279-0.

Sterpu I, Fransson E, Hugerth LW, Du J, Pereira M, Cheng L, Radu SA, Calderón-Pérez L, Zha Y, Angelidou P, Pennhag A, Boulund F, Scheynius A, Engstrand L, Wiberg-Itzel E, Schuppe-Koistinen I. No evidence for a placental microbiome in human pregnancies at term. Am J Obstet Gynecol. 2021 Mar;224(3):296.e1-296.e23. doi: 10.1016/j.ajog.2020.08.103.

2020 Publications

Companys J, Pla-Pagà L, Calderón-Pérez L, Llauradó E, Solà R, Pedret A, Valls RM. Fermented Dairy Products, Probiotic Supplementation, and Cardiometabolic Diseases: A Systematic Review and Meta-analysis. Adv Nutr. 2020 Jul 1;11(4):834-863. doi: 10.1093/advances/nmaa030.

Sandoval-Ramírez BA, Catalán Ú, Calderón-Pérez L, Companys J, Pla-Pagà L, Ludwig IA, Romero MP, Solà R. The effects and associations of whole-apple intake on diverse cardiovascular risk factors. A narrative review. Crit Rev Food Sci Nutr. 2020;60(22):3862-3875. doi: 10.1080/10408398.2019.

Rubió L, Yuste S, Ludwig I, Romero MP, Motilva MJ, Calderón-Pérez L, Pla-Pagà L, Companys J, Macià A. Application of Dried Blood Spot Cards combined with liquid chromatography-tandem mass spectrometry to determine eight fat-soluble micronutrients in human blood. J Chromatogr B Analyt Technol Biomed Life Sci. 2020 Sep 1;1152:122247. doi: 10.1016/j.jchromb.

2019 Publications

Pla-Pagà L, Companys J, Calderón-Pérez L, Llauradó E, Solà R, Valls RM, Pedret A. Effects of hesperidin consumption on cardiovascular risk biomarkers: a systematic review of animal studies and human randomized clinical trials. Nutr Rev. 2019 Dec 1;77(12):845-864. doi: 10.1093/nutrit/nuz036.

Pedret A, Valls RM, Calderón-Pérez L, Llauradó E, Companys J, Pla-Pagà L, Moragas A, Martín-Luján F, Ortega Y, Giralt M, Caimari A, Chenoll E, Genovés S, Martorell P, Codoñer FM, Ramón D, Arola L, Solà R. Effects of daily consumption of the probiotic Bifidobacterium animalis subsp. lactis CECT 8145 on anthropometric adiposity biomarkers in abdominally obese subjects: a randomized controlled trial. Int J Obes (Lond). 2019 Sep;43(9):1863-1868. doi: 10.1038/s41366-018-0220-0.

UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL EARLY BIOMARKERS OF CARDIOVASCULAR DISEASE RISK AND THEIR RELATIONSHIPS WITH BIOACTIVE DIETARY COMPOUNDS: METABOLOMIC AND GUT METAGENOMIC APPROACHES.
Lorena Calderón Pérez