GUT MICROBIOTA: A LINK BETWEEN DIET AND CARDIOMETABOLIC HEALTH

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Gut microbiota: a link between diet and cardiometabolic health

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Bulló Mònica, Professor of Nutrition and Food Science at the Department of Biochemistry and Biotechnology of Rovira i Virgili University,

I STATE:

That the present study, entitled "GUT MICROBIOTA: A LINK BETWEEN DIET AND CARDIOMETABOLIC HEALTH", presented by Ms. Serena Galiè for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of this University and it is currently up for an international distinction.

Reus, 1st May 2021

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"It's the time that you spent on your rose that makes your rose so important"

Antoine de Saint-Exupéry

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ABSTRACT

English

The role of specific foods or dietary components in shaping gut microbiota and their impact on human health represent a growing area of interest in the hallmark of precision nutrition. However, the effects of a global dietary pattern like Mediterranean diet (MedDiet) against consumption of a recognized potential prebiotic food like nuts into a habitual dietary pattern on the complex ecology of gut microbiome has not been fully elucidated.

Our first aim was then to evaluate the effects of MedDiet on gut microbiota composition and function compared to the consumption of nuts in the context of a non-MedIDiet in adults with both overweight/obesity and metabolic syndrome. Secondly, we aimed to analyze the potential intermediatory role of gut microbiota in diet-induced benefits on cardiometabolic parameters and on the overall host metabolism, by evaluating the fecal and plasma metabolomics profile of our population. The present work is conducted in the framework of METADIET study, a controlled randomized clinical trial with a crossover design.

The results illustrated in this thesis showed that consuming MedDiet is resulting in an enrichment of *Lachnospiraceae NK4A136* and a member of Ruminococcaceae family. At the same time, these changes were positively associated with improvements in cardiometabolic parameters and reflected as well in the fecal and plasma metabolomes of the host.

Overall, consuming nuts as part of MedDiet is related to greater alterations in gut microbiota interaction with the host metabolic status, thus to its ability to mediate the known beneficial effects of this dietary pattern in metabolic diseases.

RESUMEN

Castellano

El papel de determinados alimentos o componentes de la dieta en la composición de la microbiota intestinal y su impacto en la salud humana representan un área de interés creciente en el marco de la nutrición de precisión. Sin embargo, la comprensión del potencial beneficioso de la microbiota bajo el efecto de un patrón dietético saludable como la dieta mediterránea (MedDiet) frente al consumo de un alimento prebiotico como los frutos secos en una dieta habitual, queda todavía por descubrir.

Nuestro primer objetivo fue evaluar los efectos de la MedDiet sobre la composición de la microbiota intestinal y su función en comparación al consumo de frutos secos en adultos con sobrepeso/obesidad y síndrome metabólico. En segundo lugar, nos propusimos analizar el posible papel de intermediario de la microbiota intestinal en los beneficios inducidos por la dieta sobre los parámetros cardiometabólicos y sobre el metabolismo general del huésped. Para ello, evaluamos el perfil metabolómico fecal y plasmático de nuestra población. El presente trabajo se lleva a cabo en el marco del estudio METADIET, un ensayo clínico randomizado y controlado con un diseño cruzado.

Los resultados ilustrados en esta tesis muestran que seguir un patrón de dieta mediterránea se relaciona con un enriquecimiento de *Lachnospiraceae NK4A136* y de un miembro de la familia Ruminococcaceae. Al mismo tiempo, estos cambios se asocian positivamente con las mejoras en los parámetros cardiometabólicos y se reflejan también en los metabolomas fecales y plasmáticos del huésped.

En conclusión, el consumo de frutos secos como parte de la MedDiet se relaciona con mayores alteraciones en la microbiota intestinal y con el estado metabólico del huésped, y por tanto se propone como mediador de los

conocidos efectos beneficiosos de este patrón dietético en las enfermedades cardiometabólicas.

RESUM

Català

El paper de determinats aliments o components de la dieta en la composició de la microbiota intestinal i el seu impacte en la salut humana representen una àrea d'interès creixent dins el marc de la nutrició de precisió. No obstant això, la comprensió del seu paper sota l'efecte d'un patró dietètic global com la dieta mediterrània (MedDiet) davant el consum d'un aliment potencialment prebiòtic com els fruits secs en un patró dietètic habitual queda encara per descobrir.

El nostre primer objectiu va ser avaluar els efectes de la MedDiet sobre la composició de la microbiota intestinal i la seva funció en adults amb sobrepès/obesitat i síndrome metabòlic. En segon lloc, ens vam proposar analitzar el possible paper d'intermediari de la microbiota intestinal als beneficis induïts per la dieta sobre els paràmetres cardiometabòlics i sobre el metabolisme general de l'hoste, mitjançant l'avaluació del perfil metabolòmic fecal i plasmàtic de la nostra població. El present treball es duu a terme dins del marc d'estudi METADIET, un assaig clínic randomitzat i controlat amb un disseny creuat.

Els resultats il·lustrats a aquesta tesi mostren que seguir un patró de dieta mediterrània es relaciona amb un enriquiment de *Lachnospiraceae NK4A136* i de un membre de la família Ruminococcaceae. Al mateix temps, aquests canvis s'associen positivament amb les millores als paràmetres cardiometabòlics i es reflecteixen també als metabolomes fecals i plasmàtics de l'hoste.

Concloent, el consum de fruits secs com a part de la MedDiet es relaciona amb grans alteracions en la microbiota intestinal i amb l'estat metabòlic de l'hoste, i per tant, es proposa com mediador de aquest beneficiós patró de dieta cap a les malalties cardiometabòliques.

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ABBREVIATIONS

AAS, amino acids
ATP, Adult Treatment Panel
-C, cholesterol
BCAA, branched chain amino acids
BCFAs, branched chain fatty acids
BMI, body mass index
CI, confidence interval
CRC, colorectal cancer
CVD, cardiovascular disease
DBP, diastolic blood pressure
DMM, Dirichlet Multinomial Mixtures
EDTA, ethylenediaminetetraacetic acid
ELISA, enzyme-linked immunosorbent assay commercial kits
EPIC, European Prospective Investigation into Cancer and Nutrition Study
EPIRDEM, Effect of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus
FAs, fatty acids
FAMEs, fatty acid methyl esters
FFQ, food frequency questionnaire
FI, fasting insulin
FMT, fecal microbiota transplant
FOS, fructooligosaccharide
FPG, fasting plasma glucose
GC-MS, gas chromatography-mass spectrometry
GI, gastrointestinal
GLP-1, glucagon-like peptide-1
GOS, galactooligosaccharides
HDL, high-density lipoprotein
HFMDB, Human Fecal Metabolome Database
HMP, Human Microbiome Project
HOMA-IR, homeostatic model assessment of insulin resistance
HPFS, Health Professionals Follow-up Study
IBM, inflammatory bowel disorde
MCFAs, medium-chain fatty acids
MetaHIT, European Metagenomics of the Human Intestinal Tract
MetS, metabolic syndrome
IFG, impaired fasting glucose
IGT, impaired glucose tolerance
IR, insulin resistance
LC-MS, liquid chromatography-mass spectrometry
LDA, linear discriminant analysis
LDL, low-density lipoprotein
LPC, lysophosphocholine
LPE, lysophosphoethanolamine
LPS, lipopolysaccharide
MESA, Multi-Ethnic Study of Atherosclerosis Study
MedDiet, Mediterranean Diet
MDRD, Modification of Diet in Renal Disease
MU, monomeric units
MUFA, monounsaturated fatty acid
MWAS, metabolome-wide associations

NCEP, National Cholesterol Education Program
NHANES, National Health and Examination Nutrition Survey
NHS, Nurses' Health Study
NMR, nuclear magnetic resonance
OTU, Operational Taxonomical Unit
PAGln, phenylacetylglutamine
PBS, phosphate-saline buffered
PC, phosphatidylcholine
PLCO, Prostate, Lung, Colorectal and Ovarian
PREDIMED, PREvención con Dieta MEDiterránea Study
PUFA, polyunsaturated fatty acids
RESMENA, Metabolic Syndrome Reduction in Navarra
SBP, systolic blood pressure
SCFAs, short chain fatty acids
SFA, saturated fatty acids
SGCCA, Sparse Generalized Canonical Correlation Analysis
SIM, Selected Ion Monitoring mode
TG, triglyceride
TD1, type 1 diabetes
T2D, type 2 diabetes
TLR, toll-like receptor
TMA, trimethylamine
TMAO, trimethylamine N-oxide
TNF-α, tumor necrosis factor-α
UC, ulcerative colitis
VLDL, very low-density lipoprotein
WHO, World Health Organization
WMS, whole metagenome shotgun

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A. INTRODUCTION

1. GUT MICROBIOTA IN HUMAN HEALTH

In the last 15 years, gut microbiota received more and more attention from the scientific community and collective opinion thanks to the great number of studies that revealed its potential critical role in the vast majority of metabolic diseases.

Despite a major historical interest in gut microbes involved in infectious diseases, their potential relation with unsuspected diseases such as obesity, cancer, and neurodegenerative disorders started to be explored. From 2015 until now the number of publications on the gut microbiome reaches 26286 articles with a mean of almost 7 thousand articles per year in 2020. The advances in bioinformatics and the development of new emerging techniques in gut microbiota research allowed a better insight into both the composition and the functionality of the gut microbiome. Thus, with the term gut microbiota, we refer to the almost 2 kg of microorganisms that inhabit the gastrointestinal (GI) tract and co-evolved with the human ecosystem. Among these microorganisms, we could recognize bacteria, but also archaea, yeast, fungi, parasites, phages, and least but not last viruses, which are in 5:1 proportion with gut bacteria. At the same time, the evolution of *omic* sciences allowed us to explore the overall gut microbial ecology, from metagenomes to metaproteomes and metabolomes. The embracing of microbial interactions with either the host, the environment, or microbial communities itself depicts a very dynamic ecosystem which brings us to a more encompassing definition of the gut microbiome.

The cross-talk between human microbial ecosystems and the host has long been studied with regard to our immune system response[1]. Along with the classical monitoring activity of the innate immune system for

the presence of microbial pathogens, there is another important source of host-microbial interactions: the co-evolution of gut microbiota with human metabolism in the GI tract[2]. Indeed, there is stronger evidence of gut microbiome involvement in the regulation of energy homeostasis together with glucose and lipid homeostasis[3][4]. In healthy conditions, the intestine interaction with microbes is strictly monitored by a complex gut epithelial barrier made of tight junctions' proteins, a higher mucus layer thickness, and by the production of different antimicrobial signals from epithelial cells[5].

1.1. General overview of gut microbiota in human diseases

Certain genetic conditions, the influence of diet and lifestyle together with the intake of drugs trigger the loss of balance in the human metabolic state, which affects the integrity of the well-known epithelial barrier inducing a higher gut permeability thus affecting the cross-talk between the microbiome and the host[5].

The loss of the gut barrier integrity leads to a well-known phenomenon called gut dysbiosis, a condition in which the equilibrium of the symbiotic interaction between the host and microbiome is lost. In presence of gut dysbiosis, the higher concentrations of microbial toxins, together with the inactivation of antimicrobial signals and overstimulation of immune response[6],[7] cause a phenomenon called metabolic endotoxemia.

The concept of metabolic endotoxemia was firstly introduced in 2007 by Cani et al.[8], and it refers to the instauration of a low-grade inflammation caused by the loss of metabolic homeostasis and by the detrimental end products of microbial pathobionts metabolisms, which are enriched in presence of dysbiosis. The ability of the gut microbiome to face a stressful perturbation from the environment and to restore the

metabolic endotoxemia induced by gut dysbiosis defines the idea of a resilient microbiome[9]. Inversely, a poor resilient microbiome will worsen the established endotoxemia, thus causing the transition to a chronic disease condition.

Despite the absence of a clear compositional characterization of neither a healthy nor a resilient microbiome, it could be stated that microbial diversity is a key factor in order to achieve this state. Generally, a more diverse microbiome has more probabilities to shift into alternative stable states after a perturbation affected it, thus acquiring the notion of a resilient microbiome[10],[11].

The considerable high intra-individual variability in microbiota composition has been well demonstrated by multiple population studies but also by the high amount of information stored in the US NIH Human Microbiome Project (HMP) and the European Metagenomics of the Human Intestinal Tract project (MetaHIT). At the same time, the gut microbiome evolves along the lifetime of the individual, starting from a fluctuating microbiota in the early life until a more stable one in later life, which deteriorates with old age[12],[13]. In presence of such great variability and considering the complex microbial-host ecosystem, the possibility to stratify populations based on compositional patterns of the microbiome is a very attractive and promising strategy[14], as it could shed light to understand microbiome status in health and disease conditions.

Despite the lack of a reference microbiome, a huge number of studies have linked the gut microbiome with GI diseases and metabolic disorders but also more and more association studies have revealed its role in apparently unrelated diseases, such as neuropathological conditions, including Alzheimer disease[15], depression[16] and autism[17], by the well-

known gut-brain axis connection. Generally, a condition of gut dysbiosis between beneficial microbial communities and pathobionts is the common evidence in all the microbiome disease association studies[18]. The following instauration of metabolic endotoxemia together with the incapability to restore the optimal status of microbiome, therefore, leads and supports a chronic disease condition (obesity, type II diabetes, metabolic syndrome, etc).

1.2. Gut microbiota in obesity

The increasing worldwide incidence of obesity presents an unprecedented challenge to public health and economics, also in relation to the plethora of metabolic comorbidities that obesity entails, including type 2 diabetes (T2D), metabolic syndrome, cardiovascular disease (CVD), dementia and several types of cancer.

Obesity is considered a multifactorial disease characterized by an abnormal deviation from the normal energy steady-state that could potentially be treated by reducing energy intake and/or increasing energy expenditure. However, a complex architecture of coordinated mechanisms, including hypothalamic, limbic, and other central nervous systems is highly influencing that lost equilibrium in energy homeostasis. While heterogeneity in the regulation of host-derived mechanisms represents a potential contribution, the plasticity of the gut microbiome to environmental stressors suggests its key role in host metabolic regulation in obesity[19].

The presence of gut dysbiosis together with an altered microbiome function has been largely demonstrated in both animal and human clinical studies, where it has a direct role in the metabolic low-grade inflammation observed in obesity. Particularly, Cani et al. was the first

to elucidate the triggering role of the bacterial LPS in the onset of insulin resistance and therefore related metabolic diseases such as obesity and type 2 diabetes (T2D)[20]. However, there is still no consensus in research regarding its putative causative role in the pathogenesis of a multifactorial disease like obesity. Indeed, obesity was the first disease condition in which a chicken-or-egg situation arose in gut microbiota research regarding the potential causative role of gut microbiota in disease conditions. First findings of the causality link with obesity derived from microbiota transplant experiments in germ-free mice conducted by Gordon et al[21] demonstrated the microbiota translatability of an obese phenotype to lean donors. Further studies conducted by the same research group [22][23] revealed the important trigger of a high-fat and low-fiber diet as a key player in inducing obesity in germ-free mice via fecal microbiota transplant (FMT), suggesting a synergic successful effect between a low-fat-high fiber diet and a leanness microbiota transplant in obesity protection. The impact of distinct microbial communities in inducing obesity and metabolic diseases has been evaluated in different weight loss intervention studies in humans[24]-[26] and their shift has been associated with improvements in metabolic function. The first scientific observations on shifts in gut microbiota composition promoted the idea of an increased Firmicutes/Bacteroides ratio[27] as a discriminant clinical biomarker for obesity. However, further studies concluded that this evidence was contradictory and not reproducible for every study population[24],[28]. Despite the lack of a universal microbial fingerprint of gut microbiota on obesity, it has generally been recognized that a reduction in microbial richness could be predictive for weight gain, as a lower microbial richness has been observed in comparison to healthy people following a varied diet.

The importance of microbial richness in obesity was evaluated in an elegant study conducted by Le Chatelier et al.[29], in which the overall microbiome was analyzed. The taxonomical composition of gut microbiota was reproduced thanks to a bimodal distribution of bacterial genes based on their lower or higher counts and, surprisingly, the lean-obese stratification of the analyzed population only coincided with the gut microbiome variability for few bacterial species. At the same time, the identified bacterial species were acting as key players in discriminating those obese individuals characterized by an increased risk of progressing to adiposity-associated comorbidities.

Indeed, different nutritional interventions and observational studies in humans have identified potential probiotics with a protective effect on obesity, however, only for a few of them, a proof-of-concept strategy investigating their mechanistic role has been evaluated. That is the case of *Akkermansia muciniphila*, a mucin-degrading bacteria firstly isolated and characterized in 2004. It is now well accepted that supplementation with *A. muciniphila* confers cardiometabolic protection, as has been demonstrated in all the animal studies where *A. muciniphila* administration lowers body-weight and fat-mass loss, inflammation, cholesterol levels, atherosclerosis, and insulin sensitivity[30]. Notably, one of the potential mechanisms by which this bacteria acts is via specific compounds such as the protein Amuc_1100, whose immunomodulatory role has been recently identified both *in vitro* and *in vivo*[31].

Another important aspect to consider when studying the role of gut microbiota in obesity consists of its characteristic fecal metabolome. Different observational studies surprisingly observed twice the concentration of short-chain fatty acids (SCFAs) in obese individuals

compared to healthy ones[32]. SCFAs influence metabolic homeostasis by positively contributing to lipid, glucose, and cholesterol metabolisms (Figure 1). However, in obese subjects, it seems that the increased gut permeability caused by the disruption of the intestinal barrier along with a lower microbial diversity has been associated with higher concentrations of fecal SCFAs, which cannot exert their beneficial effects on energy metabolism.

1.3. Gut microbiota in Type 2 Diabetes

The rise in prevalence of obesity coincides with a higher prevalence of type 2 diabetes (T2D) which is one of the major risks for cardiovascular disease (CVD) in developing countries. In addition to what above mentioned about the great role of gut microbiota in the development of obesity, alterations in gut microbiota composition have also been observed in T2D patients, particularly linked to their impaired glucose metabolism and insulin resistance. Again, a decrease in overall microbial diversity along with the correlation between plasma levels of glucose and decreases in Firmicutes (particularly *Clostridium* species) and Proteobacteria have been observed. However, at first glance, the scientific literature about gut microbiota composition and T2D is fairly chaotic and concerns have been raised among its variability. Indeed, different taxa have been reported to be associated with T2D in different studies. Among the commonly reported findings, the genera of *Bifidobacterium, Bacteroides, Faecalibacterium, Akkermansia* and *Roseburia* were negatively associated with T2D, while the genera of *Ruminococcus, Fusobacterium*, and *Blautia* were positively associated with T2D. Consistently, different observational human case-controlstudies point out the protective role of *Faecalibaterium*

prausnitzii in T2D development[33]-[35] and few attempts have been made to propose it as a probiotic for metabolic disease.

The majority of studies investigating the potential causative role of gut microbiota in T2D are based on the analysis of gut microbiota composition in placebo-controlled trials in subjects treated with the most prescribed oral antidiabetic metformin. The ability to directly change gut microbiota composition is well known in the case of metformin and, surprisingly, FMT from metformin-treated patients to germ-free mice experienced an improvement in glucose tolerance compared to mice who received FMT from placebo-treated controls, suggesting that metformin-induced changes in gut microbiota composition represent a mediation mechanism of action of the drug in restore the energy homeostasis. These findings were further reinforced by confirming the metformin-induced effect on the increased abundance of the probiotic *A. muciniphila*[36]. The influence of metformin in analyzing gut microbiota composition encouraged researchers to re-analyze the compositional data from different human cohorts, taking into consideration the confounder effect of the antidiabetic medication. Results obtained from these studies revealed that subjects suffering from T2D were consistently characterized by a depletion of butyrate-producing taxa, which in turn causes a metabolic shift, partly alleviated by metformin-induced changes.

As we have already argued in previous section, here are multiple mechanisms that have been proposed to explain gut microbiota contribution to metabolic disease and T2D. Gut microbiota modulates low grade inflammation, interacting with dietary components, affecting gut permeability and overall glucose and insulin homeostasis. It has also been proposed that an impaired gut permeability could cause a

pancreatic ß-cells damage due to the increase of exogenous antigens, or either in relation to microbial toxins, as was demonstrated in case of type 1 diabetes (T21) development in mice[35]. In case of T2D, an impaired hepatic functionality has been related to microbial conversion of primary bile acids, mainly cholic acids, to secondary bile acids, like deoxycholic acid, by the action of bacteria belonging to *Clostridium* in the gut. Bile acids are involved in promoting glucose clearance and insulin sensitivity by activation of G-protein-coupled receptors 1 in enteroendocrine L-cells, inducing the release of glucagon-like peptide-1, which is correlated with improvements in hepatic and pancreatic function[35].

1.4. Gut microbiome in metabolic syndrome

The term "metabolic syndrome" (MetS) evolved from the very first findings of various metabolic risk factors associated with atherosclerosis in 1970, up to a generally accepted definition that emphasizes insulin resistance among the most distinguishable characteristics of MetS. The American Association for Clinical Endocrinology and the International Diabetes Federation gave two overlapping definitions of this syndrome, which include central obesity, dyslipidemia, insulin resistance, and hypertension, even if the cutoff points in these risk factors vary[37]. Therefore, the diagnostic criteria of MetS have been changing over the years, even if a consensus has been made by the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III)[38] regarding its classification. According to NCEP ATP III definition, three or more of the following clinical conditions are met in MetS:

1. waist circumference over 40 inches (men) or 35 inches (women)
2. blood pressure over 130/85 mmHg
3. fasting triglyceride (TG) level over 150 mg/dl

4. fasting high-density lipoprotein (HDL) cholesterol level less than 40 mg/dl (men) or 50 mg/dl (women)
5. fasting blood sugar over 100 mg/dl

The cluster of cardiometabolic risk factors included in MetS definition, predispose individuals to T2D and atherosclerotic vascular disease, determining common metabolic impairments involved in the pathogenesis of MetS, which is still unclear. Among these clinical shreds of evidence, another important role in MetS is played by the gut microbiome. Generally, the common presence of a low-grade inflammation together with a dysfunctional epithelial barrier in the gut is a well-recognized picture of an altered gut microbiome in metabolic diseases. Particularly, the first evidence focused its attention on the toxic effect of microbial metabolites, such as lipopolysaccharide (LPS) and trimethylamine (TMA) in the systemic circulation, as a consequence of the increased intestinal permeability. Different studies have shown that alterations of two phylotypes, Bacteroidetes spp. (*Bacteroides* and *Prevotella*) and Firmicutes spp. (*Clostridium, Lactobacillus,* and *Ruminococcus*) predominate in the pathogenesis of MetS[39],[40].

On the other side, the beneficial effects of microbial-derived SCFAs on metabolic diseases has been largely studied, for their ability to improve both dyslipidemia and glucose impairment.

SCFAs, mainly including propionate, butyrate and acetate have a particular affinity to G-protein-coupled receptors, namely FFAR2 and FFAR3, which are directly involved in promoting energy storage through regulation of satiety and intestinal motility directly in the gut, by promoting the secretion of glucacon-like peptide (GLP) and peptide YY (PYY) from intestinal L-cells[35]. However, contrasting results have been

observed about higher concentrations of SCFAs in metabolic diseases, like the study conducted by Perry et al., who found a higher production of acetate by gut microbiota could activate the parasympathetic nervous system, causing an increased glucose-stimulated insulin secretion, ghrelin secretion, hyperphagia and obesity[41] (Figure 1). Overall, these findings suggest that gut microbiota derived by-products, such SCFAs, interacts with the host in a highly context-dependent manner[32]. Recent observations in microbiome-targeted therapies with nutritional intervention, prebiotics or probiotics supplementations, and FMTs provide promising findings in the context of metabolic disease prevention. As matter of fact, gut microbiota transplantations from lean donors to subjects with metabolic syndrome have recently been shown to increase *Roseburia* and butyrate levels together with improved insulin sensitivity[42]. At the same time, dietary supplementation with fructooligosaccharides (FOS) and inulin was correlated with improved glucose tolerance, insulin resistance, and reduction in metabolic endotoxemia and proinflammatory cytokines, both in animals[43],[44] and in humans[45]. Thus, prebiotic supplementation is considered a potential preventive approach to metabolic disease management.

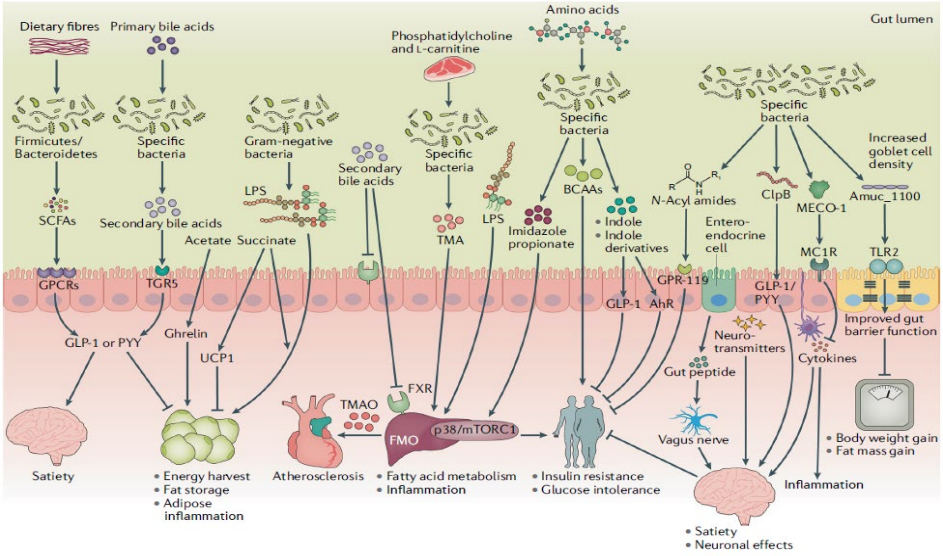

***Figure 1**: Schematic overview of the microbial messengers implicated in regulation of human metabolic homeostasis. Image taken from the Review of Fan and Pedersen*[46]

1.5. Is there any healthy gut microbiome?

The increasing interest in the role of the gut microbiome in the development of metabolic disturbances such as obesity, T2D, and metabolic syndrome is related to the introduction and refinement of next-generation sequencing techniques combined with an exponential increase of bioinformatics competencies in analyzing microbiome data. Nonetheless, the vast majority of studies exploring the causal or correlative interaction between the gut microbiome and metabolic disorders are based on associational attempts made on compositional data[47].

Despite the greater advances in computational technologies facilitating metagenome analysis, all the disease-associations studies conducted were not able to identify microbiome-based diagnostic or therapeutic

markers for clinical practice. The apparent lack of reproducibility in gut microbiome findings made it challenging to find a one-direction interpretation of gut microbiota's role in disease development. In this context, the idea of defining the boundaries of gut ecosystem variation in healthy hosts resulted in a renewed effort to identify specific confounders to one hand and to phenotypically characterize the populations based on their microbiome ecology to the other hand[48].

In fact, over the last years, different attempts have been made in order to obtain a unique compositional characterization of a healthy microbiome, but without obtaining a definitive microbiome of reference[10]. In absence of a control reference, all the microbiome association studies lack a universal microbiota-target therapeutical approach in disease conditions. The high intra-individual variability in microbiota composition represents an added difficulty in this area of research: all healthy microbiomes are different from each other, even if specific disease microbiomes similarly behave.

At the very beginning of microbiome research, an attempt to simplify the complex variability of gut microbiota in the overall population has been made, thanks to microbial enterotypes classification. This latter concept had been later abandoned and considered as a reductionist view of microbiome complexity. However, the new emerging analytical modeling approaches together with the increasing accumulation of scientific evidence and information conducted the researchers to a reformulated revival of the enterotypes concept[14].

In general, enterotypes have been described as more densely populated areas in a multidimensional space of microbial communities' ecosystem, a concept based on the conservative and reproducible clustering of a

limited number of prevalent community configurations in microbiome space. The concept of community-type microbiome stratification has recently been re-proposed and confirmed by a robust modeling approach called Dirichlet Multinomial Mixtures (DMM) which described 4 prevalent enterotypes[49]. According to their dominant taxonomic identifiers, we could distinguish Ruminococcaceae (R), *Prevotella* (P) Bacteroides1 (B1), and Bacteroides2 (B2) enterotypes. The R enterotype, dominated by Firmicutes, is prevalent in hard stools displaying higher rates of proteolytic fermentation bacteria. The P enterotype, dominated by *Prevotella* is common in loose stools and agrarian diets. The B1 enterotype is the most common healthy microbiome of western diet populations with higher amounts of *Fecalibacterium* genus, while B2 is more common in inflammatory gut diseases.

Finally, the enterotyping concept, in its new meaning as biologically relevant community characterization, could be a helpful tool in order to categorize samples in reference to a population stratification rather than a universal health microbiome.

2. METABOLOMICS IN HUMAN HEALTH

Metabolomics is the last “omic” technology involved in the systemic comprehension analysis of all the small molecules (< 1500 Dalton) of a biological environment, like a cell, a tissue, an organ or an organism. Thus, the metabolomic profile globally represents the final end-products of the host metabolism and his health status.

Metabolomics is based on high-throughput technologies of analysis, adopting high performance instrumentation, like Nuclear Magnetic Resonance (NMR) or mass spectrometry (MS) in order to lead with an extreme molecular variability in metabolites. As happens for the rest of

omics, metabolomics determinations are low-expense, rapid and automated techniques, allowing the possibility to analyze several biological samples. Indeed, metabolomics studies, compared to genomics or proteomics alone, are more suitable to detect changes in a particular phenotype of a biological environment (cell, tissue, organ). Metabolomics could be useful for both characterization of all measurable molecules in a biological sample (untargeted metabolomics) as well as the identification and quantification of all the specific set of chemically characterized metabolites (targeted metabolomics).

In both cases, metabolomics is a useful analytical tool in order to 1) get an overall comprehension of the metabolic status in human health conditions 2) obtain a metabolomic profile distinguishing a particular disease phenotype 3) combine the previous know-how from case-control metabolomics analysis in personalized and metabolites-monitored treatment approaches.

2.1. Circulating metabolome in obesity

Whether the composition of gut microbiota gives us a picture of the relevant players of the host-microbiome interaction in disease conditions, analyzing the circulating metabolome could elucidate its mechanistic role in the host metabolism. Moreover, metabolomics is a useful analytical tool in order to define and measure the metabolic profile in overweight and obesity. Obtaining a circulating metabolic fingerprint of obesity represents one of the most ambitious goals in precision medicine, as it could be a useful tool in both diagnosis, prognosis, and treatment strategies. As matter of fact, it has been long recognized that obesity comprises metabolically healthy or unhealthy obese subjects, with increased blood pressure, hyperlipidemia, hyperglycemia, hyperuricemia, and increased peripheral insulin

resistance reported in the second category[50],[51] Metabolomics techniques allow both qualitative identification and quantitative measurement of metabolites, thanks to the combination of a targeted and an untargeted approach of analysis[52]. The introduction of untargeted metabolomics strategy allows the simultaneous profiling of thousands of small molecules, in absence of a reliable standard reference.

Over the last decades, an increasing number of studies both in animals and humans have explored the metabolic fingerprint of obesity, though the vast majority of obtained results are based on targeted approaches of analysis[53]. Elevated levels of branched-chain amino acids (BCAAs) like leucine, isoleucine, and valine, together with aromatic amino acids (AAAs), like phenylalanine, tyrosine, tryptophan, and methionine in plasma levels have been detected in subjects with obesity and frequently associated with insulin resistance[54],[55]. Also, elevated plasma levels of phosphatidylcholines (PCs) species were found in morbidly or moderately obese men and women in an NMR analysis conducted by Stroeve et al.[55], while serum concentrations of lysophospholipids based on both choline and ethanolamine (LPCs or LPEs) seem to be altered in obesity, even if the direction of their correlation with BMI, weight, or waist circumference is inconsistent among studies[56]. The levels of nitrogen compounds like nucleotides, nucleosides, and their derivatives such as uridine and uric acid seem to reflect the degree of insulin resistance in obese subjects[57],[58]. At the same time, the circulating lipid profile of obese subjects is characterized by higher levels of certain fatty acids (FAs) like palmitic, palmitoleic, stearic and oleic acids. Moreover, the plasma levels of different TCA compounds seem to be altered in obesity, like higher plasma concentrations of glucose, fructose, mannose, xylose, gluconic acid, glucuronic acid, glycerol and lactate[57]-[59]. In this complex

scenario, the most relevant metabolite of certain microbial origin is represented by the trimethylamine N-oxide (TMAO), a recognized biomarker for cardiometabolic diseases, including obesity[60]. TMAO is obtained from the oxidation of TMA, a derivative of the microbial metabolism of dietary carnitine and choline, which is decreased in weight loss conditions. Right now, TMAO is a well-established microbial biomarker in blood of cardiovascular disease risk, as many studies confirmed[60]-[62].

Other microbial derivative metabolites have been recently proposed in the last years as putative biomarkers of cardiometabolic risk factors in blood and that is the case of the phenylacetylglutamine (PAGln), recently discovered in an elegant study conducted by Nemet et al[63], where a coupled *in vitro* and *in vivo* study in mice, demonstrated a direct action of PAGln on adrenergic receptors involved in thrombosis.

Furthermore, a better comprehension of the metabolomics fingerprint in obesity derives from dietary intervention studies. The effect of specific weight-loss interventions, could also be explained from a metabolomic point of view, always in the perspective of a future precision nutrition program. The beneficial effects of specific dietary interventions on the circulating metabolome allow to identify the key metabolic pathways involved in obesity and also to establish the potential dietary confounders in metabolomics analysis. For instance, the plasma lipids profile in obesity should be considered in reference to the ratio intake of polyunsaturated fatty acids (PUFAs) versus saturated fatty acids (SFAs) as it has been discussed also by Kim et al[64]. The importance of metabolomics in the dietary management of obesity has been highlighted by Stroeve et al[55], who demonstrated that the 57% of the variation in weight-loss success is predicted by baseline metabolic parameters, with

a focus respectively on plasma lipid species (particularly sphingomyelins and phosphatidylcholines) and certain amino acids (AAs) for either males or females with metabolic obesity. The comparative analysis[65] of two weight-loss intervention studies, the POUND LOST and the DIRECT studies, validated the correlations between decreased levels of certain AAs (leucine, isoleucine, and valine) as well as aromatic AAs (tyrosine and phenylalanine) with weight loss. At the same time, higher levels of glycine and linoleic acid were moderately but consistently associated with higher postprandial satiety in two different appetite assessments in overweight and obese subjects in SATIN study[66]. An altered serum profile including increased levels of FAs, AAs, and carboxylic acids is also characteristic of obese subjects after consuming a very-low carbohydrate diet[67].

2.2. Circulating metabolome in MetS

The MetS is a common pathophysiology state in different cardiometabolic disorders and is strictly associated with cardiovascular disease development. Despite the high incidence of MetS and its relation to high-incident chronic diseases, knowledge on its pathogenesis, treatment, and prevention still remains limited. The development of advanced metabolomics methods with their promising attempt to explore novel biomarkers and therapeutic targets has encouraged researchers to study the metabolomics of MetS[68].

In a Chinese cohort with MetS subjects, a different metabolic fingerprint was identified between treated (hypoglycemic medication or therapeutic lifestyle changes) and non-treated patients, with a worse pathophysiologic state in the seconds[69]. Like in obesity, serum levels of BCAA are enriched and, simultaneously, it has been reported a consequent decrease in expression of BCAA catabolic enzymes in MetS

subjects. At the same time, the circulating lipid profile of these patients is altered, according to observations in obesity. Another metabolic pathway that seems to be upregulated in MetS is the ketogenic pathway, which serves as an alternative source of energy during food deprivation. These findings were also confirmed by a Finnish cohort of subjects with MetS. Indeed, in the METSIM cohort were also found higher plasma levels of TMAO, which was also associated with carnitine, valine, acetate, and unsaturated omega-3 fatty acids levels[70]. Surprisingly, an inverse correlation was found between TMAO concentrations and abundances of *F. prausnitzii*, whose protective role in MetS has been previously explored.

A metabolomics approach on plasma samples has also been conducted on the randomized controlled clinical trial PREDIMED, a feeding clinical trial conducted on elderly subjects at cardiovascular risk. In this study, the analysis of circulating metabolites in a targeted approach revealed a specific fingerprint depicting both the adherence to Mediterranean diet intervention and its beneficial effects on CVD risk have been demonstrated. Similar to other studies, plasma lipids, acylcarnitines, amino acids and vitamins were among the 67 selected metabolites in the metabolic signature of MedDiet, which was subsequently associated to CVD risk changes and validated in 6868 participants from the US Nurses' Health Studies I and II, and Health Professionals Follow-up Study (NHS/HPFS) cohorts[71]. Similarly, a multimetabolite score obtained from a sub-cohort of PREDIMED was also associated with insulin resistance and T2D incidence in the same population[72].

2.3. The role of fecal metabolome

If circulating metabolomics could give us an idea of what is going on at a systemic level in metabolic disease conditions, another important

source of mechanistic understanding derives from the fecal metabolites, which reproduces the gut microbial metabolism and therefore, its potential interaction with host metabolism[73].

Fecal metabolomics has only recently been improved and adopted by researchers as a powerful tool for studying gut microbiome ecology, thus here we face the same analytical problems of reproducibility and lack of a gold standard healthy reference of the fecal metabolome. Moreover, due to the compliances with sample recollection, the vast majority of studies analyzing fecal metabolome have been conducted in animals. Currently, a comprehensive fecal metabolome could be obtained by the combination of liquid and gas chromatography–mass spectrometry (LC-MS, GC-MS) and nuclear magnetic resonance (NMR) technologies, with the first in a targeted analytical approach and the second ones in an untargeted analysis of metabolites. The majority of metabolites produced by gut microbiota, thus found in fecal samples include SCFAs, branched chain fatty acids (BCFAs), BCAAs, biogenic amines, secondary bile acids and gases like CO_2 and CH_4. In addition, various other metabolites in gut are derived from plant secondary metabolites, dietary choline, bacterial components, polyamine and volatile compounds. These metabolites are partially absorbed, distributed or excreted by a plethora of metabolic pathways which describes a very dynamic ecosystem. Given the high heterogeneity of these metabolites, many efforts have been done in order to develop a comprehensive technology that could allow the identification of polar, non-polar and volatile compounds.

Following an elegant systematic review of 97 studies on human fecal metabolomics along with the integration of another critical review based on volatile compounds in fecal samples, a total of 6738 metabolites has

been incorporated in the Human Fecal Metabolome Database (HFMDB)[74]. The HFMDB is integrated into the Human Metabolome Database and consists on a freely available electronic database containing detailed information about many metabolites, as well as hyperlinks to other databases, like KEGG, PubChem, ChEBI or UniProt among others, which provide both molecular/biochemistry information data and clinical shreds of evidence.

Among the most representative fecal metabolites, there are recognized ones with gut microbial origin. As mentioned before, SCFAs are saturated aliphatic organic acids that consist of one to six carbons, including acetate, propionate, butyrate and valerate which are the most abundant ones and are mainly produced by the fermentation of undigested carbohydrates from certain gut bacteria. However, even if their content is particularly high in fecal samples, they only represent 5% of the SCFAs produced in intestinal lumen, where they are rapidly absorbed by colonocytes. Their involvement in guaranteeing metabolic homeostasis is pretty known[75], even if higher fecal concentrations of SCFAs have been also observed in obesity and associated to higher cardiometabolic disease risk, probably due to their intestinal malabsorption[32].

Also, the secondary bile acids in fecal samples are directly derived from microbial conjugation of primary bile acids in the gut. While some of these bile acids (i.e chenodeoxycholic acid and cholic acid) have been inversely associated with insulin resistance, other are considered toxic, like for example deoxycholic acid in obesity. Gut microbial metabolites also include medium-chain fatty acids (MCFAs), alcohols, amino acids and derivatives, phenols and polyphenol derivatives, and toxic compound such indoles and sulfites.

In the context of clinical practice, fecal metabolomics is considered as a powerful tool for biomarker detection in different disease conditions[76]. It is worth to mention that the complex matrix of fecal material, thus its harder management, has a direct consequence on the susceptibility to cohort effects, interindividual variability and difference in analytical procedures, as mentioned above. Therefore, the associational studies conducted up to now, are characterized by the rather impossibility to find a common metabolic trend among different clinical trials based on specific diseases.

Not surprisingly, most of the clinical trials which investigated fecal metabolomics, have been focused on gastrointestinal diseases, like Chron's disease, inflammatory bowel disorder (IBM), ulcerative colitis (UC) and colorectal cancer (CRC). Particularly, a strong effort has been made for the last one, with the identification of novel fecal metabolites of microbial origin potentially associated with worsen CRC prognosis, like for example the microbial genotoxin colibactin produced by *Escherichia coli-pKs*[77].

3. EFFECT OF NUTRITION ON GUT MICROBIOTA AND METABOLOME

3.1. Diet and gut microbiota composition

Diet is thought to influence gut microbiota composition for at least 20% of its variation in humans[78]. Dietary habits act like an environmental stressor to gut microbiome ecology, thus contributing to both health and disease conditions, including metabolic disorders, but also neuro-behavioral traits. However, the impact of short-to-medium dietary interventions on gut microbiota composition has only a transient effect, because of its resilience to a stable-state microbial profile[79]. Therefore, long-term interventions are thought to have a permanent influence on

the gut microbial ecosystem, even if a consensus about its duration has not been reached yet. Also, an increasing number of studies are pointing out the importance of feeding time and rhythmicity in shaping gut microbiota communities.

The effect of acute dietary exposures has long been studied in humans, demonstrating a higher microbial fluctuation with the most extreme diets. However, these observed changes could maybe refer to the stronger effect of diet on the intestinal transit time rather than a real gut microbiome shaping, as recently discussed by Falony et al[48]. Indeed, a high fat/low fiber diet is known to induce an obesogenic gut microbiota in mice, which could at the same time be reverted after FMT in germ-free mice, adopting a low fat/high fiber diet.

The fiber content, amount and type seem to have a pivotal role in microbiota composition. The beneficial effect of dietary fiber on gut microbiome homeostasis has been elegantly evaluated into the mark of MyNewGut European project[80]. The prebiotic role of galactooligosaccharides (GOS), FOS including inulin-type fructans, and arabinoxylans and arabinoxylo-oligosaccharides (AX and AXOS) have been evaluated in reference to gut microbial saccharolytic fermentation processes leading to increased fecal abundances of probiotic species of the genera *Bifidobacterium* and *Lactobacillus* as well as higher production of SCFAs, like acetate, propionate and butyrate. Despite most of the intervention studies conducted were based on supplementation of these chemical compounds, what is most relevant in the clinical practice is the impact of fiber intake contained in specific food groups and in the context of specific dietary patterns. In this context, an increasing number of intervention clinical trials, have analyzed the impact of specific dietary styles in gut microbiota composition and function. For instance, Ayeni et al.[81], assessed the human gut microbiome variation

as a consequence of either an agrarian diet rich in fiber and Western or Westernized dietary patterns, typically represented by an animal-fat-based diet, with a lower content in dietary fibers. The investigation of the fecal microbiome and metabolome of healthy individuals in rural versus urban areas in Nigeria not only confirmed the loss of the specific microbiome trait of agrarian populations with urbanization but also confirmed these data in worldwide populations with the same rural-urbane stratification of individuals. Particularly, rural microbial communities were depleted in genera members of Lachnospiraceae and Ruminococcaceae families, such *Blautia, Lachnospira, Faecalibacterium* and *Oscillospira*, which are typical commensals of a Western gut, together with Bacteroides and Bifidobacterium. At the same time, Spirochaetes and Fusobacteria were enriched in rural microbial communities. Based on the scientific evidence derived from epidemiologic studies, a higher intake of dietary fibers has been positively associated with improvements in body weight, adiposity, glucose or lipid metabolism, with a potential protective effect against obesity and overweight. Despite the potential mediator effect of gut microbiota in these beneficial effects on health, a clear explanation of the biological mechanisms that hide behind these associations remains undefined. Nonetheless, few intervention studies in humans have observed the involvement of the enteroendocrine system at the intestinal level, with particular reference to the production of hormones like PYY and GLP-1 involved in the regulation of appetite and satiety, hence in glucose homeostasis[82].

Dietary fibers are not the only substrates for gut microbiome activity, such as other macro and micronutrients[83],[84]. An elegant review on the influence of high-protein diets on the gut microbiome revealed that their proteolytic fermentation is involved in the production of microbial

metabolites, with a different health outcome, depending on the source of protein (vegetal or animal sources)[85]. Indeed, protein catabolism in the gut is generally characterized by a negative connotation, in relation to some toxic derivative compounds, including amines, indoles, phenols and sulfurous compounds. However, not all the amino acids metabolized by gut microbiota consist of toxic products, as among the end-products of proteolytic fermentation there are also SCFAs. Moreover, to some extent, the host metabolism is able to monitories the ammonia concentration deriving from proteolytic fermentation in the gut. The bacterial metabolites from amino acids also include neurotransmitters and their precursors, which could interact with both the enteric and central nervous systems, like for example serotonin, which not only modulate gastrointestinal motility but is also involved in neuronal circuits of cognition, mood, appetite, among others.

At the same time dietary fats, depending on their amount and source, are pretty known for their modulating role in gut microbiota composition and function. A western diet rich in saturated fats is more likely to induce gut microbial dysbiosis, directly associated with metabolic disorders[86]. This effect is partly due to the contemporary lower consumption of other macronutrients, like carbohydrates, but also because of their direct effect on the secretion and composition of bile acids. Primary bile acids are released in the small intestine and converted into secondary bile acids by gut microbiota in the colon and their beneficial impact on glucose metabolism and insulin resistance has been demonstrated in animal models and humans[87]. Nonetheless, high PUFA diets do not seem to exert the same negative impact on gut microbiome homeostasis as the high intake of MUFA and saturated fatty acids.

Therefore, even if analyzing the individual impact of macro and micronutrients from food on gut microbial ecosystem could provide a better comprehension about the biological mechanisms of interaction with the host metabolism, a bigger effort is now addressed to intervention clinical trials based on the global dietary patterns and lifestyle. Among the most studied dietary patterns in relation to gut microbiome mediation in beneficial cardiometabolic health effects is the Mediterranean diet (MedDiet). Higher adherence to MedDiet has been associated with increased abundances of microbial SCFAs producers, like *Prevotella* and some fiber-degrading Firmicutes, as well as higher fecal concentrations of acetate, propionate and butyrate in an observational study conducted on an Italian cohort[88]. Parallelly, a higher MedDiet adherence was associated to enriched abundances of potential probiotic genera like *Faecalibacterium prausnitzii*, *Eubacterium* and *Roseburia* in an intervention clinical trial conducted in elderly individuals across European countries[89]. Along with MedDiet other dietary patterns have been evaluated in reference to gut microbiota fluctuations, like vegetarian or vegan diets[90],[91] Overall, plant-based diets are characterized by higher sources of fiber, thus have largely been associated with enrichment in fiber-degrading bacteria *like Ruminococcus, E. rectale* and *Roseburia*, as well as a reduction in pathobionts *Clostridium* and *Enterococcus* species.

3.2. Nutrients, food and dietary patterns on circulating metabolome

Metabolomics is rapidly maturing in nutrition, with the perspective to use it as a supportive integration tool in complex biology system research. The possibility to switch from population-based to individual-based criteria for nutrition management would allow the introduction of the concept of personalized nutrition. The development of these new

methodologic approaches, concerning the exposome, predictive health and complex pathobiology, emphasizes the central role of nutrition in integrated biosystem models of health and disease. The implementation of improved analytical tools, either with targeted or no-targeted approach, as well as bioinformatics, pathway analysis and computational methods made these technologies useful in the context of metabolome-wide association studies (MWAS) and provide a foundation for nutritional metabolomics.

Indeed, nutritional epidemiologic studies are mainly based on the use of less reliable self-reported dietary assessment methods, that are subjected to recall bias and measurement error and, at the same time, they lack objective biomarkers of all food and nutrients. On the contrary, metabolomics depends on objective measurements of metabolites that specifically relate to intake of certain nutrients and/or food and also take into account the biochemical variability among individuals, thus could be considered as a loyal reproduction of the "true exposure" to individual nutrients, food and overall diet[92].

Importantly, diet can have a dual effect on metabolome, one is the host metabolome involved in biochemical mechanisms, and the other is the food metabolome, which is characterized not only by all the derivative metabolites of the macro and micronutrients ingested but also involved all those non-nutrients compounds like pesticides and all the cooking derived compounds, which also have a biologic impact on the host-metabolism.

Generally, in nutritional metabolomics there is the dual possibility to: a) individually evaluate the metabolomic fingerprinting of the consumption of a single food in a population by using intervention studies with short-

to-medium intervention period, where strict control of dietary components could be achieved; b) obtain multimetabolite biomarker panels in relation to specific dietary patterns in observational studies with multiple measurements over time.

Regarding serum metabolomics, results obtained from different observational studies conducted on large cohorts were consistent in the identification of multiple panels of metabolites related to food groups. Validated observations identified stachydrine as a biomarker of citrus together with scyllo-and-chiro-inositol. Citrus intake was also inversely related to carnosine and arginine in another observational study, presumably reflecting the lower consumption of red meat in higher citrus consumers[93]. At the same time, alcohol consumption was related to different lipid species. Some of the strongest correlations have been observed in relation to coffee components or their downstream metabolites. Apart from these correlations, novel dietary biomarkers were identified in an elegant study conducted in a cross-sectional analysis in 502 subjects from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial, like the serum metabolites 4-vinylphenol sulfate and tryptophan beta, which were both associated to peanut consumption[94]. Again, in PLCO study, sweets consumption was positively related to phosphatidylethanolamines, whose acid moieties are mainly contained in animal fats. At the same time, the intake of eggs was positively associated with phosphatidylcholine PC38:4, while red meat has been consistently associated with phosphatidylethanolamines and phosphatidylcholines and, more specifically, chicken with 1-methylhistidine. In the mark of the metabolomics profiling of specific macronutrient intake, researchers have identified a serum metabolomic fingerprint of dietary protein intake in the Modification of Diet in Renal Disease (MDRD) Study, which provided further validity for established

biomarkers of protein intake, such as creatinine, TMAO and essential amino acids and also provided new protein biomarkers related to protein metabolism such as kynurenate and xanthurenate, which are related to tryptophan metabolic pathway[95].

However, even if exploring the dietary biomarkers associated with single nutrient or food intake, the consideration of the overall dietary pattern could provide the complexity of consumed foods, revealing the high interconnection between metabolites associated with matrices of dietary exposure. Several observational studies have evaluated the effect of specific dietary patterns on single serum metabolites, but less have explored their impact on metabolite patterns. A cross-sectional investigation on German adults of the European Prospective Investigation into Cancer and Nutrition (EPIC) Post Dam cohort identified three dietary patterns associated with respective specific serum metabolic patterns. Particularly, greater intake of bread, margarine and processed red meat was associated with higher values of amino acids, while greater intake of fruits and vegetables, together with vegetable oils, was associated to a fatty acids metabolic profile and lower levels of amino acids. Finally, the intake of tea and a miscellaneous made of yeast, spices, herbs and flavorings, condiments, soya products, dietetic products, and artificial sweeteners, was associated with lower levels of amino acids and their derivatives[96]. Different concentrations in 79% of analyzed metabolites allowed to clearly discriminate meat eaters, fish eaters, vegetarians and vegans in the Oxford Epic cohort[97]. A particular emphasis was made on the amounts of acylcarnitines and glycerophospholipids (higher in the meat-eaters group) as well as biogenic amines and amino acids (such leucine, valine, lysine, methionine, tryptophan and tyrosine) which were higher in fish eaters.

At the same, time different studies evaluated the correlation among specific dietary scores and the metabolomic profiling, such as the Healthy Eating Index, the Mediterranean Diet Score, the WHO Healthy Dietary Indicators and Baltic Sea Diet score. In the study conducted by Playdon et al.[98], there was a consistency between the metabolomic fingerprints associated to the different healthy scores, with the lysolipids pathway representing the most relevant metabolite class associated with diet quality. The effect of the Mediterranean diet on circulating metabolomics was evaluated in a plasma fingerprint in RESMENA study[99] and in a plasma lipidomic analysis in the PREDIMED trial[100]. In both studies the most relevant metabolites associated with nutritional intervention were phospholipids and lysophospholipids.

3.3. Nutrients, food and dietary patterns on fecal metabolome

Despite the development of advanced analytical tools for metabolomics analysis, feces remain merely an unexplored biologic specimen. Fecal metabolomics studies are far fewer than metabolomics studies on other biofluids, such as blood, urine or cerebrospinal fluid. In part, the reason resides in the very complex and heterogenous matrix of stool, whose solid fraction is characterized by organic material in a fraction from 84 to 93% and a remaining 25-54% constituted by microbial biomass, represented in both living and dead form. However, feces also contain large and small molecules produced as a result of food consumption, digestion, intestinal absorption and microbial fermentation. Indeed, human gut microbiota can directly deliver compounds from their metabolome, which are absorbed and that contribute to the host metabolome (such as amino acids, bile acids, short-chain fatty acids, vitamins and energy substrates identified in the circulating metabolome). At the same time, the gut microbiota can change food

components and make them bioavailable for either themselves or for the host. Thus, studying fecal metabolome could provide this additional information regarding dietary biomarkers strictly related to gut microbiota metabolism, as well as giving information about the nutrient's absorption in the intestine. Up to now, 6736 fecal metabolites have been described, comprising 5.9% of the total number of characterized metabolites. Their study is extremely important in relation to their function as non-invasive diagnostic biomarkers since they specifically represent a real-time picture of intestinal processes and could be used in diagnosis/prognosis and prevention of different disease conditions.

Fecal metabolomics is indeed a promising tool for the identification and monitoring of the prebiotic effect of certain dietary components, by measuring those recognized microbiota-derived metabolites which have a recognized role host metabolism and energy homeostasis. One of the most studied dietary components influencing gut microbiota metabolism, is the dietary fiber. Despite the controversy around its definition, the Codex Alimentarius in 2008 uniformly coincided in attributing to this name all the carbohydrate polymers with three or more monomeric units (MUs) which are neither digested nor absorbed in the human intestine. Therefore, non-starch polysaccharides from fruits, vegetables, cereals and tubers, as well as resistant oligosaccharides and resistant starch all belongs to dietary fiber which are all potentially fermentable by gut microbiota. The fermentation of dietary fiber, generally called saccharolytic fermentation, has been attributed to the production of different

classes of metabolites in the intestinal lumen[80]. Among them, SCFAs are the most relevant for their crucial role in promoting energy homeostasis of the host. However, contrasting results regarding their beneficial role on metabolic homeostasis are derived from observational studies in obese populations[32], such their higher concentrations in feces are presumably reflecting the intestinal inability to absorb and use them as a source of energy. As consequence, SCFAs are maybe a class of metabolites whose concentration in feces or plasma could also depict and monitor different metabolic processes in the host (Figure 2). Beside the SCFA, another recognized class of microbial-derived metabolites is represented by BCFAs deriving from the proteolytic fermentation of dietary proteins by gut microbiota. BCFA are produced from the microbial degradation of BCAAs and AAAs, which also contributes to the release of toxic compounds such p-cresol and indole into the gut lumen[85]. A reduction in fecal concentration of BCFAs is then considered beneficial for gut health[101], as their production is concomitant with the synthesis of detrimental metabolites for the intestinal epithelium[102].

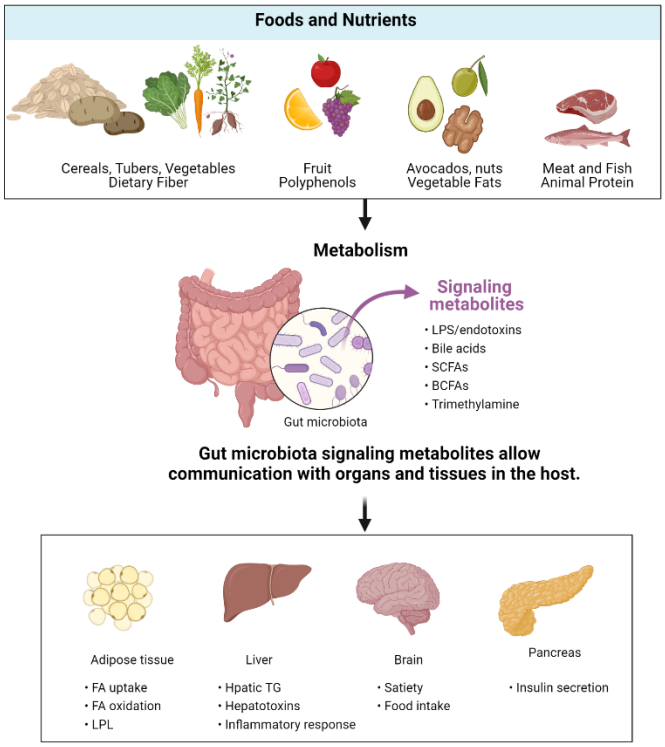

***Figure 2**: Schematic representation of the diet contribution to the metabolism of gut microbiota and its communication with organs and tissues of the host by signaling molecules*

Among the large number of diet-derived metabolites which are metabolized by gut microbiota, there are aglycons, daidzen, genistein and glycetein. Also, it is well known the gut microbiota's ability to metabolize polyphenols to phenolic compounds such as benzoate and also derivatives of hydroxyphenylacetic and hydropropionic acids, which were also measured in fecal samples of subjects undergoing a high-soy diet in healthy adults[100]. The role of fecal phenolic compounds derived

from gut microbial fermentation of dietary polyphenols, particularly high in the Mediterranean diet, has been evaluated in a Spanish cohort by Gutierrez-Diaz et al[103], which also identified another group of phenolic compounds, including phenyl acetic acid, mostly derived from the intestinal fermentation of aromatic amino acids, like phenylalanine, presumably associated with a pro-inflammatory status related to an unhealthy lifestyle and obesity.

Fecal bile acids are commonly introduced as an integrative metabolomics analysis in nutritional intervention studies where the role of gut microbiota is evaluated as a primary outcome. As we mentioned before, the gut microbiota has a central role in the enzymatic conjugation of primary to secondary bile acids in the colon, thus their measurement as potential biomarkers of intestinal bile acid absorption are particularly relevant.

Within this framework, the impact of a restricted caloric intervention on both plasma and fecal metabolites in a study conducted on post-menopausal women confirmed the interaction of gastrointestinal bile acids, especially increased lithocholic acid and reduced levels of deoxycholic acid, with specific plasma metabolic pathways involved in the metabolic homeostasis and with the adipose tissue gene expression[104].

3.4. The importance of multi-omics in nutrition and human health

Nutrition is one the most powerful tools available for influencing both metabolic processes in the human body and the intestinal microbiome ecology. Thus, nutritional interventions represent a promising strategy in the personalized treatment of metabolic diseases. However, generalized nutritional interventions in the last decades have not been

able to decrease the incidence of chronic diseases[105]. Therefore, new personalized strategies, specifically targeting patient-specific molecular processes associated with improvements in repristinate the individual metabolic homeostasis, are the new strategies of precision nutrition. For this reason, the nutritional approach should take into account the influence of different environmental factors together with the implementation of a multi-omics approach in order to have a global vision of the individual health status, by analyzing from one side the host genome, transcriptome, proteome and metabolome and from the other side, the meta-omics world of its gut microbiota[106]. Starting from the accomplishment of the Human Genome Project, a cumulative number of associational studies have attempted to define the genetic factors that could explain the inter-individual variability of the metabolic response to specific diets. In this sense, even if different genes and polymorphisms have been confirmed as relevant factors for the heterogenous response to nutrient intake, the clinical evidence supporting these associations is still too weak to be incorporated into personalized nutritional interventions. As consequence, precision nutrition could not only rely on nutrigenetics but has to take advantage of the interplay between all the other omics, like metabolomics and metabonomics.

As happened for genomics, the enormous expansion of research on microbiome ecology is also mainly attributed to the development of advanced high-throughput functional genomics techniques. The newest advances in DNA sequencing have empowered worldwide researchers of a culture-independent tool to explore the microbial community composition. Metagenomics via shotgun sequencing is the preferential technique in order to have deeper taxonomic profiling of microbiomes, but this is not the only type of information we could obtain from this method. In fact, thanks to the whole metagenome shotgun (WMS),

functional profiling of the analyzed microbiome is also achieved. Nowadays, the term metagenomics addresses not only the genomes of microbes but also the environmental and host factors, thus better incorporating the concept of microbiome ecosystem in a dynamic point of view. Hence, modifications of the environment represent valuable information for the coevolution processes of microbiomes with the host organism. In order to have a better view of the microbiome interactions with the host, incorporating a multi-omics approach of analysis represent the new research frontier for microbiome studies.

In the first multi-omics approaches, metatranscriptomics and metaproteomics were the leading omic sciences introduced in the context of microbial ecology[107]. Thanks to their introduction, we moved from a predicted functional profile obtained with metagenomics to the real functional activity of microbiome ecology, analyzing its actual gene expression, either in the form of RNA transcripts or proteins. However, a global comprehension of the functional profile of the crosstalk host-microbiome could only be achieved with the integration of metabolomics sciences, which allow the measurement of the ending products of that cross-talk between the host and the gut microbiome (**Figure 3**).

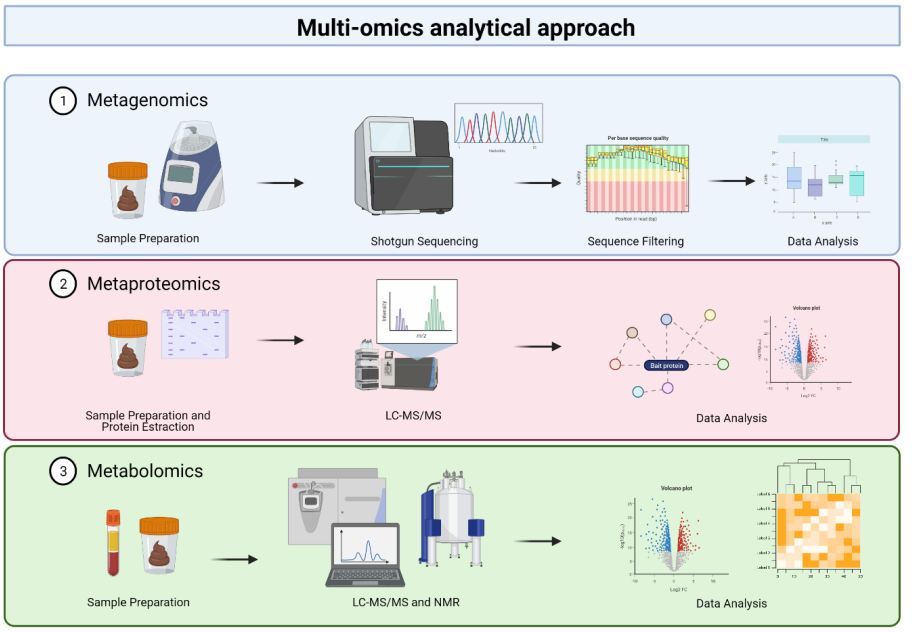

***Figure 3**: Schematic representation of a multi-omics approach including 1) a metagenomics analysis to get the taxonomical composition of gut microbiota form stool samples; 2) a metaproteomics approach of gut microbiota proteomics and 3) metabolomics analysis from plasma or fecal samples to get the metabolomics fingerprint of the host-microbial cross talk.*

Finally, the real challenge in a multi-omics approach is the possibility to integrate these different omics data, in order to obtain a global fingerprinting for precision nutrition. In this context, there are still few studies conducted in humans to evaluate the effect of a specific dietary intervention on a multi-omics profile, due to the real technical and financial challenges in developing such an analytical approach in large populations. Untargeted fecal and urine metabolomics approach together with a microbial taxonomic multi-omics analysis was adopted to grouping different dietary habits between Indian and Chinese

adults[108]. The interplay of these 3 omics datasets revealed that specific discriminating bacteria between the analyzed populations were associated with certain microbial metabolites identified both in fecal and urine samples, especially belonging to the amino acids class. Among the multi-omics studies conducted in animal models, Guirro et al.[109] confirmed some previous observations on the role of a high-fat diet in inducing an obesogenic phenotype with a distinct metaproteomics and metagenomics phenotype.

B. JUSTIFICATION

The relevance of gut microbiota ecology in human health has been extensively demonstrated in several epidemiologic studies and clinical trials. The presence of distinct bacterial communities is linked to specific chronic diseases, including obesity and metabolic syndrome. Clearly, the impact of diet in shaping gut microbiota composition and in modulating its cross-talk with the host is crucial. Although the mature gut microbiota is fairly resilient, it can be altered within individuals by an external stimulus like diet, influencing host physiology and diseases processes. When studied in isolation, different macro and micronutrients have revealed a potential prebiotic effect in regulation of obesity-related-complications. However, scientific evidences on their beneficial role on probiotic strains are still unclear and nutritional interventions with a holistic approach are needed to elucidate their role. In this regard, one obvious limitation to studying health effects of individual nutrients is that those are rarely consumed in isolation. Thus, experimental manipulation of an individual macronutrient or specific food invariability alters intake of other dietary components and foods that may have an interaction effect on gut microbiota. In this context, a regular intake of nuts, which are a nutrient-dense food, has been largely associated to improvements in cardiometabolic health. Also, because of their high content in fiber and polyphenols, their prebiotic effect started to be explored in different randomized clinical feeding studies. However, the potential contribution of other food groups and the overall dietary manipulation of other macronutrients intake in these studies, cannot provide a real picture of the impact of including nuts intake into daily individual dietary patterns. In fact, specific dietary patterns have been studied for their potential prebiotic effect, with a particular emphasis on Mediterranean diet, which typically includes nuts, among a variety of foods like fruits, vegetables, whole grains, legumes, olive oil and limited

red meat. Numerous epidemiologic studies and clinical trials have largely demonstrated the role of MedDiet in reducing the cardiometabolic risk factors and multiple related chronic diseases. Although only a few of these studies have evaluated the gut microbiota role in mediate these beneficial effects, existing data are promising towards the effect of MedDiet on favorable gut microbial profiles and metabolites production.

Therefore, the comparison of including a ·superfood· like nuts either into an unhealthy dietary pattern or in the context of Mediterranean diet could exert more information about the importance of the overall interaction between dietary components in the ability to shape gut microbiota composition and exert beneficial effects on cardiometabolic parameters. At the same time, adopting a multi-omics approach of analysis would allow a better comprehension of the microbial function and metabolic cross-talk with the health status of the host.

C. HYPOTHESIS

HYPOTHESIS

Following a Mediterranean Diet, compared to the consumption of a single healthy food in the context of a non-Mediterranean diet, will modulates gut microbiota composition and activity as a mechanism to improve metabolic risk markers associated with cardiometabolic disorders.

D. OBJECTIVES

OBJECTIVES

The main objective of this thesis was to evaluate the effects of a full dietary pattern (Medieterranean Diet) compared to a single healthy food (nuts) in the context of a non Medieterranean Diet, on changes in gut microbiota composition and function in individuals with obesity and metabolic syndrome.

In order to better analyze the specific role of gut microbiota in cardiometabolic health and its modulation by diet, we designed the following specific objectives:

1) To evaluate the effect of interventions on gut microbiota composition (16S rRNA) and function (fecal metabolomics) and its modulation of classic cardiometabolic risk factors
2) To evaluate the effects of interventions on plasma metabolomics profile and relate it with gut microbiota composition and cardiometabolic improvements
3) To evaluate the network of host-microbial interactions and thei potential involvement in cardiometabolic risk factors

E. METHODOLOGY

1. THE METADIET STUDY

METADIET study is a controlled randomized clinical trial with a crossover design for 2 dietary interventions based on either MedDiet or nuts supplementation for a period of 2 months each and with a wash-out period of 1 month between interventions.

1.1 Study Design

Eligible participants were 50 community-dwelling subjects aged between 25-60 years and with a body mass index (BMI) between 25-35 kg/m^2 and metabolic syndrome according to ATPIII diagnosis criteria following a non-MedDiet. Participants were randomized, using a random algorithm generator and instructed to either following a MedDiet or consuming 50 g/day of mixed nuts in the context of their regular non-MedDiet. Subjects were excluded if they meet the following criteria:

- Type II Diabetes (T2D)
- Secondary obesity or related diseases
- Non-controlled hypertension
- LDL-cholesterol>160 mg/dL
- Triglycerides levels<400 mg/dL
- MedDiet score≥9
- Regular intake of nuts (≥90 g/week)
- Several chronic diseases (inflammatory, infectious, chronic obstructive pulmonary, neoplasia, endocrine or hematological diseases)
- Leucocytes levels>11 (x10^3/μL)
- Specific pharmacological treatments (anti-inflammatory, corticoids, hormones or antibiotics)

- Changes in body weight (>5 kg in the last 3 months)
- Alcohol or drug abuse and consumption of prebiotics, probiotics or laxatives

Participants were recruited from primary care centers affiliated to the University Hospital of San Joan de Reus (Spain) and through public advertisements in different media. The Institutional Review Board of the University Hospital of San Joan de Reus (Spain) approved the study protocol which accomplishes the ethical standards of the Declaration of Helsinki. The trial was registered in the ISRCTN with identifier ISRCTN88780852, https://doi.org/10.1186/ISRCTN88780852.

1.2. Interventions

Once the participants were included in the study, they were randomly assigned to one of the two nutritional interventions for a period of 2 months. At baseline, sociodemographic information, lifestyle data, 3-day dietary records and anthropometric parameters together with biochemical analysis on fasting blood samples were recollected.

Participants in MedDiet were strictly followed with biweekly visits with nutritionists, in which they were instructed with written material containing daily and seasonal menus and recipes in order to accomplish their adherence to Mediterranean dietary pattern and MedDiet recommendations following the 17-item MedDiet score from PREDIMED Plus study. Particularly, dieticians emphasized the consumption of at least 2 servings of vegetables and 3 fruits per day; ≥3 servings of legumes, ≥5 servings of whole-grain cereals/pasta, ≥3 servings of fish/seafood per week and the use of extra virgin olive oil as the main culinary fat. They were also instructed to reduce the consumption of red meat and processed foods ≤1 serving/week, use of butter and margarines

(<1/week), white bread (≤1/day) and sugary beverages or sugar-sweetened fruit juices (<1/week).

On the contrary, participants following nuts supplementation were instructed on how to daily include 50 g of mixed nuts (5 units of walnuts, 10 of almonds and 13 of hazelnuts were recommended) in their habitual recipes and packages of walnuts, hazelnuts and almonds were provided them free. During the nuts intervention, dietitians did not provide any other dietary recommendation rather than written culinary advices to include nuts in regular meals with soups, creams or as side food.

Once participants ended up their first intervention period, they went through one month of wash out period in which they did not follow any dietary recommendation. After that, the subjects were switched to the other group of intervention during 2 more months. Since their enrollment in the study, the participants were strictly followed by nutritionists with detailed dietary instructions, according to their group, and they were also provided with a questionnaire to asses eventual side effects related to diet.

At the beginning and at the end of each intervention period biochemical analysis on fasting blood samples were conducted, as well as collection of biological samples, including feces and blood.

1.3. Specific measurements

Individual examination on anthropometric were conducted every month until the end of the study and biweekly in case of biochemical parameters.

1.3.1. Anthropometry, body composition and blood pressure

During the individual examination, weigh, height and waist circumference were measured with subjects wearing light clothes and no shoes, by using calibrated scales and a wall-fixed stadiometer. Body composition was also measured by using a bio-electrical impedance analysis (Human-Im-Scan, Dietosystem, Spain). Blood pressure was measured in the non-dominant arm. Using a semiautomatic oscillometer (Omron HEM-70CP, Hoofddorp, Netherlands) in duplicate with a five-minute interval between each measurement.

1.3.2. Dietary and physical activity assessment

Participants were counselled for nutritional adherence with a biweekly nutritional visit and dietary intake at baseline and at the end of each intervention was estimated by calculating a 3-day dietary record including two work days and a weekend day. Energy and nutrient intake were calculated by using validated Spanish tables of composition[110][111]. Adherence to nutritional interventions were assessed by using a validated 17-item MedDiet score[112] and by counting the empty nuts-packaging returned. Eventual side effects related to diet were asked to participants at each visit. Physical activity was evaluated by using the validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire.

1.3.3. Collection and storage of biological samples

Fasting blood samples were collected at the beginning and at the end of each intervention period, centrifuged and aliquoted till its use. Fecal samples were collected at the same time pints. Participants were instructed to collect fecal samples in hermetic sterile flasks the day before the visit of examination. Fresh fecal samples were immediately stored by participants in their home freezer at -20°C and delivered to

the lab within 1-2 days after collection by using a cooler bag and ice blocks. Once in the lab, fecal samples were immediately stored in different aliquots of about 250 mg each at -80°C.

1.3.4. Biochemical Analysis

Analysis of routinely biochemistry and inflammation parameters were conducted on fasting blood samples. Circulating glucose, insulin and serum lipid profile were analyzed with common automated enzymatic methods. Routine standard methods were used for lymphocytes and platelets counts. LDL-cholesterol was estimated using the Friedewald formula in subjects with triglycerides<400mg/dL. The IL-6 and zonulin levels were measured by commercial ELISA (Deltaclon SL, Spain and ImmunDiagnostik, Germany, respectively). The homeostatic model assessment of insulin resistance was estimated by the HOMA-IR method:

$$HOMA - IR = (Glucose\ (mg/dL) * Insulin\ (mU/mL))/405$$

1.3.5. 16S rRNA sequencing and data processing

250 mg of fecal samples were used for DNA microbial extraction by using QIAmpPowerFecal DNA kit (Qiagen, Germantown) following manufacturer instructions. A previous additional step of 5 minutes lysis of fecal matrix with garnet beads was achieved using FastPrep-24-5G homogenizer (MP Biomedicals), in order to improve the quality of Gram-bacteria DNA extraction. Microbial DNA concentration was then measured using Qubit 2.0 Fluorometer. 16S rRNA gene was amplified by using Ion Metagenomics kitTM (Life Technology, Carlsbad, California) which was based on two primers set: a) V2, V4, V8 and b) V3, V6-7, V9. A total amount of 50-100 ng of the amplicons combination from both PCR was then used to create libraries via the Ion Plus Fragment Library kit

(Life Technology, Carlsbad, California) with sample indexing using the Ion Xpress™ Barcode Adapters 1-64 kit (Life Technology, Carlsbad, California). Adapter-ligated and nick-repaired libraries were purified by using CleanNGS kit (CleanNA, Waddinxveen, Netherlands). The libraries were amplified (Ion Plus Fragment Library kit) and quantified with Bioanalyzer (Agilent Technologies, Santa Clara, California) and the kit Agilent DNA 7500 Reagents (Agilent technology, Santa Clara, California). Finally, equimolar amounts of all the libraries (60 μM) were prepared and sequenced by using the Ion-PGM™ System, including 520 and 530 Kit-Chef (Life Technology, Carlsbad, California) for template preparation and chip loading reagents and Ion S5 Sequencer for sequencing. Four (3 Ion 530 Chip and one Ion 520 Chip) loaded chips were used in order to sequence a total amount of 180 16S rRNA libraries, obtaining 300000 reads per sample.

The fastaq sequences obtained for each sample were pre-processed and adapted with an in-house script in order to split only forward reads into 6 subsets of 6 hypervariable regions (V2, V3, V4, V6-7, V8, V9). Finally, only forward reads from V4 hypervariable region were used for all the analysis included in this thesis, as they were the most representative in our population. Quality control, length filtering at 280 bp and denoising of sequences were performed in QIIME (Quantitative Insight into Microbial Ecology) 2, version 2020.9. After the denoising step, achieved by using the denoise-pyro method of DADA2 plugin in QIIME2, reads were converted into ASVs (amplicon sequence variants) and their taxonomic assignment was performed by using a naive bayes classifier trained on the last version of reference database Silva 132. Finally, the OTU (Operational Taxonomic Unit) table obtained, was then filtered at 10 cut-off prevalence at the taxonomic level of genus by using an in-house

script inside phyloseq package in R in order to remove OTUs with a prevalence ≤ 10% within and between samples.

1.3.6. Fecal metabolomics analysis

1.3.6.1. Targeted fecal metabolomics

An amount of approximately 1 g of frozen fecal samples was lyophilized in sterile glass vials with a hermetic vacuum seal. Lyophilized samples where then stored at -80 °C prior to the metabolomics analysis. Nuclear Magnetic Resonance (NMR) and liquid chromatography coupled to triple quadrupole mass spectrometry (LC-qTOF) were used for the profiling of a total of 94 metabolites in a targeted approach. NMR-based approach was uses to profile 37 metabolites including SCFAs, alcohols and organic acids. LC-qTOF platform was used for the analysis of 16 bile acids and 41 amino acids. In case of NMR analysis, an amount of 10-15 g of lyophilized fecal material was homogenized with a hydrate solution of phosphate-buffered-saline (PBS) buffer in order to separate the mixture into an upper brown clear phase and a precipitate with insoluble compounds like lipids, protein and cellular debris. For NMR measurement 200 µL of fecal water upper phase and 400µl of PBS in D2O (pH=7.4, 0.05M, TSP 1.48 mM for diluted concentration of 1 mM) was placed into a 5mm o.d. NMR tube.

1H NMR spectra were recorded at 300 K on an Advance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5 mm PBBO broadband gradient probe. Aqueous samples were measured and recorded in procno 11. For aqueous extracts one-dimensional 1H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY) presaturation sequence (RD-90°-t1-90°-tm-90° ACQ) to suppress the residual water peak, and the mixing time was set at 100 ms. Solvent presaturation with irradiation

power of 125 μW was applied during recycling delay (RD = 5 s) and mixing time. The 90° pulse length was calibrated for each sample and varied from 9.60 to 10.38 μs. The spectral width was 9.6 kHz (15 ppm), and a total of 256 transients were collected into 64 K data points for each 1H spectrum. The exponential line broadening applied before Fourier transformation was of 0.3 Hz. The frequency domain spectra were phased, baseline-corrected and referenced to TSP (δ= 0 ppm) using TopSpin software (version 3.6, Bruker). The acquired 1H NMR were compared to references of pure compounds from the metabolic profiling AMIX spectra database (Bruker®), HMDB, and Chenomx databases for metabolite identification. In addition, we assigned metabolites by 1H–1H homonuclear correlation (COSY and TOCSY) and 1H–13C heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house. After pre-processing, specific 1H NMR regions identified in the spectra were integrated using the AMIX 3.9 software package. Curated identified regions across the spectra that were integrated using the same AMIX 3.9 software Package, were exported to excel spreadsheet in order to evaluate robustness of the different 1H NMR signals and to give relative concentrations. Bile acids extraction started from approximately 5 mg of dry fecal matter and mixed with 20 μL of internal standard, 800 μL of 0.1M NaOH and a steel bead. Samples were agitated with a bullet blender for 3 minutes at speed 8 and vortexed for 5 minutes. Samples were subsequently incubated at 60 ºC for 1 hour. A volume of 600 μL of water was added and the samples were centrifuged for 10 minutes at 15000 rpm and 4 ºC. Supernatants were transferred to a new tube and centrifuged one more time. The resulting supernatants were loaded to a SPE cartridge (Oasis HLB 30 mg sorbent) previously conditioned with 1 mL of methanol and 1 mL of water. Cartridges were washed with 1 mL of water, 1 mL of hexane and 1 mL of water. Then, cartridges were dried

spectroscopy (NOESY) presaturation sequence (RD–90°–t1–90°–tm–90° ACQ) to suppress the residual water peak, and the mixing time was set at 100 ms. Solvent presaturation with irradiation power of 125 μW was applied during recycling delay (RD = 5 s) and mixing time. The 90° pulse length was calibrated for each sample and varied from 9.60 to 10.38 μs. The spectral width was 9.6 kHz (15 ppm), and a total of 256 transients were collected into 64 K data points for each 1H spectrum. The exponential line broadening applied before Fourier transformation was of 0.3 Hz. The frequency domain spectra were phased, baseline-corrected and referenced to TSP (δ= 0 ppm) using TopSpin software (version 3.6, Bruker). The acquired 1H NMR were compared to references of pure compounds from the metabolic profiling AMIX spectra database (Bruker®), HMDB, and Chenomx databases for metabolite identification. In addition, we assigned metabolites by 1H–1H homonuclear correlation (COSY and TOCSY) and 1H–13C heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house. After pre-processing, specific 1H NMR regions identified in the spectra were integrated using the AMIX 3.9 software package. Curated identified regions across the spectra that were integrated using the same AMIX 3.9 software Package, were exported to excel spreadsheet in order to evaluate robustness of the different 1H NMR signals and to give relative concentrations.

1.3.6.2. Untargeted fecal metabolomics

The untargeted approach was focused on the detection of phenolic compounds, fatty acids and acylcarnitines. After a previous homogenization step with an internal standard reference for the desired class metabolites, the chromatographic separation was achieved with a gradient elution using milli-Q water (0.05% formic acid) and methanol

(0.05% formic acid) on a reverse phase AQUITY BEH C18 (100x2.1mm) column from Waters®. The qTOF mass spectrometer operates in scan mode both in positive and negative electrospray ionization on two separate chromatographic runs. In addition, a target tandem mass spectrum (MS/MS) was acquired for QC sample at 20 eV for deconvoluted features in order to identify the proposed metabolites. For data processing, MassHunter qualitative and quantitative were used. MassHunter qualitative analyses were used for metabolite profiling using "find by formula" algorithm from an in-house database created from human fecal metabolome database from HMDB. The resulting tentative matched entities were refined manually by comparing the experimental MS/MS spectra, retention time with the information on database or pure standards when available, also published in relation to the main phenolic metabolites, fatty acids and other fecal metabolites. After metabolite screening a total of 133 annotates compounds were found and used in MassHunter quantitative analysis for chromatographic peak extraction and integration. The area of each metabolite was normalized by the area of the corresponding internal standard compound based on their structural similarity and retention time proximity. Finally, the matrix containing the semi-quantitative information for these metabolites was normalized by the exact sample weight.

1.3.7. Plasma metabolomics analysis

Fasting plasma samples were collected from subjects at baseline and at the end of each intervention and stored at -80 °C. In total, 378 metabolites were analyzed with a targeted approach. LC-QqQ is an LC-MS/MS analysis which combined an HPLC to a QqQ/MS 6470 from Agilent Technology and that was used to analyze TMAO and derivatives, acylcarnitines, amino acids and serotonin. LC-qTOF was used for the

lipidomics analysis by Folch extraction. Determination of total fatty acids profile together with most organic acids and sugar metabolites, especially focusing on TCA cycle, was obtained by GC-qTOF analytical platform.

In case of TMAO determinations, aliquots of 50 µl were mixed with 10 µl of internal standard, 75 µl of 50mM tert-Butyl bromoacetate in ACN and 10 µl of 70% ammonium hydroxide. Samples were vortexed for 1 min and derivatized for 30 minutes at room temperature. A volume of 50 µl of 1% Formic acid in ACN was added. Samples were vortexed and centrifuged for 5 minutes at 15000 rpm and 4ºC. Supernatants were transferred to glass vials for their analysis. Mobile phase A was 10% acetonitrile/90% water and B was 90% acetonitrile/10% water, and both with 10 mM ammonium formate and 0.125% formic acid. The column temperature was set at room temperature and the injection volume was 1 µl.

In case of acylcarnitines quantification, 30 µl of plasma were mixed with 270 µL of 100% methanol containing the set of labelled internal standards. The mixture was vortexed for 15 s and centrifuged during 10 min at 4700 rpm at 4ºC. The supernatant was transferred into a new plate and injected on LC-MS/MS. The extraction was carried out with a semi-automated process using Agilent Bravo Automated Liquid Handling Platform. The chromatographic separation was performed with an isocratic gradient of mobile phase B was 100% methanol with 0.1% formic acid over 13 minutes. The column temperature was set at 20 ºC and the injection volume was 1 µL.

For the extraction of more hydrophobic lipids, a liquid-liquid extraction with chloroform:methanol (2:1) based on Folch procedure was performed by adding four volumes of chloroform:methanol (2:1)

containing internal standard mixture (Lipidomic SPLASH) to serum. Then, the samples were mixed and incubated at -20ºC for 30 minutes. Afterwards, water with NaCl (0.8 %) was added and mixture was centrifuged at 15,000 rpm. Lower phase was recovered, evaporated to dryness and reconstituted with methanol:methyl-tert-butyl ether (9:1) and analyzed by UHPLC-qTOF (model 6550 of Agilent, USA) in positive electrospray ionization mode. The chromatographic method consists on an elution with a ternary mobile phase containing water, methanol and 2-propanol with 10mM ammonium formate and 0.1% formic acid. The stationary phase was a C18 column (Kinetex EVO C18 Column, 2.6 µm, 2.1 mm X 100 mm) that allows the sequential elution of the more hydrophobic lipids such as lysophospholipids, sphingomyelins, phospholipids, diglycerides, triglycerides and cholesteryl esters, among others.

The identification of lipid species was performed by matching their accurate mass and tandem mass spectrum, when available, to Metlin-PCDL from Agilent containing more than 40,000 metabolites and lipids. In addition, chromatographic behavior of pure standards for each family and bibliographic information was used to ensure their putative identification.

Sample preparation was based on [113] to obtain volatile fatty acid methyl ester derivatives (FAMEs). Briefly, 50 µl of plasma samples were mixed with IS solution, chloroform and methanolic HCl and incubated at 80 ºC for 2 hours. Afterwards, obtained FAMEs were extracted by a liquid-liquid extraction using hexane before to be injected on GC-MS system.

Chromatographic analysis was based on [113] to determine the 37 FAMEs included in Food Industry FAME Mix. Briefly, FAMEs were separated

on HP-88 (100 m x 250 µm x 0.25 µm) column using a temperature program between 140 and 240 ºC at 1mL/min using He as carrier gas. Ionization was carried out by electronic impart (70 eV) and mass analyzer operates on Selected Ion Monitoring mode (SIM).

Serotonin was analyzed starting from 50 µL of plasma, which were aliquoted to a 1.5 ml Eppendorf tube and mixed with 5 µL of internal standard (Serotonin-d4 at 1 µg/mL) and 245 µL of acetonitrile. Samples were vortexed and centrifuged for 5 minutes at 15000 rpm and 4ºC. Supernatants were transferred to glass vials for analysis. The analytical column used for serotonin was Luna Omega 1.6 µm Polar C18 (100 x 2.1 mm) (Phenomenex, Torrance, CA).

The chromatographic separation was performed with a linear gradient to 99% mobile phase B of 0.1% formic acid in acetonitrile. The column temperature was set at 35 ºC and the injection volume was 5 µL.

Amino acids determination started from 8 µL of plasma mixed with 32 µL of internal standard (Metabolomics amino acid mix labeled standard from Cambridge Isotopes) in MeOH. Samples were vortexed and centrifuged for 5 minutes at 15000 rpm and 4ºC. Supernatants (30 µL) were transferred to a new tube and evaporated in a SpeedVac at 45 ºC. The analytical column used was ACQUITY UPLC HSS T3 Column, 1.7 µm, 2.1 mm x 150 mm (Waters, Milford, MA, USA). Samples were reconstituted with 30 µL of borate buffer and were derivatized using AccQ-Tag reagent from Waters following manufacturing protocol. The chromatographic separation was performed with a linear gradient to 70% mobile phase B (0.1% formic acid in acetonitrile) over 13 minutes and an isocratic gradient to 87% of mobile phase A (0.1% formic acid in water) over 2 minutes. The column temperature was set at 40 ºC and the injection

volume was 2 µL. The MRM transitions for amino acids and metabolites in all cases used as precursor ion- the [derivatized amino acid + H]+ and the main daughter ion was 171 m/z. The chromatographic behavior and presence of possible interferences was based on the methodology described by Wang et al.

1.4. Statistical Analysis

Normality in variables for biochemical, anthropometric and nutritional parameters were checked with Lilliefors test. Descriptive characteristics of patients have been presented as means and 95% CI or medians with 25%-75% interquartile range for quantitative variables and as percentages for categorical variables. Changes in biochemical, anthropometric and nutritional variables were used to compare the two nutritional interventions, by using linear mixed model analysis of variance with intervention group and sequence of intervention as fixed factors and subjects as random effect in order to take into account the multilevel structure of the cross-over design of this study (repeated measurements), as well as the potential carry over effect. Moreover, we used baseline values as covariates and subjects as random effect in the linear regression mixed model. To account for multiple testing, we have multiple adjusted both the p values for treatment and for its interaction with intervention sequence with the use of Benjamini-Hochberg false discovery rate (FDR) method.

For the analysis on gut microbiota composition, we used the OTU table previously obtained and filtered at 10% cut off point of OTU prevalence within and between samples, in order to calculate α and ß microbial diversity, by using the functions available in “phyloseq” R package (version 1.30.0). The α diversity was measured by using different index, including observed diversity, Shannon index, Inverse Simpson index and

Phylogenetic distance, either considering final and initial time points of each nutritional intervention or changes values between MedDiet and nuts supplementation group. At the same time, ß microbial diversity was calculated with "adonis" function in R ("vegan" package, version 2.5-6) using Bray-Curtis dissimilarity within and between interventions. At the same time the Bacteroides/Firmicutes ratio was calculated with bfratio function ("microbiome" package, version 1.9.19) and paired Wilcoxon test was applied to compare this ratio between and within groups of intervention.

The compositional analysis of gut microbiota was achieved with a collapsed OTU table at genus taxonomic level and converting the absolute in relative abundances. The relative abundances of taxa at genus level were subjected to a linear discriminant effect size analysis in LEfSe (LEfSe, http://huttenhower.sph.harvard.edu/galaxy/). LEfSe determines the features, such as operational taxonomic units, most likely to explain differences between classes by coupling standard tests for statistical significance (Kruskal Wallis and Wilcoxon tests) together with additional analysis to measure the magnitude of the observed phenomenon by ranking the biological consistency and effect size relevance. Linear discriminant analysis (LDA) scores of 2 and a p-value for Wilcoxon test of 0.05 were used to identify genera that were enriched with respect to baseline time-points in each dietary intervention or to the other diet in the comparison of genus abundances at final time point.

Generalized linear regression models for repeated measures were used to evaluate the associations between the discriminant microbial genera between interventions identified with LEfSe and significant changes in cardiometabolic parameters, by using glm function ("stas" package in R, version 3.6.2). In order to address potential confounding factors, we

have run a sensitivity analysis, evaluating the potential confounder effect of weight and waist circumference by adding them as covariates in the regression model.

Regarding both the fecal and plasma metabolomics profiling, a previous data cleansing was achieved by removing metabolites with high number of missing values (>20%) and metabolites with less than 20% missing values were imputed with "missforest" function of "randomForest" package in R, version 4.6-14). Therefore, the concentration of metabolites was normalized with rank-based inverse normal transformation. Due to the high dimensionality and collinear nature of the data, in both cases, the fecal and circulating metabolites fingerprinting, a logistic regression analysis with elastic net penalty (alpha=0.5) was implemented in the "glmnet" (R package, version 1.40-4) to select the metabolites associated with dietary interventions.

The associations between changes in the selected fecal metabolites of the previous regression model and the significant changes in cardiometabolic risk factors were evaluated with a linear regression analysis with elastic penalty by using the first as independent variables and each cardiometabolic risk factor as dependent variable.

In case of the plasma metabolomic profile, we have evaluated the correlation network between changes in the selected metabolites from the previous regression model and changes in 151 microbial genera, previously centered log-ratio transformed using "clr" (in "composition" R package, version 1.40-4).

The plasma metabolomic profile identified was also converted to a weighted multimetabolite score in order to evaluate its predictive

potential on improvements observed in insulin, glucose and insulin resistance levels.

A cross-sectional multi-omics analysis, including plasma metabolomics, fecal metabolomics and 16S rRNA sequencing data was achieved by using a Sparse Generalized Canonical Correlation Analysis (SGCCA) in an unsupervised mode, in order to selected the most relevant variables from three omics datasets. The tuning procedure to identify the optimal number of components for each omic dataset was performed by a separated performance analysis on a dual-omics dataset including fecal and plasma metabolites with the performance function of spls models (perf function in "mixOmics" package in R, version 6.14.0). A network analysis was implemented by network function with a cut-off point of correlation of 0.6. The network was further analyzed in Cytoscape software (version 3.8.2) in order to better visualize the presence of connected components and the relevant associations between fecal and plasma metabolites and microbial genera.

All statistical analysis were performed using R, version 3.6.2 and all tests were two-sided with a significance defined as $p<0.05$.

F. RESULTS

1. PUBLICATION 1

Title: Effects of the Mediterranean diet or nut consumption on gut microbiota composition and fecal metabolites and their relationship with cardiometabolic risk factors

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Journal: Molecular Nutrition and Food Research

IF: 5.309; Food Science and Technology; Q1

Abstract:

Scope To examine whether a Mediterranean Diet (MedDiet) compared to the consumption of nuts in the context of habitual non-MedDiet exerts a greater beneficial effect on gut microbiota and fecal metabolites, thus contributing to explain major benefits on cardiometabolic risk factors.

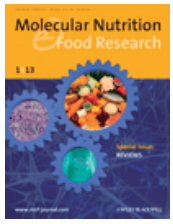
Methods and Results Fifty adults with Metabolic Syndrome were randomized to a controlled, crossover 2-months dietary-intervention trial with a 1-month wash-out period, following a MedDiet or consuming nuts (50gr/day). Microbiota composition was assessed by 16S rRNA gene sequencing and metabolites were measured using NMR and LC-qTOF platforms in a targeted metabolomics approach.

Decreased glucose, insulin and HOMA-IR was observed after the MedDiet compared to the nuts intervention. Relative abundances of

Lachnospiraceae NK4A136 and un uncultured genera of Ruminococcaceae were significantly increased after the MedDiet compared to nuts supplementation. Changes in *Lachnospiraceae NK4A136* were inversely associated with insulin levels and HOMA-IR, while positively and negatively with changes in cholate and cadaverine, respectively.

Conclusions Following a MedDiet, rather than nuts, induced a significant increase in *Lachnospiraceae NK4A136* and improved the metabolic risk. This genera seems to affect the bile acid metabolism and cadaverine which may account for the improvement in insulin levels.

Molecular Nutrition and Food Research



Effect of the Mediterranean diet or nut consumption on gut microbiota composition and fecal metabolites and their relationship with cardiometabolic risk factors

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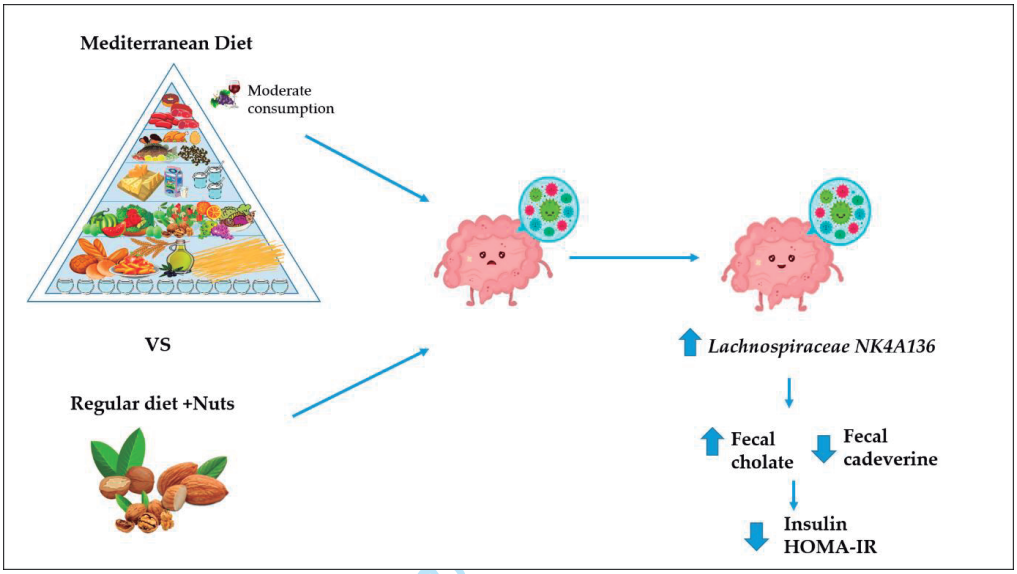
Keywords:	Mediterranean diet, nuts, gut microbiota, fecal metabolites, insulin resistance

Graphical Abstract



Graphical abstract Text

Following a Mediterranean diet (MedDiet) rather than a regular diet supplemented with nuts increases the abundance of *Lachnospiraceae NK4A136* which is associated with MedDiet benefits on insulin metabolism, partially explained by fecal cholate increases and cadaverine decreases.

Effects of the Mediterranean diet or nut consumption on gut microbiota composition and fecal metabolites and their relationship with cardiometabolic risk factors

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Abbreviations: HOMA-IR: homeostatic model assessment of insulin resistance; LDA: linear discriminant analysis; MedDiet: Mediterranean diet; NMDS: non-metric multi-dimensional scaling, T2D: Type 2 Diabetes.

Keywords: Mediterranean diet, nuts, gut microbiota, fecal metabolites, insulin resistance

Abstract

Scope To examine whether a Mediterranean Diet (MedDiet) compared to the consumption of nuts in the context of a habitual non-MedDiet exerts a greater beneficial effect on gut microbiota and fecal metabolites, thus contributing to explain major benefits on cardiometabolic risk factors.

Methods and Results Fifty adults with Metabolic Syndrome were randomized to a controlled, crossover 2-months dietary-intervention trial with a 1-month wash-out period, following a MedDiet or consuming nuts (50 g/day). Microbiota composition was assessed by 16S rRNA gene sequencing and metabolites were measured using NMR and LC-qTOF platforms in a targeted metabolomics approach.

Decreased glucose, insulin and HOMA-IR was observed after the MedDiet compared to the nuts intervention. Relative abundances of *Lachnospiraceae NK4A136* and un uncultured genera of Ruminococcaceae were significantly increased after the MedDiet compared to nuts supplementation. Changes in *Lachnospiraceae NK4A136* were inversely associated with insulin levels and HOMA-IR, while positively and negatively with changes in cholate and cadaverine, respectively.

Conclusions Following a MedDiet, rather than nuts, induced a significant increase in *Lachnospiraceae NK4A136* and improved the metabolic risk. This genera seems to affect the bile acid metabolism and cadaverine which may account for the improvement in insulin levels.

1) Introduction

The role of specific foods or dietary components in shaping gut microbiota and fecal metabolites and their impact on human health is widely recognized. However, beyond the traditional view of the health effects of single foods, regardless of the dietary context in which they are consumed, the interest of full dietary patterns becomes an area of growing interest.

Consumption of plant-based foods has been associated with both lower cardiometabolic risk factors and a diverse microbiota profile, with a greater abundance of probiotic species compared to the intake of animal-based foods[1],[2]. Within plant-based foods, nuts, a complex matrix of nutrients rich in fiber, unsaturated fatty acids and other phytochemical compounds, have demonstrated a favorable impact on gut microbiota. Regular consumption of almonds and pistachio was related to an increased amount of butyrate producers in humans[3]. Similarly, supplementation of almonds and walnuts in rats was associated with increased *Lactobacillus spp.* and *Bifidobacterium spp.*[4] or *Roseburia*[5], respectively. Changes in gut microbiota related to circulating urolithins[6], urine hippurate, p-cresol sulfate and dimethylamine[7], and fecal secondary bile acids[8] have been reported after nuts consumption.

Nuts are typically included in Mediterranean Diet (MedDiet), a dietary pattern widely recognized as a powerful nutritional strategy to improve cardiometabolic health. The MedDiet is also rich in other types of foods such as whole grains, vegetables, legumes, fruits, and olive oil that, at least individually, seems to be also related to a diverse microbiome profile. Higher adherence to MedDiet has been positively associated with changes in specific bacteria and derived metabolites[9]. However, few studies have examined the effect of the MedDiet on gut microbiota composition and fecal metabolites together with their potential benefits on cardiometabolic health. An ancillary analysis of the CORDIOPREV study (CORonary Diet Intervention with Olive oil and cardiovascular PREVention) has demonstrated the restoration of some microbiota species after 2 years following a MedDiet in obese adults with Metabolic Syndrome [10]. More recently, a 12-month MedDiet intervention in elderly subjects displayed

changes in specific taxa associated with lower frailty, improved cognitive function, and lower inflammation[11]. Although the effects of consuming either a MedDiet or including nuts in an habitual non-MedDiet on gut microbiota composition is poorly explored, less is known about dietary modulation of fecal metabolites which could help to provide a functional readout of microbial activity[12].

Therefore, we examined whether following a MedDiet modifies gut microbiota composition and fecal metabolomics profile as well as cardiometabolic risk factors compared to a non-MedDiet supplemented with nuts. We also analyzed whether changes in gut microbiota and fecal metabolites are associated with changes in cardiometabolic risk factors.

2) Experimental Section

Study Design and population

METADIET is a randomized, controlled, crossover 2-months dietary-intervention trial, with a 1-month wash-out period. Eligible participants were community-dwelling subjects aged 25-60 years with a body mass index (BMI) between 25-35 kg/m^2 and Metabolic Syndrome according to the ATPIII diagnosis criteria[13]. Exclusion criteria were: Type 2 Diabetes (T2D); secondary obesity or related pathologies; non-controlled hypertension; LDL-cholesterol>160mg/dL; triglycerides <400mg/dL; 17-item MedDiet score[14]; regular intake of nuts ≥ 90 g/week); several chronic diseases (inflammatory, infectious, chronic obstructive pulmonary, neoplasia, endocrine, or hematological diseases); leucocytes>11x10E9; specific pharmacological treatments (anti-inflammatory, corticoids, hormones or antibiotics); changes in body weight (>5kg in the last 3 months); alcohol or drug abuse and consumption of prebiotics, probiotics or laxatives. Subjects who met the inclusion criteria were randomly assigned to one of the two sequence intervention periods using a computer-generated random-number table. After 1 month of "wash-out" period, participants crossed the interventions for the other 8 weeks. Half of the participants followed a MedDiet intervention for 8 weeks, while the other half continued with their habitual non-MedDiet supplemented with 50 g/day of mixed nuts (almonds, hazelnuts, and walnuts, provided free) (supplementary **Figure S1**). The trial was registered in the ISRCTN (ISRCTN88780852) on the 7th of April 2017, https://doi.org/10.1186ISRCTN88780852.Written informed consent was obtained for all participants.

Dietary interventions

Several face-to-face interviews with trained dietitians were scheduled at the beginning, after 15 days, at 1 month and at the end of each intervention period. During the MedDiet intervention period participants were encouraged to adhere to the 17-item MedDiet score used in the

PREDIMED Plus study[14]. Participants received written material and all recommendations to follow the MedDiet, emphasizing in the consumption of at least 2 servings of vegetables and 3 fruits per day; ≥3 servings of legumes, ≥5 servings of whole-grain cereals/pasta, ≥3 servings of fish/seafood per week and the use of extra virgin olive oil as the main culinary fat. They were also instructed to reduce the consumption of red meat and processed foods ≤1 serving/week, use of butter and margarines (<1/week), white bread (≤1/day) and sugary beverages or sugar-sweetened fruit juices (<1/week). Participants were provided with biweekly menus and seasonal recipes to facilitate the adherence to the MedDiet intervention. During the nuts intervention period, dietitians did not provide any other dietary advice rather than the consumption of 50 g/day of mixed nuts that were provided by free, and written culinary advices to include nuts in regular meals with soups, creams or as side food.

Nutritional data were collected in each sampling visit using 3-day dietary records, nutrient and energy intakes were calculated using Spanish food composition tables [15],[16]. Adherence to the interventions was assessed by the validated 17-item MedDiet score[14] and counting the empty nuts-packaging returned in several visits along the intervention.

Anthropometry and blood pressure

Weight, height, and waist circumference were determined with calibrated scales and a wall-fixed stadiometer, BMI was then calculated. Blood pressure was measured in duplicate using a validated semiautomatic oscillometer (Omron HEM-705P, Holland).

Biological samples, collection and storage

Fasting blood samples were collected at baseline and at the end of each intervention period. Glucose, lipid profile and lymphocytes were measured using standard enzymatic automated methods. LDL-cholesterol was estimated using the Friedewald formula in subjects with triglycerides>400mg/dL. Circulating insulin, IL-6 and zonulin levels were measured by commercial ELISA (Deltaclon SL, Spain and ImmunDiagnostik, Germany, respectively). The

homeostatic model assessment of insulin resistance (HOMA-IR) was estimated[17]. Participants were instructed to collect stool samples in hermetic sterile-flasks and freeze them immediately at -20°C. Frozen samples were delivered to the laboratory within 1-2days after collection and stored in different aliquots at -80°C.

16S rRNA gene sequencing and data processing

Fecal DNA extraction was performed with QIAmpPowerFecal DNA kit (Qiagen, Germantown). A first additional 5-minute lysis step using FastPrep-24-5G Homogenizer (MP Biomedicals) was conducted. The 16S rRNA gene region was amplified with Ion Metagenomics kit™ (Life Technology, Carlsbad, California) by 2 separated PCR reactions using two primer sets: V2, V4, V8 and V3, V6-7, V9. 50-100 ng of combined amplicons were processed to obtain DNA libraries using Ion Plus Fragment Library kit and Ion Xpress Barcodes Adapters, 1-64 (Life Technology, Carlsbad, California). Adapter-ligated and nick-repaired libraries were purified by using CleanNGS kit (CleanNA, Waddinxveen, Netherlands). The libraries were amplified (Ion Plus Fragment Library kit) and quantified with Bioanalyzer (Agilent Technologies, Santa Clara, California) and the kit Agilent DNA 7500 Reagents (Agilent technology, Santa Clara, California). Finally, equimolar amounts of all the libraries (60 µM) were sequenced in 4 different runs with Ion 520 and Ion 530 Kit-Chef (Life Technologies, Carlsbad, California, EUA) and a 530 chip for sequentiation (Ion Torrent platform). The sequencing data were pre-processed with an adapted in-house script[18] in order to split only forward reads of each sample data into 6 subsets of 6 hypervariable regions. Reads from V4 region were used for this study. Quality control, length filtering at 280bp and denoising of sequences other than taxonomy assignment were performed in QIIME2 software package using the latest version of Silva 132 as 16SrRNA gene classifier database. Finally, we used a priori cut off value of 10% of prevalence at genus level on the absolute abundances of the OTU table obtained from QIIME2, in order to remove OTUs with a prevalence ≤10% within and

between samples. We then transformed the filtered OTU table in relative abundances in all the samples, irrespective of treatment and sequence of intervention by using phyloseq package in R (version 1.34.0).

Fecal Metabolomics analysis

Fecal samples were lyophilized previous to the metabolomics analysis. The 94 metabolites are listed in supplementary **Table S1**. Nuclear magnetic resonance (NMR) and liquid chromatography coupled to triple quadrupole mass spectrometry (LC-qTOF) were used for the fecal metabolome analysis in a targeted approach. NMR was used to profile metabolites including SCFAs (short chain fatty acids), alcohols and organic acids. 10-15mg of lyophilized dry fecal matter was homogenized and separated into aqueous phase, insoluble compounds and protein and cellular debris. 200µl of fecal aqueous phase and 400µl of PBS in D2O (pH=7.4, 0.05M, TSP 1.48mM for diluted concentration of 1mM) were placed into a 5mm o.d. NMR tube. 1H-NMR spectra were recorded at 300K on an Advance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20MHz using a 5mm PBBO broadband gradient probe. The acquired NMR was compared to references of pure compounds from the metabolic profiling AMIX spectra database (Bruker®), HMDB, and Chenomx databases for metabolite identification. We assigned metabolites by 1H–1H homonuclear correlation (COSY and TOCSY) and 1H–13C heteronuclear (HSQC) 2D-NMR experiments and by correlation with pure compounds run in-house. After pre-processing, specific 1H-NMR regions identified in the spectra were integrated using the AMIX 3.9 software package. Liquid chromatography coupled to triple quadrupole mass spectrometry (LC-qTOF) was used to determine bile acids, amino acids and its derivatives. The chromatographic separation of bile acids was performed on a Kinetex EVO C18 (150x2.1mm) column and bile acid species were assigned by direct comparison with commercial standards. The chromatographic separation of amino acids was performed on a ACQUITY UPLC HSS T3 Column, and assigned by direct comparison with

commercial standards. The chromatographic behavior and presence of possible interferences were based on the methodology previously described[19].

Statistical analyses

The sample size was estimated to detect a difference of 3-fold changes in microbiota genus (*Roseburia, Oscillospira* and *Prevotella*) between the dietary interventions, accepting an alfa risk of 0.05 and a beta risk of 0.1 in a bilateral contrast[20].A total of forty-five participants were required considering 10% withdrawal. Normality was checked by Lilliefors test. Descriptive data of participants are presented as means and 95%CI or medians with 25%-75% interquartile range for quantitative variables, and as percentages for categorical variables. Changes in anthropometric, biochemical and nutritional data were analysed by using linear mixed-model analysis of variance with intervention groups and periods modelled as fixed factors, baseline values as covariates and subjects as a random effect. To account for multiple testing, we adjusted P for treatment and P for treatment * period of the crude and multivariable-adjusted associations with the use of the Benjamini-Hochberg false discovery rate (FDR) procedure. To check for possible carry-over effect, we adopted a linear model of regression analysis of variance with intervention group and period modelled as fixed factors and subjects as random effect. For the microbiome analysis, α-diversity indices were calculated in R with "phyloseq" package (version 1.30.0). Adonis test was performed in R with "Adonis" function ("vegan" package, version 2.5-6) using β-diversity calculated as Bray-Curtis dissimilarity within and between interventions. Non-metric Multi-Dimensional Scaling (NMDS) plots of Bray-Curtis dissimilarity were generated. Bacteroides/Firmicutes ratio was calculated with bfratio function in "microbiome" package in R (version 1.9.19) and paired Wilcoxon test was applied to compare this ratio between and within interventions. The relative abundances of taxa at genus level were subjected to a linear discriminant analysis effect size analysis (LEfSe, http://huttenhower.sph.harvard.edu/galaxy/)[21]. LEfSe determines the features, such as

operational taxonomic units, most likely to explain differences between classes by coupling standard tests for statistical significance (Kruskal Wallis and Wilcoxon tests) together with additional analysis to measure the magnitude of the observed phenomenon by ranking the biological consistency and effect size relevance[21]. Linear discriminant analysis (LDA) scores of 2 and a p-value for Wilcoxon test of 0.05 were used to identify genera that were enriched with respect to baseline time-points in each dietary intervention or to the other diet in the comparison of genus abundances at final time point. Fitted linear mixed-effects models of regression analyses, with the selected genera from LEfSe analysis and the cardiometabolic risk factors significantly changed as fixed terms and subjects as random effect was also conducted by using lmer function of "lme4" package in R, version 1.1-2.6. An additional test to evaluate the p-value of the random effect was conducted with "rand" function in "lmerTest" package of R (version 3.1-3). To address potential confounding effects of weight and waist circumference changes on the association between genera and cardiometabolic risk factors we conducted a sensitivity analysis by adding them as covariates. From a total of 94 fecal metabolites profiled, 1 metabolite was removed due to the high number of missing values (>20%) and metabolites with less than 20% missing values were imputed using the random forest imputation approach[22] ("missForest" function of "randomForest" R package version 4.6-14). The concentrations of metabolites were approximated to a normal distribution with the rank-based inverse normal transformation. Due to the high dimensionality and collinear nature of the data, logistic regression with elastic net penalty (alpha=0.5) was implemented in the "glmnet" (R package, version 3.0-2) to select the metabolites that are associated with the dietary interventions. The same package was used and logistic regression with elastic net penalty was implemented for estimating the associations of changes in metabolites identified in the previous model (independent variables) with significant changes in cardiometabolic risk factors (each cardiometabolic risk factor was the dependent variable and treated as dichotomic variable

according to the median). The associations between higher metabolites' concentrations (independent variables) and changes in microbial genera (each genera was the dependent variable) were analyzed implementing elastic net linear regression models. For this purpose, we used changes in relative abundances of the genera significantly increased or decreased after dietary interventions which were normalized using "clr" function of the R package "compositions" (R package, version 1.40-4). All analyses were performed using R, version 3.6.2. All tests were two-sided, and significance was defined as $p<0.05$.

3) Results

A total of 50 participants wererandomized to the two dietary interventions. Of them, 38 were finally included in the analyses due to the fact that six droppedout for personal reasons and other six were excluded because of the unavailability of gut microbiota data (supplementary **Figure S2**). No significant differences in participants' baseline characteristics were observed between the two interventions (**Table 1**). The mean±SD for the 17-items MedDiet score was 11.37±2.39 [increase, 4.49 (95% CI, 3.5-5.5] after MedDiet and 9.18±2.41[increase, 0.5 (95% CI, -0.05-1.0)] at the end of nuts supplementation; between group differences 3.48 (95% CI, 2.41-4.56) $p< 0.001$. No significant differences in total energy and macronutrients intake were observed between interventions. Following a MedDiet resulted in a higher increase in fruit and fish and a decrease in alcohol and potatoes consumption compared to the non-MedDiet supplemented with nuts period. As expected, nuts consumption was significantly increased in the nuts intervention group (supplementary **Table S2)**. Significant differences in changes in glucose, insulin and HOMA-IR were observed between the MedDiet and the nuts supplementation periods (**Table 1**).

Effect of dietary interventions on gut microbiota composition

There were no significant differences neither in estimated alpha-diversity indices (supplementary **Figures S3**, **S4**, and **S5**) nor in beta-diversity (supplementary **Table S3**).

Additionally, no clear discrimination of microbial composition within each intervention was found in NMDS plots based on Bray-Curtis dissimilarity (supplementary **Figure S6, S7, S8**). The ratio of relative abundances between Bacteroidetes and Firmicutes was not significantly different between or within the interventions (data not shown). Differences in the gut microbiota composition revealed significant enrichment in *Lachnospiraceae NK4A136* genera and uncultured genera of Ruminococcaceae family after the MedDiet intervention compared to the non-MedDiet+nuts (LDA score of 2.0 and $p<0.05$, **Figure 1**). The within-group analysis revealed a significant increase in *Roseburia* and *Oxaobacter* while *Ruminococcaceae UCG014* and *Lactococcus* decreased (LDA score of 2.0 and $p<0.05$, **Figure 2**). No significant changes were observed after the MedDiet intervention.

Effect of dietary interventions on fecal metabolites

Positive associations were found between changes in 4 metabolites' concentrations and the MedDiet intervention, (**Figure 3**) with the highest effect on homocitrulline followed by acetate, cadaverine and malate changes. Inverse associations were also observed between changes in 9 metabolites and the nuts supplementation intervention. The highest effect of nuts intervention was found for changes in tryptophan followed by taurine, hyodeoxycholic acid (HDCA), methionine sulphoxide, serotonin, cholate, alanine, glycerol and valine.

Associations between gut microbiota composition, fecal metabolites and cardiometabolic risk factors

Increases in relative abundances of *Lachnospiraceae NK4A136* were significantly associated with decreases in insulin and HOMA-IR (**Table 2**). Similar results were found after adjusting analyses for adiposity measures except for the association with HOMA-IR (**Table S**). No associations were found between genera differentially abundant after the nuts intervention and cardiometabolic risk factors. Regarding fecal metabolites, decreased concentrations of fecal HDCA, cholate-bile acid, cholic acid (CA) and β-aminoisobutyric acid (BAIBA) were

associated with increased glucose and insulin circulating levels (**Table 3 and Table S5**). Increased concentrations of fecal cadaverine and acetate and decreased concentrations of methionine and serotonin were associated with increased insulin levels (**Table 3**). Decreased BAIBA was also associated to increased HOMA-IR. Associations between changes in the relative abundance of genera and fecal metabolites changing during the interventions are shown in **Table 4**. Increased *Lachnospiraceae NK4A136* observed in the MedDiet intervention was positively associated with increases in the concentrations of cholate-bile acid and negatively with cadaverine. Also, increases in *Ruminococcaceae UCG014* were positively associated with cholate-bile acid and HDCA, whereas increases in *Lactococcus*, were inversely associated with HDCA and serotonin. In contrast, increases in *Roseburia* and *Oxalobacter*, were positive and negatively associated to changes in cadaverine, respectively.

4) Discussion

In the present study we demonstrated for the first time that participants with Metabolic Syndrome following a Mediterranean dietary pattern, as opposed to a non-MedDiet diet supplemented with nuts, significantly changed specific microbiota genera and fecal metabolites that could partially explain the reduction of glucose, insulin levels, and HOMA-IR. Our findings imply that the quality of the diet may account for the metabolic benefits of the MedDiet, independent of changes in adiposity.

Strong evidence suggests that following a MedDiet or consuming nuts exerts beneficial effects on metabolic risk markers[23],[24]. Results of our study highlight the importance of a full dietary pattern like the MedDiet instead of a specific healthy food on improving metabolic health. In line with previous studies, our findings suggest that medium-term dietary interventions do not induce major changes on alpha and beta-diversity indices[25]. However, following a MedDiet compared to a non-MedDiet supplemented with nuts, displayed a significant increase in *Lachnospiraceae NK4A136* genera and uncultured genera of the Ruminococcaceae family.

Other genera from the Lachnospiraceae family have been previously associated to a higher MedDiet adherence[26]–[28].

The MedDiet intervention was also associated with increased fecal SCFA acetate and the organic acid malate, together with decreased fecal bile acids (HCDA and cholate) supporting some[25],[26] but not all[29] previous findings. Although we failed to find any significant association between changes in *Lachnospiraceae NK4A136* and the fecal SCFAs, this genus belongs to a family of anaerobic bacteria involved in the fermentation of plant polysaccharides to SCFA. A study conducted in T2D mice treated with a flavonoid-rich extract demonstrated an increase in this genera abundance[30]. This genus was positively correlated with fecal acetate, butyrate and total SCFAs in children[31]. Inconsistencies exist in the literature about the role of fecal SCFAs on metabolic health. In a recent cross-sectional study among 160 adults circulating rather than fecal SCFA were associated with peripheral insulin sensitivity[32]. Our study showed positive associations of changes in fecal acetate and butyrate with changes in insulin levels and this is in accordance with the findings of a recent cross-sectional analysis of 441 community-dwelling adults in which fecal acetate and butyrate were significantly correlated with insulin levels but not glucose and HOMA-IR[33]. An increase in *Lachnospiraceae NK4A136* in mice has been correlated with a decrease in glucose levels, improved glucose tolerance and reduced inflammatory status by activation of the IRS1/PI3K/AKT and inhibition of the JNK1/2 insulin pathway[30]. Similarly, to these findings, we also observed that increased *Lachnospiraceae NK4A136* genus was associated with decreased insulin levels and HOMA-IR. The reduction in fecal bile acids after the MedDiet, reflects the replacement of foods from animal origin with plant-based foods. Beyond their role to facilitate the intestinal absorption of dietary fat, bile acids may act as signaling molecules to control several metabolic pathways including regulating glucose metabolism[34] through the regulation of multiple receptors. This could explain the inverse association between changes in

fecal bile acids with glucose and insulin levels. At the same time, fecal bile acids are elevated in patients with type 2 diabetes and uncontrolled hyperglycemia, while they are decreased upon insulin treatment, supporting a modulatory role of insulin on bile acid metabolism[35]. Interestingly, increases in *Lachnospiraceae NK4A136* were associated with increases in fecal cholate. Previous studies have revealed positive correlations between Lachnospiraceae but also Ruminococcaceae families with secondary fecal bile acids[36]. These correlations are likely due to the ability of these taxa to perform 7α-dehydroxylation from primary to secondary bile acids to provide an energy advantage to the bacteria. Therefore, it could be speculated that in a dynamic situation with increasing *Lachnospiraceae NK4A136*, the positive association we found between this genus and the fecal cholate was due to an increased reabsorption of cholate at intestinal level to convert to secondary bile acids. Changes in *Lachnospiraceae NK4A136* were also inversely associated with cadaverine, which has been found to promote insulin secretion[37]. In our study, increased cadaverine was associated with increased insulin levels. Whether increases in *Lachnospiraceae NK4A136* abundance play a role in the link between the MedDiet and improvements in insulin levels by decreasing fecal cadaverine concentrations needs further investigation.

Significant increases in *Roseburia* and *Oxalobacter* genera were observed after nuts consumption. Our results are similar to those observed in two different walnuts feeding studies conducted either in a healthy population or in subjects at risk for cardiovascular disease showing enrichment of *Roseburia* after 3- or 6-weeks of intervention[8],[38]. An increase in *Oxalobacter* was also observed after nuts consumption in our study. Nuts are rich in oxalate, the main substrate for *Oxalobacter formigens,* and consumption of almonds has been related to a higher concentration of gastric oxalate[39]. Simultaneously, we found a significant decrease in *Ruminococcaceae UCG014 group* and *Lactococcus* after nuts supplementation. *Ruminococcaceae UCG014 group* increased after a methionine-choline deficient diet in

mice[40] and after a high-fat-diet supplemented with quercetin and resveratrol[41] in rats, but no evidences are available in human studies.

Our study has limitations. First, the relatively small sample size and the high variability in gut microbiota composition among individuals made it challenging to find a microbial profile characterized by more genera discriminating both dietary interventions. Also, the crossover design of this study does not allow to completely discard a residual carry-over effect (despite the lack of statistical significance after testing the carry-over effect over the main outcomes) as well as the potential bias deriving from the unfeasible blinding. Furthermore, we have to take into consideration the known "Anna-Karenina Principle" in human microbiota, which essentially points out that individual gut microbiota composition under specific medical conditions is not able to vary in a common direction after an external stimulus like dietary intervention is applied[42]. So, the lack of a distinct microbial diversity between intervention groups observed in our study could be related to the absence of a common gut microbial behavior. Another important limitation is the use of 16S rRNA sequencing which, in contrast to shotgun metagenomics, does not allow to identification of bacterial species. Finally, conducting a targeted metabolomics analysis limits the perspective to discover new metabolites potentially associated with the identified genera and future untargeted metabolite profiling could be a complementary approach. Our study has also strengths that deserve to be mentioned such as the crossover, randomized, controlled design, able to balance the intra-individual variability in gut microbiota. Also, the long time of the wash-out period avoided the potential carry-over effect.

In conclusion, the present study documented for the first time that following a MedDiet rather than the consumption of nuts in the context of a non-MedDiet, induced a significant increase in *Lachnospiraceae NK4A136* abundance and improved the metabolic risk profile. This genus seems to affect the fecal bile acids metabolism and cadaverine which may account for

improvements in insulin and glucose metabolism. Further intervention studies are needed to understand the effects of different healthy dietary patterns on gut microbiota composition and functionality for the prevention and/or management of cardiometabolic diseases.

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Conflict of interest statement

The authors declare that they have no competing interests.

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Microbiota and Associations with Cardiovascular Risk Factors: Follow-up of a Randomized, Controlled, Feeding Trial in Adults at Risk for Cardiovascular Disease. *J Nutr*. **2020**,*150*, 806 DOI:10.1093/jn/nxz289.

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Table 1. Study participants' baseline and changes of anthropometric and biochemical parameters

Characteristics	All	Mediterranean Diet		Nuts		MedDiet vs Nuts (changes)	
	Baseline (38)	Baseline	Change	Baseline	Change	p value (treat)	p value (treat* per)
Age (years)	51.37 (49.19, 53.55)	51.37 (49.19, 53.55)	--	51.37 (49.19, 53.55)	--	--	--
Weight (Kg)	85.1 (81.54, 88.66)	84.71 (79.71, 89.70)	-0.79 (-1.37, -0.21)	85.5 (80.3, 90.7)	0.02 (-0.48, 0.51)	0.131	0.414
Waist Circumference (cm)	101.83 (98.77, 104.9)	102.71 (99.24, 106.19)	-1.35 (-2.62, -0.08)	100.96 (95.83, 106.08)	1.07 (-2.01, 4.15)	0.583	0.900
SBP (mmHg)	134.88 (131.12, 138.64)	134.74 (129.76, 139.71)	-0.78 (-4.54, 2.98)	135.03 (129.26, 140.8)	-1.88 (-6.23, 2.47)	0.836	0.978
DBP (mmHg)	84.87 (82, 87.74)	84.87 (81.51, 88.23)	-0.82 (-2.81, 1.18)	84.87 (80.11, 89.63)	-2.45 (-5.71, 0.81)	0.583	0.900
Total Cholesterol (mg/dL)	210.21 (200.09, 220.33)	210.21 (196.66, 223.76)	-7.37 (-14.16, -0.57)	210.21 (194.81, 225.61)	-3.66 (-10.51, 3.19)	0.686	0.414
LDLc (mg/dl)	132.53 (123.99, 141.07)	133.32 (122.11, 144.52)	-5.79 (-12.38, 0.8)	131.74 (118.55, 144.93)	-3.5 (-9.74, 2.74)	0.836	0.414
HDLc (mg/dL)	50.58 (47.04, 54.11)	51.32 (46.01, 56.62)	-1.16 (-3.24, 0.93)	49.84 (45.05, 54.64)	0.16 (-1.51, 1.82)	0.283	0.978
VLDLc (mg/dL)	27.11 (23.64, 30.57)	25.58 (20.54, 30.62)	-0.42 (-3.2, 2.36)	28.63 (23.84, 33.42)	-0.66 (-4.00, 2.69)	0.991	0.900
Triglycerides (mg/dL)	135.97 (118.6, 153.35)	128.63 (103.39, 153.87)	-2.42 (-16.11, 11.27)	143.32 (119.22, 167.42)	-3.13 (-19.9, 13.63)	0.991	0.900
Glucose (mg/dL)	101.05 (96.76, 105.34)	101.53 (95.36, 107.70)	-4.05 (-7.48, -0.63)	100.58 (94.45, 106.7)	1.97 (-0.93, 4.88)	0.032	0.900
Insulin (mcUI/mL)	12.54 (10.54, 14.54)	12.99 (10.64, 15.35)	-1.65 (-3.3, 0)	12.08 (8.79, 15.38)	2.61 (-0.05, 5.26)	0.032	0.414
HOMA-IR	3.16 (2.61, 3.71)	3.28 (2.63, 3.93)	-0.56 (-1.05, -0.06)	3.04 (2.13, 3.95)	0.88 (0, 1.76)	0.032	0.414
Lymphocytes (x10^3/µL)	2.20 (2.02, 2.38)	2.16 (1.94, 2.38)	-0.13 (-0.35, 0.09)	2.24 (1.95, 2.52)	-0.01 (-0.12, 0.10)	0.448	0.900
IL-6 (pg/mL)	2.93 (2.29, 3.57)	2.65 (1.76, 3.53)	0 (-0.62, 0.62)	3.23 (2.3, 4.17)	-0.15 (-0.62, 0.32)	0.836	0.414
Zonulin (ng/mL)	40.16 (38.22, 42.1)	40.4 (38.82, 41.97)	1.31 (-0.88, 3.51)	39.92 (36.31, 43.52)	1.07 (-1.32, 3.46)	0.991	0.978
17-items MedDiet score	7.13 (6.49, 7.77)	6.95 (6.22, 7.67)	4.13 (3.28, 4.98)	7.32 (6.26, 8.38)	0.26 (-0.99, 1.52)	<0.016	0.414

All values are given as means (95% CI). Changes in anthropometric and biochemical parameters between dietary interventions were analyzed by using linear mixed-models analysis of variance with intervention groups and periods modelled as fixed factors, baseline values as covariates and subjects as random effect. Abbreviations: SBP; systolic blood Pressure, DBP; diastolic blood pressure, LDLc; low density lipoprotein cholesterol, HDLc; high density lipoprotein cholesterol, VLDLc; very low-density lipoprotein cholesterol, HOMA-IR; homeostatic model assessment of insulin resistance, MedDiet; Mediterranean Diet. [1]Adjusted with the Benjamini-Hochberg False Discovery Rate method.

Table 2. Associations between changes in relative abundances of *Lachnospiraceae NK4A136* and uncultured genera of Ruminococcaceae with changes in cardiometabolic risk factors

	Lachnospiraceae NK4A136						*Ruminococcaceae uncultured*					
	Fixed effects			Random effect			Fixed effects			Random effect		
	B coeff	SE	p-value	ICC grp	SD	p-value	B coeff	SE	p-value	ICC grp	SD	p-value
Glucose (mg/dL)	-0.0003	0.0002	0.176	0.097	9.73e-03	0.143	-0.0001	0.0001	0.181	0.112	9.70e-03	1
Insulin (mcUI/mL)	-0.0008	0.0003	0.017	-0.061	9.71e-03	0.119	0.0000	0.0001	0.966	-0.071	2.35e-07	1
HOMA-IR	-0.0023	0.0011	0.036	-0.066	9.70e-03	0.129	-0.0001	0.0003	0.848	-0.077	2.36e-07	1

Generalized linear regression model for the changes in relative abundances of *Lachnospiraceae NK4A136 group* and *Ruminococcaceae uncultured genera*. Abbreviations: HOMA-IR; homeostatic model assessment of insulin resistance; ICC grp; intra-class coefficient of correlation; SD; standard deviation; SE; standard error.

Table 3. Associations between changes in fecal metabolites and changes in cardiometabolic risk factors.

Cardiometabolic risk factors	Metabolites	Coefficient	Metabolites	Coefficient
Glucose			Cholate	-0.059
			HDCA	-0.076
Insulin	Acetate	0.528	Methionine	-0.167
	Cadaverine	0.491	Serotonin	-0.414
			HDCA	-0.427
			Cholate	-0.463

Abbreviations: HDCA; Hyodeoxycholic acid,

Table 4. Fecal metabolites significantly associated with cardiometabolic risk factors ranked from the highest to the lowest elastic net positive or negative regression coefficients for genera significantly changed across and within interventions

MedDiet vs Nuts		**Nuts**		
Lachnospiraceae NK4A136	***Ruminococcaceae UCG014***	***Lactococcus***	***Roseburia***	***Oxalobacter***
Cholate 0.134	Cholate 0.459	HDCA -0.398	Cadaverine 0.368	Cadaverine -0.271
Cadaverine -0.333	HDCA 0.322	Serotonin -0.807		Cholate -0.430
				HDCA -0.491

Abbreviations: HDCA;Hyodeoxycholic acid

Figure legends

Figure 1. Plots of LEfSe results for between-dietary interventions comparisons of significant relative abundances at genus level. The 2 histograms reproduce the differential composition in changes of relative abundances foreach of the two genera detected as statistically and biologically different between MedDiet and nuts interventions. The horizontal continue and dotted black line respectively represent the mean and median of changes in relative abundances for that selected genera.

Figure 2. Plot of LEfSe results for relative abundances comparisons within the nuts intervention at genus level. LEfSe scores (in absolute value) can be interpreted as the degree of consistent difference in relative abundances between genera before (indicated as pre-treatment) and after (indicated as post-treatment) the nutritional intervention. The histograms thus identifies which clades among all those detected as statistically and biologically differential explain the greatest differences between communities of these two classes.

Figure 3. Changes in fecal metabolites ranked from the lowest to the highest elastic net positive or negative regression coefficients for the Mediterranean dietary intervention

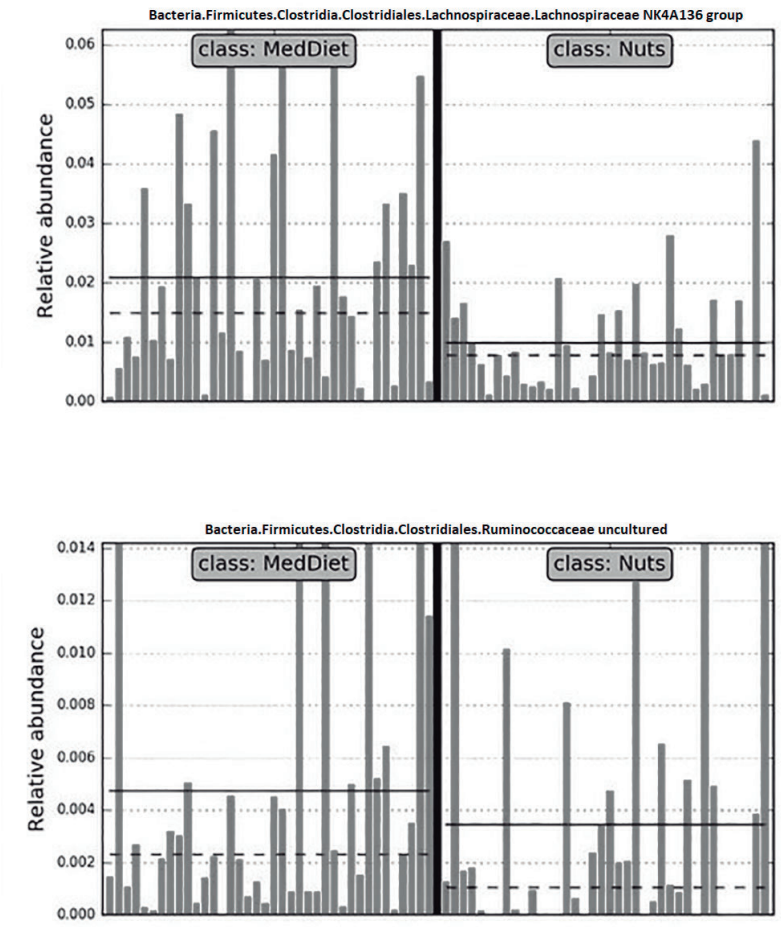

Figure 1. Plots of LEfSe results for between-dietary interventions comparisons of significant relative abundances at genus level.

42x51mm (600 x 600 DPI)

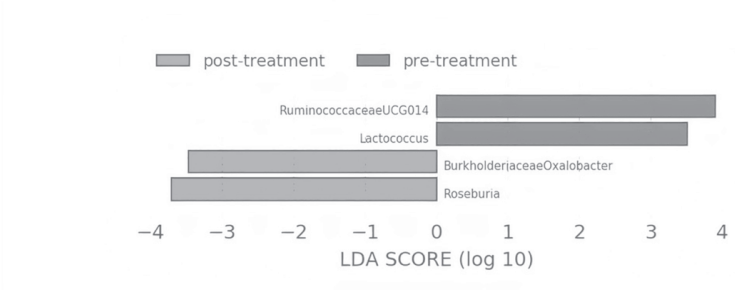

Figure 2. Plot of LEfSe results for relative abundances comparisons within the nuts intervention at genus level.

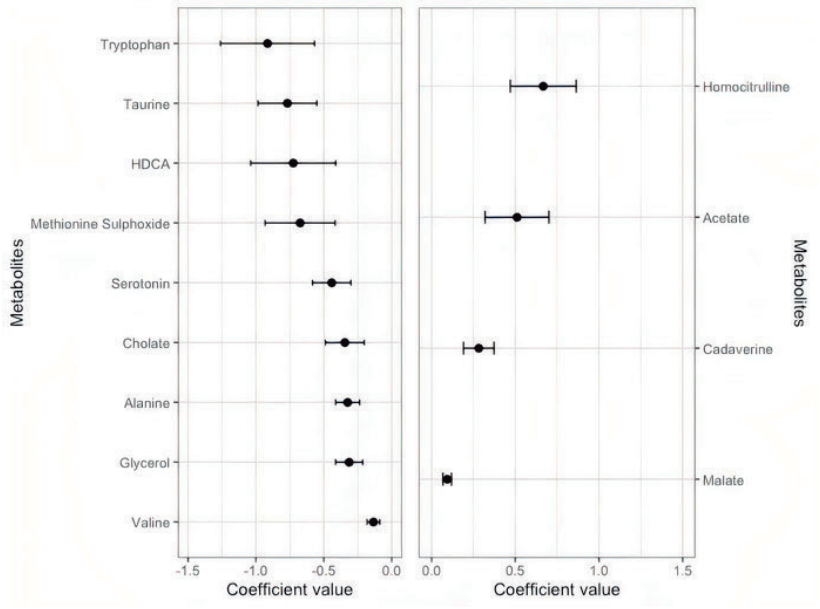

Figure 3. Changes in fecal metabolites ranked from the lowest to the highest elastic net positive or negative regression coefficients for the Mediterranean dietary intervention

58x43mm (300 x 300 DPI)

2. PUBLICATION 2

Title: Effects of the Mediterranean Diet on plasma metabolites, and their relationship with insulin resistance and gut microbiota composition in a crossover randomized clinical trial

Authors: Serena Galié, Jesús García-Gavilán, Christopher Papandreou, Lucía Camacho-Barcía, Pierre Arcelin, Antoni Palau, Antoni Rabassa, Mònica Bulló

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Abstract:

Background & Aims The Mediterranean Diet (MedDiet) may decrease the cardiometabolic risk through modulation of metabolic pathways. Furthermore, the interplay between MedDiet, metabolites and microbial metabolism may improve our understanding on the metabolic effects of this diet. We aimed to evaluate the effect of the MedDiet compared to nuts supplementation on circulating metabolites and their relationship with cardiometabolic health. We further examined whether changes in the metabolomic profiles were associated with changes in gut microbiota composition in a multi-omics integrative approach.

Methods Forty-four adults with Metabolic Syndrome (MetS) participated in a randomized, controlled, crossover 2-months dietary-intervention trial with a 1-month wash-out period, consuming a MedDiet or a non MedDiet plus nuts (50gr/day). Fasting blood and fecal samples were collected at the beginning and at the end of each intervention. Plasma

metabolites (m=378) were measured using targeted metabolomics. Associations of these metabolites with the interventions were assessed with elastic net regression analyses. Gut microbiota composition was assessed by 16SrRNA sequencing. A sparse least regression analysis combined with a canonical correlation analysis was conducted between the plasma selected metabolites and genera in order to identify the relevant dual-omics signatures discriminating the dietary interventions.

Results Changes in 65 circulating metabolites were significantly associated with the MedDiet (mainly lipids, acylcarnitines, amino acids, steroids and TCA intermediates). Importantly, these changes were associated with decreases in glucose, insulin and HOMA-IR. The network analysis identified two main clusters of genera with an opposite behaviour towards selected metabolites, mainly PC species, ChoE(20:5), TGs and medium/long-chain acylcarnitines.

Conclusion Following a MedDiet, rather than consuming nuts in the context of a non-MedDiet was associated with a specific plasma metabolomic profile, which was also related to metabolic improvements in adults with MetS. The identified correlated network between specific bacteria and metabolites suggests an interplay between diet, circulating metabolites and gut microbiota.

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Effects of Mediterranean Diet on plasma metabolites and their relationship with insulin resistance and gut microbiota composition in a crossover randomized clinical trial

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Effects of Mediterranean Diet on plasma metabolites and their relationship with insulin resistance and gut microbiota composition in a crossover randomized clinical trial

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Background & Aims The Mediterranean Diet (MedDiet) may decrease the cardiometabolic risk through modulation of metabolic pathways. Furthermore, the interplay between MedDiet, metabolites and microbial metabolism may improve our understanding on the metabolic effects of this diet. We aimed to evaluate the effect of the MedDiet compared to nuts supplementation on circulating metabolites and their relationship with cardiometabolic health. We further examined whether changes in the metabolomic profiles were associated with changes in gut microbiota composition in a multi-omics integrative approach.

Methods Forty-four adults with Metabolic Syndrome (MetS), (aged 37-65) participated in a randomized, controlled, crossover 2-months dietary-intervention trial with a 1-month wash-out period, consuming a MedDiet or a non MedDiet plus nuts (50 g/day). Nutritional data were collected at the beginning and the end of each intervention period using 3-day dietary records, as well as fasting blood and fecal samples. Plasma metabolites (m=378) were profiled using targeted metabolomics. Associations of these metabolites with the interventions were assessed with elastic net regression analyses. Gut microbiota composition was assessed by 16S rRNA sequencing. A sparse least regression analysis combined with a canonical correlation analysis was conducted between the plasma selected metabolites and genera in order to identify the relevant dual-omics signatures discriminating the dietary interventions.

Results Changes in 65 circulating metabolites were significantly associated with the MedDiet (mainly lipids, acylcarnitines, amino acids, steroids and TCA intermediates). Importantly, these changes were associated with decreases in glucose, insulin and HOMA-IR. The network analysis identified two main clusters of genera with an opposite behaviour towards selected metabolites, mainly PC species, ChoE(20:5), TGs and medium/long-chain acylcarnitines.

Conclusion Following a MedDiet, rather than consuming nuts in the context of a non-MedDiet was associated with a specific plasma metabolomic profile, which was also related to metabolic

improvements in adults with MetS. The identified correlated network between specific bacteria and metabolites suggests an interplay between diet, circulating metabolites and gut microbiota.

The trial was registered in the ISRCTN with identifier ISRCTN88780852, https://doi.org/10.1186/ISRCTN88780852.

Keywords: Mediterranean diet, nuts, insulin resistance, plasma metabolome, gut microbiota

Abbreviations: CVD; cardiovascular disease, GC-QTOF-MS; gas chromatography time-of-flight mass spectrometry, HOMA-IR; homeostasis model assessment of insulin resistance, LC-Q-TOF-MS; liquid chromatography quadrupole time-of-flight mass spectrometry, LC-QqQ-MS; liquid chromatography with triple-quadrupole mass spectrometry, LPC; lysophosphocholine, LPE; lysophosphoethanolamine, MedDiet; Mediterranean diet, MetS; metabolic syndrome, PC; phosphocholine, T2D; type-2 diabetes, TG; triglyceride, TMA; trimethylamine, TMAO; trimethylamine N-oxide, TCA; tricarboxylic acid

1. Introduction

Diet may contribute to the risk of chronic conditions through the modulation of several biological pathways [1]. Therefore, in the era of precision nutrition, a better understanding of the biological consequences following a dietary intervention is required to build a functional readout of the major dietary factors related to health and disease. Circulating metabolites reflect individual dietary intakes, endogenous metabolic processes and other sources of variability in metabolism as the interplay with gut microbiota, which objectively allows assessing the complex metabolic responses to dietary patterns. Previous dietary interventions integrating nutrition with metabolomics have focused on a single nutrient or specific food [2], without accounting for all interactions and failing to consider important aspects such as the substitution effects of other nutrients and the synergistic or antagonistic interactions between nutrients within a dietary pattern.

Accumulating data from epidemiological and clinical trials have demonstrated that consuming a Mediterranean Diet (MedDiet) is a useful dietary strategy for the prevention of cardiovascular diseases (CVD), obesity and type 2 diabetes (T2D) [3]. The cardiometabolic benefits of this dietary pattern could be partially explained by its effects on insulin resistance [4], oxidative stress and inflammation improvements. However, the metabolic pathways through which the MedDiet exerts its beneficial effects has been little investigated. Previous randomised trials have identified changes in specific metabolites after the consumption of foods typically included in the MedDiet such as nuts [5]. On the contrary, few intervention studies exist on the effects of the MedDiet, which is also rich in other types of foods such as vegetables, fruits, legumes, whole grains, red wine and extra-virgin olive oil, on circulating metabolites [6], while the relationship between diet-induced changes in metabolomic profiles and cardiometabolic risk factors remains largely unknown. Furthermore, the role of gut microbiota in influencing host circulating metabolites in the context of dietary interventions has not been explored yet.

In the present study, we examined changes in plasma metabolites after following a MedDiet compared to the consumption of a single healthy food such as nuts in the context of a non-MedDiet among participants with Metabolic Syndrome (MetS) and whether these changes can be mediated by differences in gut microbiota composition in a multi-omics integrative approach.

2. Material & Methods

2.1 Study design and population

METADIET is a randomized, controlled, crossover, non-blinded dietary-intervention trial (**Supplemental Figure 1**) in which eligible participants were community-dwelling adults aged 25-60 years, with overweight/obesity (body mass index (BMI) 25-35 kg/m^2) and MetS according to harmonized ATPIII diagnosis criteria [7], and who regularly consumed a non-MedDiet (scoring <9 in the MedDiet score used in PREDIMED-Plus trial and volunteers with ≥9 were excluded from the trial [8]). The primary outcome of METADIET was evaluating changes in gut microbiota composition after following either a MedDiet or a regular diet supplemented with nuts (unpublished results), while changes in plasma metabolites and cardiometabolic risk factors were secondary outcomes. The exclusion criteria were: following a MedDiet considered as a punctuation ≥9 in the PREDIMED-Plus MedDiet score tool [8], presence of T2D; several chronic diseases (inflammatory, infectious, chronic obstructive pulmonary, neoplasia, endocrine, or hematological diseases), regular consumption of nuts (≥90 g/week); changes in body weight (>5 kg in the last 3 months); non-controlled hypertension; LDL cholesterol >160 mg/dL; triglycerides <400 mg/dL; use of anti-inflammatory, corticoids, hormones or antibiotics; alcohol, smoking or drug abuse; consumption of prebiotics, probiotics or laxatives. Participants were recruited from primary care centers affiliated with the University Hospital of Sant Joan and through advertisements in different media. Written informed consent

was obtained from all study participants. The Institutional Review Board approved the study protocol which accomplishes the ethical standards of the Declaration of Helsinki.

Participants who met the inclusion criteria were randomized, using a computer-generated random-number table, either to follow a MedDiet or to consume 50 g/day of mixed nuts (almonds, walnuts, hazelnuts provided free) in the context of their regular non-MedDiet for 2 months. After a 1-month washout period with no treatment, participants crossed over to the other treatment condition for the last 2 months of the study. During the MedDiet period, trained dietitians instructed participants in personalized face-to-face interviews to follow a Mediterranean dietary pattern. Participants were encouraged to adhere to the 17-point scale MedDiet [8] used in PREDIMED Plus study, emphasizing daily consumption of at least 2 servings of vegetables and 3 fruits, and weekly consumption of ≥3 servings of legumes, ≥5 servings of whole-grain cereals and pasta≥3 servings of fish and seafood and the use of extra virgin olive oil as the main culinary fat. Also, a decreased consumption of red meat and processed foods to less than 1 serving/week, and reduced use of butter and margarine, white bread and sweetened beverages was recommended. Participants were provided with biweekly menus and seasonal recipes to facilitate adherence to the MedDiet intervention. During the nuts intervention period, dietitians did not provide any other dietary advice rather than the consumption of 50 g/day of mixed nuts that were provided by free, and also written culinary advice to include nuts in regular meals with soups, creams or as side food. Adherence to the interventions was assessed either by the validated 17-item MedDiet score [8] or counting the empty nuts-packaging returned at the beginning and at the end of each intervention period, and during two intermediate control visits scheduled after 15 and 30 days from the beginning of each intervention period. Nutritional data was collected in each sampling visit using 3-day dietary records and nutrient and energy intakes were calculated using Spanish food composition tables [9,10]. Biological samples were collected at the beginning and at the end of each

intervention period (**Supplemental Figure 1**). Anthropometric data, blood pressure and biochemical measurements were conducted following regular protocols in the clinical practice and are detailed in the **Supplemental methods.**

2.2 Plasma metabolomics profiling

A total of 378 metabolites were quantified using a multiplatform approach including gas and liquid chromatography coupled to high-resolution mass spectrometry (LC-qTOF-MS, LC-QqQ-MS and GC-QTOF-MS) (**Supplemental Table 2**). The analytical procedures are specified in the **Supplemental methods.** Information about the mean and SD of the metabolites used in the present is shown in **Supplemental Table 2.**

2.3 16S rRNA sequencing and data processing

A detailed description of fecal samples collection, microbial DNA extraction and metagenomic analysis can be found in the **Supplemental methods**. Briefly, microbial DNA from fecal samples collected at the beginning and at the end of each intervention, was performed with QIAmpPowerFecal DNA kit (Qiagen, Germany) with an additional lysing step (FastPrep-24 5G Homogenizer, MPBiomedicals). Hypervariable region V4 from 16S rRNA was amplified using the Ion Metagenomics kit^{TM} (Life Technology, Carlsbad, California).

2.4 Statistical analyses

Descriptive data of participants are presented as means and 95%CI for quantitative variables, and as percentages for categorical variables. Changes in anthropometric, biochemical and nutritional data were analyzed by using linear mixed-models analysis of variance with intervention groups and periods modelled as fixed factors, baseline values as covariates and subjects as a random effect. To account for multiple testing, we adjusted P for treatment and P for treatment * period of the crude and multivariable-adjusted associations with the use of the Benjamini-Hochberg false discovery rate (FDR) procedure [11]. An FDR-adjusted P-value < 0.05 was considered to be statistically significant. The effect size was estimated via the

calculation of Cohen's, which shows the magnitude of the difference in changes in biochemical and anthropometrical parameters between MedDiet and nuts interventions (i.e. Cohen's *d* value of 0.2, 0.5 and 0.8 indicate small, medium and large effect size respectively) [12]. From a total of 378 plasma metabolites profiled, 5 metabolites with missing values >20% were removed and for those with less than 20% we used the random forest imputation approach [13] ("missForest" function of "randomForest" R package version 4.6-14). Metabolites were approximated to a normal distribution with the rank-based inverse normal transformation. Due to the high dimensionality and collinear nature of the data, logistic regression with elastic net penalty was implemented in the "glmnet" (R package, version 3.0-2) (alpha=0.5) to select the metabolites associated with the dietary interventions. We performed 10-fold cross-validation (CV) to find the optimal value of the tuning parameter that results in a mean squared error within 1-SD of the minimum [14]. The performance of the model was examined based on parameters of lambda.min. For reproducibility purposes, regression coefficients were reported using 9-10 iterations of the 10-fold CV elastic regression approach in the whole dataset. We also performed a sensitivity analysis adjusting for energy intake. A multi-metabolite score was calculated as the weighted sum of the selected metabolites with weights equal to regression coefficients from the elastic net regression model. Linear regression models were fitted to examine the association between the derived score and changes in glucose, insulin and HOMA-IR adjusting for age, sex, changes in body weight, dietary interventions and value for the respective outcome traits at the baseline examination. All analyses were performed using R statistical package 3.6.1 (www.r-project.org) (R Development Core Team, 2012). Metabolites selected from the elastic net models were mapped into a metabolic network to identify key pathways and enrichment analysis by using MetaboAnalyst 4.0.

Integration of metabolomics and 16S rRNA sequencing data was performed using DIABLO analysis in mixOmics package of R (version 6.10.9, for which we applied a full design matrix

with a value of 1 to seek for linear combinations of variables from each omic dataset that are maximally correlated between the two dietary interventions. Changes in relative abundances of 151 genera (normalized using clr function from the R package "compositions" package, version 1.40-4) and changes in the 65 selected metabolites were used. The block.splsda function with full weighted design and 10-fold CV was used to identify the optimal number of components for each omic dataset. Model performance was also evaluated using a 10-fold CV approach in order to choose the best number of latent components to be included for each omic dataset. We then evaluated the highly correlated omic variables which were able to discriminate the MedDiet from the nuts supplementation diet. Each model was built on 80% of participants (training) and the remaining 20% was used as the test set. A global overview of the correlation structure at the component level was represented with the plotDiablo function. A circosplot representing the correlations between variables of different types represented on the side quadrants was obtained with the circosplot function. Also, a network analysis was implemented by network function, in order to display the association network for regularized canonical correlation analysis and sparse partial least square regression.

3. Results

Of the 50 participants initially included in the study, 6 dropped out for personal reasons resulting in 44 participants with available metabolomic data (**Figure 1**). No significant differences in participants' baseline characteristics were observed between the two interventions (**Table 1**). Medium effect sizes (Cohen's $d = 0.5$) were observed for glucose, insulin and HOMA-IR, whereas large effect size (Cohen's $d > 1$) was observed for the MedDiet score. The mean±SD for the 17-items MedDiet score was 11.59±2.41 [increase; 4.61 (3.74, 5.48)] after the MedDiet and 9.30±3.54 [increase; 0.36 (-0.82, 1.55)] after the nuts supplemented diet. Significant decreases in the levels of glucose, insulin and the values of HOMA-IR were observed in the MedDiet compared to the nuts supplementation diet (**Table**

1). Significant differences in total lipids, saturated fatty acids (SFA), and protein intake were observed between interventions. (**Supplemental Table 3**). A higher increase of fruits, legumes, fish and a decrease of alcohol consumption was found after the MedDiet compared to the regular diet supplemented with nuts, whereas nuts consumption was significantly increased in the nuts intervention group (**Supplemental Table 3**).

Table 2 shows 65 selected metabolites ranked from the highest to the lowest elastic net positive and negative regression coefficients for the MedDiet. Thirty-five metabolites were positively associated with the MedDiet; hydroxyyperoxide-eicosapentanoic acid (HpEPE), testosterone, phosphatidylcholines (PC) (40:6, 35:1), trimethylamine (TMA), succinic acid, cholesterol esters (ChoE) (17:0, 20:5), taurolithocholic acid, amino acids (threonine, cystathione, histidine, phenylalanine), lysophosphatidylcholines (LPC) (19:0-sn1, 22:6, 16:1), carnitine species (C5-OH, C5:1, C18:2, C2:0, C16:0-OH), triglycerides (TG) (56:6, 56:7, 56:5) and lysophosphoethanolamine (LPE) 20:5-sn1. Among the 29 metabolites negatively associated with the MedDiet, the highest regression coefficients were found for LPE18:3-sn1 followed by sphingomyelin (SM) (36:0, 41:1, 32:2), 9-OxoODE, carnitines (C12:0, C5-M-DC, C12:0-OH-a), taurine and its bile acid, taurocholic acid, linolenic acid-iso2 and -iso1, dehydroepiandrosterone sulfate, androsterone sulfate-iso4, androsterone sulfate-iso2, PCs (33:2, 34:1e, 32:0, 34:2, 32:2), TG 47:0, ChoE 22:5, dihomo-y-linolenic acid-iso2 and hydroxyoctadecadienoic acid (HODE)-iso1, hydroperoxyoctadecadienoic acid (HpODE).

In order to take into account the significant difference in energy intake, we have conducted a sensitivity analysis which shown that 29 metabolites of the 65 previously selected from the unadjusted model were associated to the dietary interventions (**Supplemental Table 4**). The enrichment analysis of the selected metabolites revealed that most of them were involved in amino acids metabolism. The metabolic pathways with the highest impact were related to taurine, glycine, serine and threonine metabolism, phenylalanine and tryptophan biosynthesis,

together with tricarboxylic acid (TCA). A moderate impact was also found for the metabolism of alpha-linolenic acid, glycerophospholipid and unsaturated fatty acid (**Figure 2**). The linear regression models revealed significant inverse associations of the multi-metabolite score with changes in glucose, insulin and HOMA-IR (**Table 3**).

In **Figure 3** we plotted the multi-omic analysis including the selected metabolites from the elastic net regression and the 153 genera depicting gut microbial composition. This analysis includes a total of 38 subjects with available data after filtering procedures in gut microbiota (**Supplemental Figure 1**). No significant differences in the general characteristics of participants included in both analyses were observed (**Supplemental Table 1)**. Considering a correlation cut-off value of 0.2 we were able to find two main clusters of genera differentially correlated with a set of metabolites. The first cluster consisted of an uncultured genus of Lachnospiraceae, *Ruminococcaceae UCG002*, *Lachnoclostridium* and some genera from Prevotellaceae family which were positively correlated with changes of C16-OH, C12:0, C12-OH, PC35:1, PC40:6, and TGs 56:6, 46:7, 56:5 and also with ChoE 20:5. On the contrary, negative correlations with changes in phosphoethanolamine and taurine were found for this cluster. The second cluster, constituted by an uncultured genus from Christensenellaceae family, *Oxalobacter,* Clostridiales family XII, *Ruminococacceae UCG009, Terrisporobacter*, and a genus from Clostridiales family, presented the same associations of the first cluster but with an opposite sign.

4. Discussion

In the present study, we identified changes in several plasma metabolites associated with following either a MedDiet or a non-MedDiet supplemented with nuts in participants with overweight/obesity and MetS. These metabolites mainly included lipid species and acylcarnitines but also amino acids, steroids and TCA intermediates. Importantly, metabolites changes induced by the MedDiet, compared to a single healthy food like nuts in the context of

a regular diet, were associated with improvements in participants' metabolic risk profile, independent of changes in body weight and dietary interventions indicating their promising use in understanding the biological mechanisms behind the effects of the MedDiet.

The effect of the MedDiet on circulating metabolites has been poorly investigated. In a case-cohort study nested within the Prevention with Mediterranean Diet (PREDIMED) trial, participants following a MedDiet had a 1-year significant reduction in several lipid species, mainly PCs, PEs, ChoE and TGs, compared to participants in the low-fat diet control group, although only changes in ChoE remained statistically significant after correcting for multiple comparisons [15]. Similarly, in the Metabolic Syndrome Reduction in Navarra (RESMENA) trial conducted among 72 subjects with obesity and at least two features of MetS, who received an energy-restricted MedDiet or a low-fat diet, displayed significant changes in several lipid species, mainly PCs, LPCs and SMs after 2 months [16]. A more recent cross-sectional study that identified a metabolic profile of adherence to the MedDiet revealed that out of 67 metabolites, 45 were lipids [17]. Accordingly, in our study, following the MedDiet compared with non-MedDiet supplemented with nuts was associated with decreases in PCs and SMs. Previous observational studies have suggested a relationship between elevated levels of PC and increased risk of coronary artery disease and mortality [18], but in the PREDIMED study polyunsaturated PCs with at least 5 double bonds conferred CVD protection [19]. PCs are direct substrates for the formation of SMs by SM-synthase [20], which are hydrolyzed by sphingomyelinases activated by inflammatory cytokines and oxidative stress, to produce ceramides [21]. Whether increases in PC40:6 induced by the MedDiet are related to metabolic benefits requires further investigation. ChoE and TGs with >56 carbon atoms and >3 double bonds have been inversely associated with T2D [22] and in our study were increased after the MedDiet. These lipids could represent a pathway through which the MedDiet may have favorably affected insulin resistance.

The increase in plasma α-tocopherol after the end of the MedDiet suggests a good adherence to this dietary pattern [23]. Furthermore, given that the MedDiet and nuts are high in unsaturated fats, it is not surprising that a large proportion of lipid species consist of PUFAs [17]. Therefore, the increases in plasma LPE20:5 and LPC22:6 after the MedDiet may reflect the trend to a higher intake of fish, since plasma EPA and DHA has been identified as the most suitable biomarker of acute changes in 20:5(n-3) and 22:6(n-3) fatty acids intake [24]. Furthermore, an EPA-derived lipoxygenase metabolite, HpEPE, was highly associated with the MedDiet. Our results are in line with a previous study in which higher fish intake was associated with higher levels of highly unsaturated lipid metabolites containing 22:6 and 20:5 (n-3) [17]. An involvement of the metabolism of lysophospholipids in cardiometabolic health has been suggested, although, findings from recent lipidomic studies of LPC have been controversial [25,26]. In a previous *in vitro* study, HpEPE was found to inhibit platelet aggregation [27]. On the contrary, a positive association with the nuts supplemented diet was found for α-linolenic acid (ALA), which could also explain the increases in LPE18:3 and LPC18:3 after following this intervention. ALA is an essential n-3 fatty acid, mainly deriving from the diet, especially from nuts that has been related to cardiovascular health benefits [28]. Metabolites produced by the linoleic acid metabolism such as 9-OxoODE, 13-HpODE and its catabolic product 13-HODE increased after the nuts intervention, whereas 15-HETE produced by arachidonic acid increased after the MedDiet. Further studies are needed to understand the role of these metabolites on inflammation regulation.

Changes in different acylcarnitines were found to be associated with the dietary interventions indicating a different role of these dietary patterns on the fatty-acids β-oxidation and energy metabolism [29]. Two long-chain and three short-chain acylcarnitines were associated with the MedDiet, while one short- and two medium-chain acylcarnitines were associated with the nuts intervention. Previous studies have suggested that pathways related to acylcarnitine metabolism

can be influenced by diet. In a previous cross-sectional study, a metabolite profile composed of medium- and long-chain acylcarnitines was inversely associated with the Western dietary pattern [30]. On the other hand, long-chain acylcarnitines were associated with veganism in the EPIC-Oxford cohort [31]. In the PREDIMED study, high short- and medium-chain acylcarnitines levels were associated with a higher risk of CVD [32], while short- and long-chain acylcarnitines with higher T2D risk [33].

Besides the impact of the study dietary interventions on lipid metabolism and fatty acid oxidation, changes in amino acid metabolism were also observed in our study. Following the MedDiet was associated with increases in plasma amino acids such as phenylalanine, histidine, cystathionine and threonine. Previous studies have suggested that a Western dietary pattern or an omnivorous diet are associated with increased levels of amino acids like phenylalanine [30,34]. This could be due to the higher content of phenylalanine in animal proteins. In our study, participants assigned to the MedDiet intervention may have increased circulating amino acids concentrations possibly due to the trend for a higher fish consumption as compared to the nuts group. On the other hand, the sulfur-containing amino acid, cystine, derivative of cysteine mainly found in almonds [35] was associated with the nuts intervention group.

Increased plasma concentrations of TCA-related metabolites (i.e., succinate) after the MedDiet compared to nuts group is a novel finding. Recent evidence suggests that succinate is not only a TCA intermediary metabolite but also a by-product of gut microbial carbohydrate and amino acid fermentation [36] with pleiotropic functions, such as resolution of inflammation associated with obesity [37]. Regarding other gut microbiota-derived metabolites, increases in TMA concentrations were found after the MedDiet possibly due to the higher fish consumption observed following this dietary pattern than the non-MedDiet supplemented with nuts. TMA can be generated in the colon from choline or L-carnitine or metabolic retro-conversion of trimethylamine N-oxide (TMAO) to TMA by the gut microbiota [38]. In a study with 40 healthy

young men, consumption of meals producing TMAO provided directly from fish led to a significant increase in postprandial plasma TMA levels. However, eggs or beef consumption, providing choline and L-carnitine, respectively, resulted only in negligible TMA increases [39]. At the same time, the MedDiet was associated with increases in taurolithocholic acid, while the regular diet plus nuts with increases in taurine and its conjugated bile acid (taurocholic acid). Recent animal studies showed an increase in taurocholic acid in mice fed with a high-saturated fat diet [40], which may result in microbiota dysbiosis that can perturb immune homeostasis. Similarly, in our study, the higher intake of saturated fatty acids in the nuts group may explain these findings. In addition, previous in vitro studies have demonstrated that treatment with taurolithocholic acid significantly increased nitric oxide production [41], while taurocholic acid exerted an arrhythmogenic effect [42].

Another novel finding of our study was increased in testosterone after MedDiet and increases in three androsterone sulfate species following the nuts supplemented diet. Whether both diets provide sufficient nutrients for precursors of steroidogenesis [43] is a hypothesis that needs to be examined. Interestingly, obese men with low testosterone concentrations are more prone to metabolic disturbances such as insulin resistance [44]. A recent *in vivo* study also found a relationship between lower plasma concentrations of sulfated steroids, such as dehydroepiandrosterone sulfate and impaired glucose tolerance [45].

The dual-omic analysis demonstrated a correlation between a cluster enriched by medium-and long-chain acylcarnitines, PCs, ChoE and TGs with >56 carbon atoms and >3 double bonds and a cluster of bacteria belonging to Firmicutes (Lachnospiraceae, *Lachnoclostridium* and Ruminococcaceae) and genera from Prevotellaceae family. Genera from the Lachnospiraceae family were associated to a higher MedDiet adherence [46] whereas higher abundances of *Lachnoclostridium* were reported after a 3 months MedDiet intervention [47]. Similarly, plant-based foods diets were associated with a Prevotella enterotype [48]. On the other hand, the

second cluster, constituted of *Oxalobacter* and genera from Christensenellaceae family together with *Ruminococcaceae UCG009* and genera from Clostridiales family may reflect the effect of the nuts supplemented diet on these microbes. Nuts are rich in oxalate, the main substrate for *Oxalobacter formigens*, and their consumption has been related with a higher concentration of gastricoxalate [49]. Christensenellaceae is reported higher in relative abundance in humans with an omnivorous diet, relative to vegetarians [46]. Potential mechanisms through which the identified microbiota may affect circulating acylcarnitines, PCs, TGs and ChoE may involve short-chain fatty acids (SCFAs). Some of the genera (uncultured Lachnospiraceae, *Lachnoclostridium*, *Ruminococcaceae UCG002*, uncultured Prevotellaceae and Christensenellaceae) identified in our study are involved in production of SCFAs, which can be absorbed into the bloodstream and affect lipid metabolism [50].

Our study has several strengths such the crossover, randomized, controlled design, thus balancing the potential intra-individual variability in metabolites and gut microbiota with a longer washout period to avoid a potential carryover effect, even if we cannot completely discard due to the crossover design of the study. With regards to limitations, the relatively small sample size and the high intra-variability of gut microbiota composition could limit to reach statistically significance for some genera. Also, the lack of blinding, despite its unfeasibility, represents a potential bias that should be taken into account. Furthermore, the 16S rRNA analysis does not allow to identify bacterial species, leaving the taxonomy at genus level. Finally, diablo analysis is one of the computational methods for inferring correlation networks which is computationally challenging since sensitivity, specificity and precision have not been evaluated in reference to either real or theoretical data sets and these limitations should be accounted when interpreting our findings.

5. Conclusion

In conclusion, our study demonstrated that following a MedDiet, rather than the consumption of a non-MedDiet supplemented with nuts, was associated with changes in the plasma metabolome that were associated with insulin resistance improvements among participants with MetS. Medium- and long-chain acylcarnitines, two PCs, and TGs and ChoE with >56 carbon atoms and >3 double bonds appeared to be related with a specific gut microbiota composition supporting an interplay between diet, circulating metabolites and gut microbiota.

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Author Contribution

MB designed the research and was the coordinator of subject's recruitment; MB, SG, JG-G, LC-B, PA, AP-G, AR recruited participants and conducted the research, SG, JG-G and LC-B obtained data and conducted the bioinformatics analysis; SG, JG-G and CP conducted the statistical analysis; SG, CP, MB, drafted the paper; all the authors revised the manuscript for important intellectual content, and read and approved the final version.

Conflict of interest statement

The authors declare that they have no competing interests.

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Table 1. Baseline and changes in biochemical and anthropometrical parameters (n=44)

Characteristics	Mediterranean Diet		Nuts		MedDiet vs Nuts (changes)		
	Baseline	Change	Baseline	Change	p treat [1]	p treat*period[1]	Effect size Cohen's *d*
Age	50.73 (48.65, 52.80)	0 (0, 0)	50.73 (48.65, 52.80)	0 (0, 0)	N/A	N/A	
Weight (Kg)	84.81 (83.01; 86.61)	-0.7 (-1.21, -0.19)	84.43 (82.64; 86.22)	-0.02 (-0.47, 0.42)	0.156	0.580	
Waist Circumference (cm)	102.79 (101.49; 104.10)	-1.1 (-2.22, 0.02)	101.77 (100.35; 103.19)	0.74 (-1.93, 3.41)	0.617	0.726	
SBP (mmHg)	134.85 (133.14; 136.56)	-1.4 (-5.14, 2.35)	132.48 (130.74; 134.21)	-1.73 (-6.61, 3.16)	0.894	0.580	
DBP (mmHg)	85.26 (84.13; 86.39)	-0.99 (-2.99, 1.01)	84.35 (82.96; 85.75)	-2.17 (-5.53, 1.19)	0.617	0.639	
Total Cholesterol (mg/dL)	215.09 (210.09; 220.09)	-7.82 (-14.99, -0.64)	212.41 (207.10; 217.72)	-0.16 (-9.04, 8.73)	0.189	0.639	
LDLc (mg/dl)	136.93 (132.86; 141.00)	-6.66 (-13.33, 0.02)	133.27 (128.95; 137.60)	-2.65 (-8.31, 3.01)	0.474	0.381	
HDLc (mg/dL)	50.48 (48.71; 52.24)	-1.23 (-3.13, 0.68)	50.61 (48.84; 52.39)	0.02 (-1.56, 1.61)	0.565	0.793	
VLDLc (mg/dL)	27.68 (25.77; 29.59)	0.07 (-2.47, 2.61)	28.52 (26.91; 30.14)	-1.44 (-4.55, 1.67)	0.617	0.681	
Triglycerides (mg/dL)	138.66 (129.12; 148.20)	0.07 (-12.44, 12.58)	142.84 (134.75; 150.94)	13.25 (-29.51, 56.01)	0.617	0.681	
Glucose (mg/dL)	100.55 (98.61; 102.48)	-3.18 (-6.53, 0.17)	97.45 (95.77; 99.14)	2.25 (-0.47, 4.97)	0.046	0.580	0.53 (0.10, 0.96)
Insulin (mcUI/mL)	14.46 (13.33; 15.58)	-1.44 (-3.1, 0.21)	12.44 (11.33; 13.54)	2.35 (0.03, 4.66)	0.046	0.210	0.56 (0.12, 0.99)
HOMA-IR	3.65 (3.32; 3.98)	-0.42 (-0.96, 0.12)	1.01 (2.73; 3.29)	0.8 (0.03, 1.56)	0.046	0.210	0.55 (0.11, 0.97)
Zonulin (ng/mL)	40.08 (38.93; 41.23)	0.52 (-1.67, 2.7)	40.45 (39.51; 41.40)	1.04 (-1.04, 3.11)	0.770	0.830	
17-items MedDiet score	6.98 (6.61; 7.35)	4.61 (3.74, 5.48)	8.93 (8.46; 9.40)	0.36 (-0.82, 1.55)	0.001	0.381	-1.22 (-1.53, -0.89)

All values are given as means (95% CI). Changes in anthropometric and biochemical parameters between dietary interventions were analyzed analyzed by using linear mixed-models analysis of variance with intervention groups and periods modelled as fixed factors, baseline values as covariates and subjects as random effect. Cohen's *d* indicates the effect size of MedDiet vs. Nuts group. Abbreviations: SBP; systolic blood pressure, DBP; diastolic blood pressure, LDLc; low density lipoprotein cholesterol, HDLc; high density lipoprotein cholesterol, VLDLc; very low-density lipoprotein cholesterol, HOMA-IR; homeostatic model assessment of insulin resistance, MedDiet; Mediterranean Diet, N/A; not available.

[1] Adjusted with the Benjamini-Hochberg False Discovery Rate method.

Table 2. Plasma metabolites ranked from the highest to the lowest elastic net positive or negative regression coefficients for the MedDiet intervention.

Metabolites	Coefficient	Metabolites	Coefficient
HpEPE	0.958	LPE18:3-sn1	-0.649
Testosterone	0.579	SM36:0	-0.563
PC40:6	0.537	9-OxoODE	-0.517
TMA	0.518	C12:0	-0.334
Succinic acid	0.407	C5-M-DC	-0.320
ChoE(17:0)	0.388	Taurine	-0.284
Taurolithocholic acid	0.380	Linolenic acid-iso2	-0.283
Threonine	0.356	SM41:1	-0.282
LPC19:0-sn1	0.306	Dehydroepiandrosterone sulfate	-0.281
C5-OH	0.304	Hydroxyproline trans	-0.236
3-Phosphoglyceric acid	0.291	PC33:2	-0.235
LPC 22:6	0.278	Linolenic acid-iso1	-0.229
Cystathione	0.273	androsterone sulfate-iso4	-0.222
Histidine	0.231	PC34:1 e	-0.218
C5:1	0.219	Taurocholic acid	-0.215
C18:2	0.215	PC32:0	-0.213
Glycoursodeoxycholic acid	0.205	TG47:0	-0.198
Phenylalanine	0.199	ChoE(22:5)	-0.191
C2:0	0.197	PC34:2	-0.188
Glycerol-1-phosphate	0.172	dihomo-y-linolenic acid-iso2	-0.188
TG56:6	0.166	androsterone sulfate-iso2	-0.178
PC38:2	0.161	C12_0-OH-a	-0.172
alpha-tocopherol	0.145	HODE-iso1	-0.140
TG56:7	0.139	HpODE	-0.134
15-HETE	0.131	Cystine	-0.112
ChoE(20:5)	0.128	9.12.13-TriHOME	-0.109
LPE20:5-sn1	0.095	SM32:2	-0.106
Fumaric acid	0.089	Phosphoethanolamine	-0.096
3-Hydroxybutyric acid	0.088	LPC18:3-sn1	-0.078
PC35:1	0.088	PC32:2	-0.059
Nervonic acid	0.075		
C16:0-OH	0.058		
cis-10 heptadecenoic acid	0.041		
LPC16:1	0.026		
TG56:5	0.025		

Abbreviations: ChoE: cholesterol ester; HETE: hydroxyeicosatetranoic acid; HpEPE: hydroxyperoxy-eicosapentaenoic acid; HpODE: hydroperoxyoctadecadienoic acid; LPC: lysophosphatidylcholine; LPE: lysophosphoethanolamine; OxoODE: oxo-octadecadienoic acid; PC: phosphatidylcholine; SM: sphingomyelin; TG: triglycerides; TMA: trimethylamine; TriHOME: trihydroxyoctadecenoic acid

Table 3. Linear regression analysis examining the associations of 1-SD increase of multi-metabolite score with changes of main cardio-metabolic parameters.

Changes	(Mean±SE)	p value
Glucose (mg/dL)	-0.72± 0.34	0.020
Insulin (mcUI/mL)	-0.57± 0.24	0.010
HOMA-IR	-0.56 ± 0.23	0.010

Values are given as means ± standard error. Model was adjusted for sex, age, weight changes, dietary interventions and value for the respective outcome traits at the baseline examination.

Figure legend

Figure 1: Flow Diagram of the study design METADIET

Figure 2. Metabolite set enrichment analysis

Over representation analysis (ORA) was implemented in the enrichment analysis of MetaboAnalyst 4.0 using the hypergeometric test to evaluate whether a particular metabolite set is represented more than expected by chance within the metabolites previously selected. On the left there is a bar-plot representation of this test and on the right the same information is plotted in a network with a clustering of the identified metabolic pathways

Figure 3. DIABLO graphical and numerical outputs on dual-omics analysis

a) Sample scatterplot from plotDiablo displaying the first component in each data set (upper diagonal plot) and Pearson correlation between each component (lower diagonal plot); b) Loading plot of each feature selected on the first component of both data sets, with color indicating the class with a maximal mean expression value for each feature; c) Circos plot of the final dual-omics signature; d) Relevance network visualization of the selected features at a correlation cut off value of 0.2.

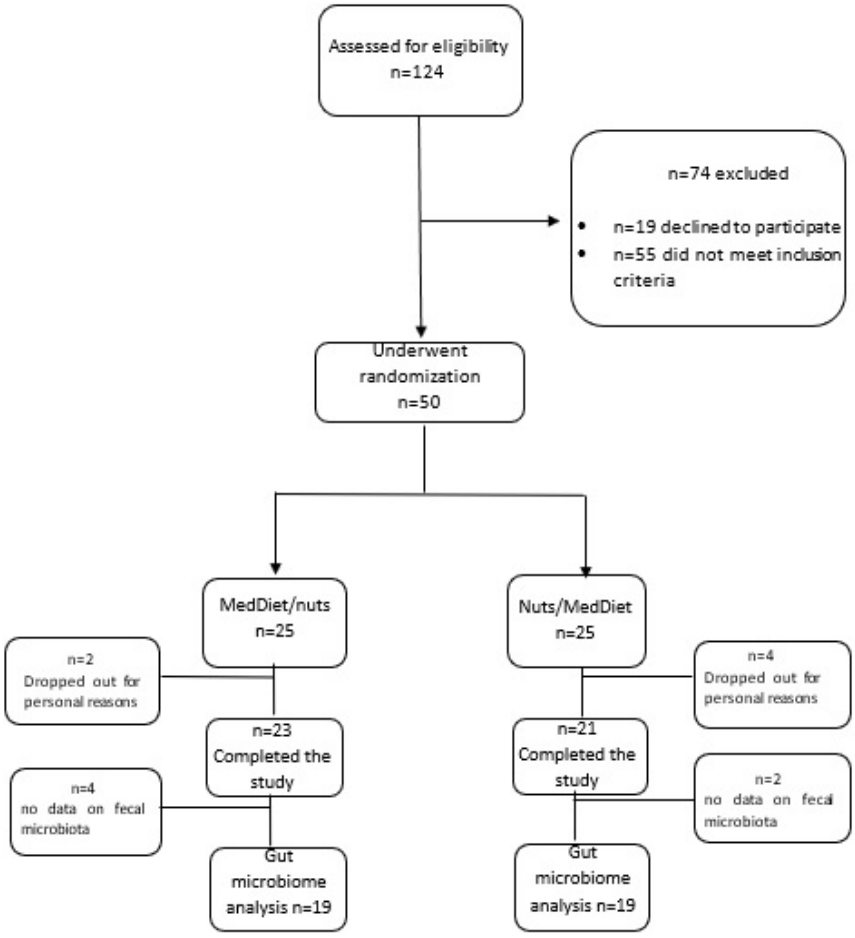
Assessed for eligibility
n=124
n=74 excluded
n=19 declined to participate
n=55 did not meet inclusion criteria
Underwent randomization
n=50
MedDiet/nuts
n=25
Nuts/MedDiet
n=25
n=2
Dropped out for personal reasons
n=4
Dropped out for personal reasons
n=23
Completed the study
n=21
Completed the study
n=4
no data on fecal microbiota
n=2
no data on fecal microbiota
Gut microbiome analysis n=19
Gut microbiome analysis n=19

Figure 1

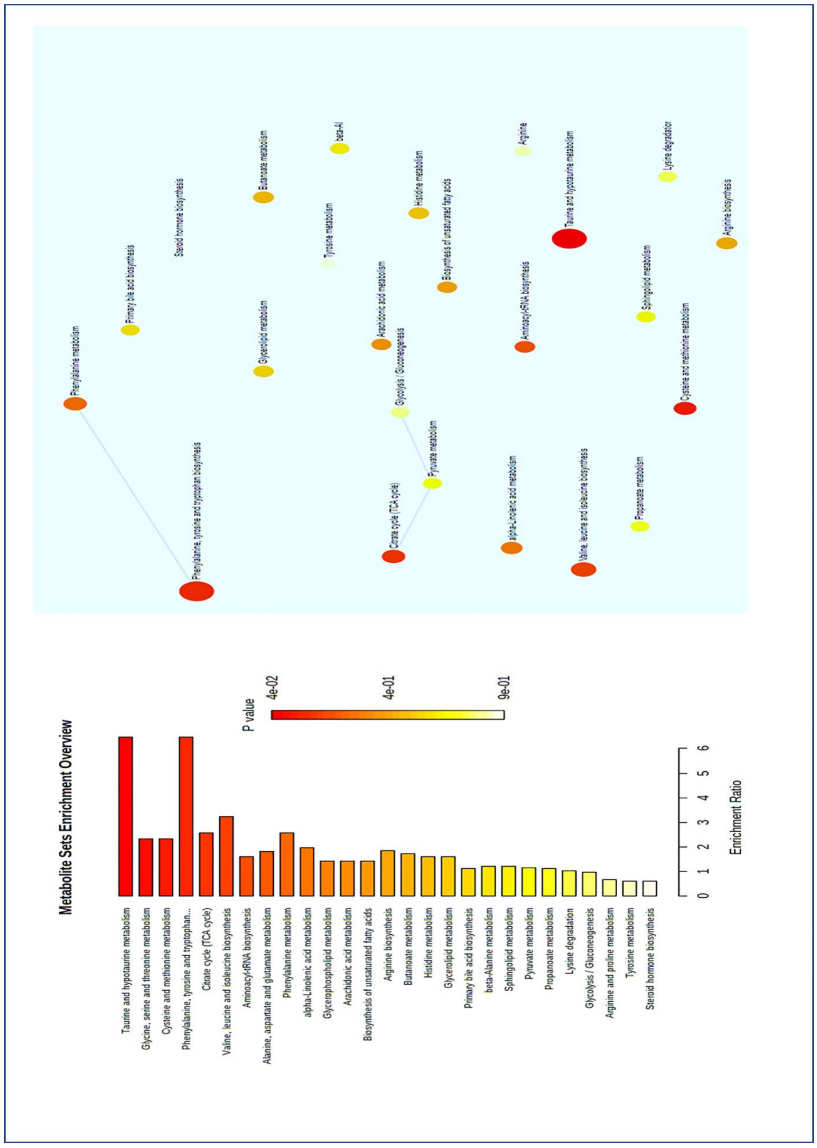
Metabolite Sets Enrichment Overview
P value
4e-02
4e-01
9e-01
Enrichment Ratio
0 1 2 3 4 5 6
Taurine and hypotaurine metabolism
Glycine, serine and threonine metabolism
Cysteine and methionine metabolism
Phenylalanine, tyrosine and tryptophan...
Citrate cycle (TCA cycle)
Valine, leucine and isoleucine biosynthesis
Aminoacyl-tRNA biosynthesis
Alanine, aspartate and glutamate metabolism
Phenylalanine metabolism
alpha-Linolenic acid metabolism
Glycerophospholipid metabolism
Arachidonic acid metabolism
Biosynthesis of unsaturated fatty acids
Arginine biosynthesis
Butanoate metabolism
Histidine metabolism
Glycerolipid metabolism
Primary bile acid biosynthesis
beta-Alanine metabolism
Sphingolipid metabolism
Pyruvate metabolism
Propanoate metabolism
Lysine degradation
Glycolysis / Gluconeogenesis
Arginine and proline metabolism
Tyrosine metabolism
Steroid hormone biosynthesis
Phenylalanine metabolism
Primary bile acid biosynthesis
Steroid hormone biosynthesis
Phenylalanine, tyrosine and tryptophan biosynthesis
Glycerolipid metabolism
Butanoate metabolism
Tyrosine metabolism
beta-Ala
Arachidonic acid metabolism
Glycolysis / Gluconeogenesis
Histidine metabolism
Biosynthesis of unsaturated fatty acids
Citrate cycle (TCA cycle)
Pyruvate metabolism
alpha-Linolenic acid metabolism
Aminoacyl-tRNA biosynthesis
Arginine
Valine, leucine and isoleucine biosynthesis
Taurine and hypotaurine metabolism
Propanoate metabolism
Sphingolipid metabolism
Lysine degradation
Cysteine and methionine metabolism
Arginine biosynthesis

Figure 2

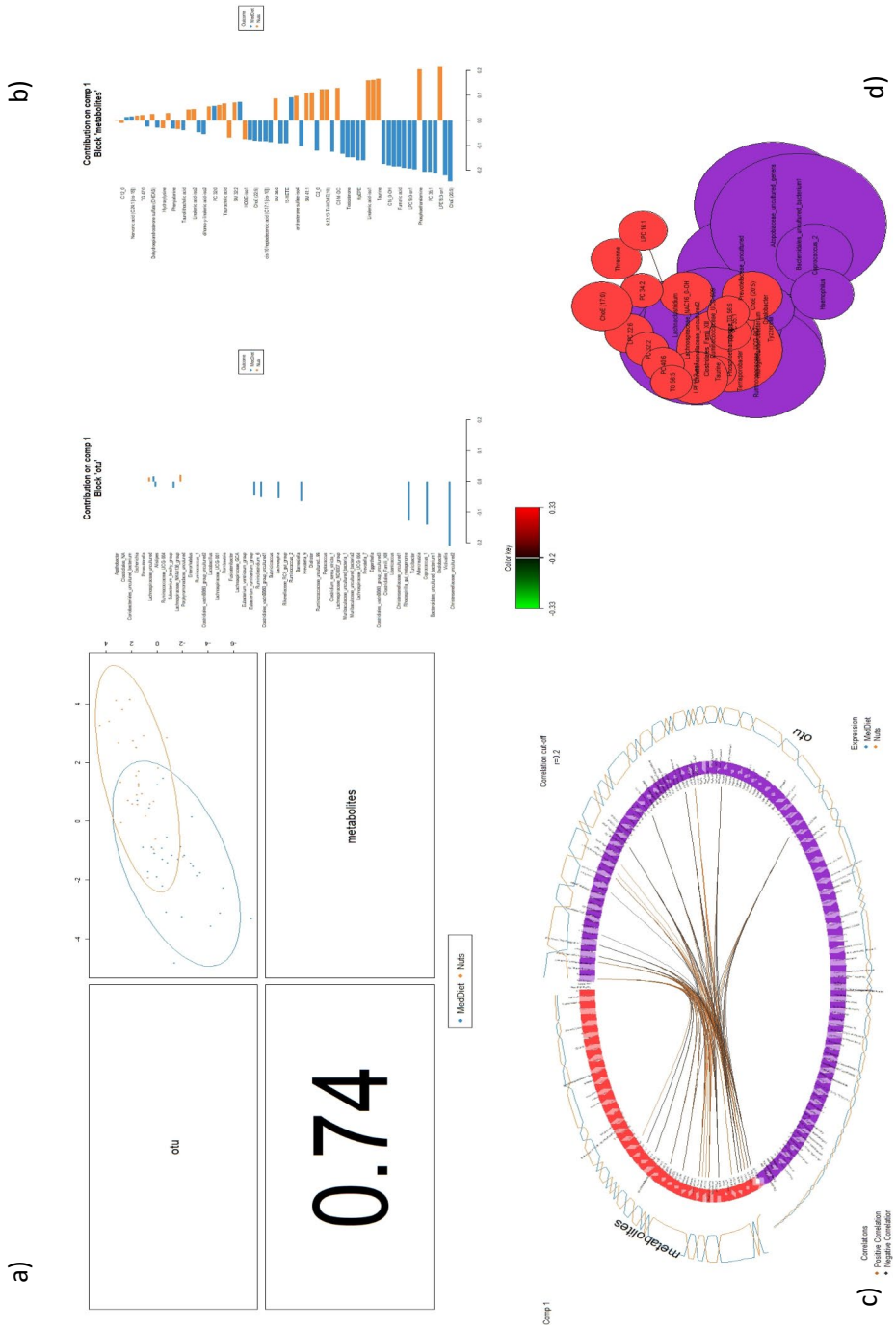
a)
otu
metabolites
0.74
MedDiet
Nuts
b)
Contribution on comp 1
Block 'otu'
Contribution on comp 1
Block 'metabolites'
Color key
0.33
-0.2
-0.33
c)
Correlation cut-off
r=0.2
otu
metabolites
Comp 1
Expression
MedDiet
Nuts
Correlations
Positive Correlation
Negative Correlation
d)

Figure 3

3. PUBLICATION 3

Title: Examining the interaction of the gut microbiome with host metabolism, and its relationship with cardiometabolic risk in adults with metabolic syndrome: cross-sectional analysis in the METADIET study

Authors: Serena Galiè, Christopher Papandreou, […] Mònica Bulló

Abstract:

The high variability in gut microbiota composition comes up with a greater metabolic potential that remains all to be discovered. The role of the host metabolic activity, related or not to microbial communities, on cardiometabolic health is a growing area of interest. This study aimed to unravel the network of host-microbial interactions and correlate the host metabolome with different cardiometabolic parameters.

A total of 47 adults with overweight/obesity and metabolic syndrome from the METADIET study were included in this cross-sectional analysis. Microbiota composition (151 genera) was assessed by 16SrRNA gene sequencing, fecal (m=203) and plasma (m=373) metabolites were quantified with NMR and LC-qTOF. An unsupervised sparse generalized canonical correlation analysis was used to select the most relevant correlated features from the 3 datasets and a network of correlations was constructed to identify the most relevant microbiota-metabolites interactions.

Five different clusters of microbiota-metabolome cross-talk were identified. The most relevant metabolites from feces were primary and secondary bile acids, propionate, microbial-derived

13-methylmiristic acid, arachidonic acid derivatives and linear or branched-chain aromatic amino acids, as well as serum levels of different sphingomyelins, and lysophospholipids and medium to long-chain acylcarnitines. In these metabolic cross-talks, an intermediate role between the gut and the serum metabolome was mainly attributed to members of Ruminococcacea and Christencellaceae families.

Examining the interaction of the gut microbiome with host metabolism, and its relationship with cardiometabolic risk in adults with metabolic syndrome: Cross-sectional analysis in the METADIET study

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Introduction

The metabolic variability derives from a complex and dynamic interaction between endogenous metabolism, environmental factors and the gut microbial ecosystem. Although gut microbiota is considered quite stable in adulthood, several modifiable factors can modulate its composition and activity. Whether these changes could compromise health is not well understood yet[1]. Therefore, a better understanding on how microbial communities affect or modify the complex environment of the human organism is a promising approach for the design of preventive and therapeutic strategies in different conditions[2]. In this sense, previous studies have suggested a role of some gut microbiota-derived metabolites, like bile acids and short-chain fatty acids (SCFAs), branched-chain amino acids (BCAAs), trimethylamine N-oxide, tryptophan and indole derivatives in the host-microbial cross-talk[3,4]. Furthermore, several fecal and circulating metabolites have been associated with clinical features associated with cardiometabolic risk[5], but it remains difficult to determine whether they are fully microbiota-derived or if other sources including diet or the host itself, are also involved. Therefore, an integration of metagenomics and metabolomics information has proven to be helpful in enriching the knowledge on microbiota-host interactions[6], however, there arelimited multi-omic analyses conducted right now. Two recent studies confirmed a host-microbial cross-talk by analyzing the correlations of fecal and blood metabolites with gut microbiome

composition analyzed by using both 16S rRNA sequencing and whole metagenomic shotgun sequencing in a large sample of UK adults (TwinsUK)[7,8]. Our previous findings in the framework of METADIET study identified a plasma metabolomic signature of 65 plasma metabolites[9] as a reflection of the Mediterranean diet (MedDiet) intervention compared to nuts supplementation, which was also associated with improvements in cardiometabolic risk factors and changes in a certain microbial cluster.

Therefore, the aim of this study was to decipher the network of correlations between microbial genera, fecal and plasma metabolites using a multi-omics integrative approach, in adults with overweight/obesity and metabolic syndrome (MetS).

Materials and Methods

Study design

This is a cross-sectional analysis nested within the METADIET study. The METADIET is a randomized, controlled, crossover, dietary-intervention trial comparing MedDiet *vs* nuts supplementation in the context of a habitual non-MedDiet conducted in 50 adults with overweight/obesity and MetS. Detailed information of the clinical trial can be found elsewhere[9]. Community-dwelling adults aged 30-65 years, with a body mass index (BMI) of 25-34.9kg/m^2 who met at least three of the five diagnostic criteria of MetS and who regularly consumed

a non-MedDiet were included in the study. Subjects were excluded if they suffered from type 2 diabetes, chronic diseases, had secondary obesity or related pathologies, non-controlled hypertension, LDL-cholesterol>160mg/dL, triglycerides>400mg/dL, followed specific pharmacological treatments (anti-inflammatory, corticoids, hormones or antibiotics), were alcohol or drug abusers and consumed prebiotics, probiotics or laxatives. Written informed consent was obtained from all study participants. The Institutional Review Board approved the study protocol which accomplishes the ethical standards of the Declaration of Helsinki.

Anthropometric and biochemical measures

General data on weight, height, waist circumference and blood pressure were determined using standard clinical procedures. Blood and fecal samples were collected before any intervention and in fasting conditions. Glucose and lipid profile were measured using standard enzymatic automated methods. LDL-cholesterol was estimated using the Friedewald formula in subjects with triglycerides<400mg/dl. Circulating insulin levels were measured by commercial ELISA (Deltaclon SL, Spain). The homeostatic model assessment of insulin resistance (HOMA-IR) was estimated[10]. Participants were instructed to collect stool samples in hermetic sterile-flasks and freeze immediately at -20°C. Frozen

samples were delivered to the laboratory within 1-2 days after collection and stored at -80°C.

Fecal metabolomics profiling

The fecal metabolomics profiling included 226 metabolites derived from a dual analytical approach (**Supplementary Table S1**). Ninety-four metabolites were quantified by a targeted analysis, using Nuclear Magnetic Resonance (NMR) and liquid chromatography coupled to triple quadrupole mass spectrometry (LC-qTOF), while 132 metabolites were analyzed and semi-quantified with an untargeted approach. Particularly for the targeted analysis, NMR-based metabolic profiling of 37 metabolites included SCFAs, alcohols and organic acids, while LC-qTOF was used for determination of 16 bile acids and 41 amino acids. The analytical procedures are specified in the **Supplementary methods**.

Plasma metabolomics profiling

378 metabolites were analyzed by different analytical platforms (**Supplementary Table S2**). LC-QqQ was used to analyze TMAO and derivatives, acylcarnitines, amino acids and serotonin. LC-qTOF was used for the lipidomic analysis. Measurements of total fatty acids together with organic acids and sugar metabolites, mainly belonging to TCA cycle, were obtained by a GC-qTOF analytical platform. A further description of the analytical procedures can be found in the **Supplementary methods.**

16S rRNA sequencing

Fecal DNA extraction was performed using QIAmpPowerFecal DNA kit (Qiagen, Germantown) with a previous 5-minute lysis step (FastPrep-24-5G Homogenizer, MP Biomedicals). The 16S rRNA gene was amplified (Ion Metagenomics kitTM (Life Technology, Carlsbad, California)) performing 2 separated PCR reactions with two primer sets (to amplify different hypervariable regions of 16S rRNA: V2, V4, V8 and V3, V6-7, V9. Amplicons were processed to obtain DNA libraries (Ion Plus Fragment Library kit and Ion Xpress Barcodes Adapters, 1-64 (Life Technology, Carlsbad, California)) and adapter-ligated and nick-repaired libraries were purified (CleanNGSkit, CleanNA, Waddinxveen, Netherlands). The libraries were further amplified (Ion Plus Fragment Library kit) and quantified with Bioanalyzer (Agilent DNA 7500 Reagents, Agilent Technologies, Santa Clara, California) Equimolar amounts of all the libraries (60 µM) were sequenced in 4 different runs with Ion 520 and Ion 530 Kit-Chef (Life Technologies, Carlsbad, California, EUA) in a S5 sequencer from Ion Torrent platform. Fastaq data from sequencing were pre-processed with an adapted in-house script[11] in order to split only forward reads of each sample data into 6 subsets of 6 hypervariable regions. Forward reads from V4 region were used for this study. Quality control, length filtering at 280bp and denoising of sequences other than taxonomy assignment were performed in QIIME2 software package using the latest version of Silva 132 as 16SrRNA gene classifier database. Finally,

a further filtering step of OTU (Operational Taxonomy Units) table at 10% prevalence cut-off at the taxonomic level of genera was achieved in R (using phyloseq package functions).

Statistical analysis

Twenty-three fecal metabolites were removed from the analysis due to the high number of missing values (>20%), and a total of 203 metabolites were included. In those metabolites with less than 20%, missing values were imputed using the random forest imputation approach ("missForest" function of "randomForest" R package version 4.6-14). The concentrations of metabolites were approximated to a normal distribution with the rank-based inverse normal transformation.

Five over 378 plasma metabolites were removed because of the high number of missing values (>20%) and the remaining missing values were imputed using the same approach as above. Rank-based inverse normal transformation was used to normalize their concentrations.

Baseline values of absolute abundances of OTU table at the taxonomic level of 151 genera were center-normalized with clr function from "composition" package on R (version 1.4-40). Due to the high dimensionality and collinear nature of the data, a Sparse Generalized Canonical Correlation Analysis (SGCCA) in an unsupervised mode was conducted to select the most relevant variables from three omics datasets (fecal metabolites, plasma metabolites, 16S rRNA).

The tuning procedure to identify the optimal number of components for each omic dataset was performed by a separated performance analysis on a dual-omics dataset including fecal and plasma metabolites with a performance function of spls models (perf function in mixOmics package in R, version 6.14.0). A network analysis was implemented by network function with a cut-off correlation value of 0.6[12]. The network was further analyzed in Cytoscape software (version 3.8.2) in order to better visualize the presence of connected components and the relevant associations between fecal and plasma metabolites and microbial genera. A prefuse force-directed layout with a color grouping visualization based on the nature of components was selected to better features the clusters of the network. A prefuse force-directed layout in Cytoscape is based on the force-directed-layout algorithm, which uses repulsive forces between nodes and attractive forces between adjacent nodes. All analyses were performed using R, version 3.6.2. All tests were two-sided, and significance was defined as $p<0.05$.

Results

Of the 50 participants initially included in METADIET study, 3 were excluded because of the unavailability of either 16S rRNA sequencing data or fecal metabolomics, resulting in a final total number of 47 participants. The anthropometric and biochemical characteristics of participants are shown in

Table 1. The mean age of participants was 50.6 ± 7.13 years, and the mean BMI was 30.5 ± 2.28 Kg/m^2.

Tri-omics network of correlations between gut microbiota, fecal and circulating metabolites

Figure 1 shows the network deriving from the correlation network analysis with a cut-off point value of 0.6. Ninety-four nodes and 263 edges characterized the resulting network, which displays the most relevant correlations deriving from the sparse generalized canonical correlation analysis between the multi-omics datasets. The network clearly shows 5 separated clusters of hubs, including a total number of 9 genera, 31 fecal metabolites and 41 plasma metabolites. In one cluster plasma levels of phosphocholines (PCs 32:2, 32:1, 34:4, 30:0), triglycerides (TGs 46:1, 46:2, 48:1, 48:2, 48:3), lysophosphatidylcholine (LPC) 14:0, lysophosphatidylethanolamine (LPE) 14:0 and lysophosphatidylinositol (LPI) 14:0 were negatively correlated with a genera from Desulfovibrionaceae family. Regarding the other 4-omics clusters, one cluster was mainly constituted by fecal primary bile acid chenodeoxycholic acid (CDCA) and its derivative secondary bile acid ursodeoxycholic acid (UDCA), fecal propionate, fecal derivatives of arachidonic acid, as well as fecal LPC16:0 and LPC20:4, that were negatively correlated with 3 uncultured genera from Ruminococcaceae family and *Christensenellaceae R7 group*. These same microbial genera were at the same time positively correlated with different sphingomyelin (SM) species in plasma.

Another cluster was mainly constituted by fecal amino acids positively correlated with plasma species of LPCs (16:0, 17:1, 18:2, 18:1, 18:3, 20:5, 22:6), LPIs (18:1, 18:2, 20:4). Plasma levels of LPC 22:6 were also negatively correlated with *Subdoligranulum* genera. The third cluster was constituted by plasma levels of medium/long-chain mostly saturated acylcarnitines (C8:0, C10:0, C12:0, C12:1, C14:1, C14:2) positively correlated with fecal levels of spermidine and cadaverine. Plasma levels of C10:0, C12:0, C14:1, C12:0-OH) were also negatively correlated with 2 genera from Ruminococcaceae family (*Ruminococcaceae UCG09 group* and *Acetanaereobacterium).* The last cluster was formed by different fecal species of fatty acids (myristic acid, methyladipic acid, capric acid and dodecanoic acid) that were positively correlated with plasma levels of different androsterones, including dehydroepiandrosterone sulfate (DHEAS) and with a member of Firmicutes (*Anaerotruncus).* Better visualization of the direction of each correlation is shown in **Supplementary Figures S2, S3 and S4.**

Discussion

While the vast majority of studies on the role of gut microbiota in human health are mainly focused on the identification of specific taxa associated to health outcomes, the potential of gut microbiota metabolism is still unclear. In this secondary analysis conducted in the METADIET study, we identified a network of correlations between certain microbial genera and specific fecal and circulating metabolites that clustered in five different groups of interaction

between host and microbiome metabolism. The identified clusters suggest a potential host-microbiota crosstalk highly related to previous evidences about specific circulating metabolites and cardiometabolic health[13]. A microbial cluster mainly constituted by Ruminococcaceae and Christencellaceae families seem to play an intermediate role in the correlation between circulating levels of SMs and cholesterol metabolism. Indeed, plasma levels of SMs have been previously related to cardiovascular disease risk[14] and cholesterol homeostasis[15], particularly affecting LDL ability to bind surface receptors and subsequent internalization[15]. A growing body of evidence regarding their favorable interaction with sterols indicates SMs as a potential key regulator of cholesterol distribution within cellular membrane. Interestingly, *Christensenellaceae R7 group* and *Ruminococcaceae UCG002,* which we found to be positively correlated with the identified plasma levels of SMs species, have both been negatively associated to VLDL diameter in an observational study conducted in two separated cohorts in Netherlands[16]. Similarly, other studies have also found negative associations of Christensenellaceae family with total cholesterol, LDL and apolipoprotein particles[17,18] suggesting its role in overall cholesterol homeostasis. At the same time, we observed negative correlations between *Christensenellaceae R7 group* and *Ruminococcaceae UCG002* with fecal levels of bile acids UDCA and CDCA. In humans, UDCA is considered a secondary bile acid derived from microbial

conversion of the primary bile acid CDCA into lithocholic acid (LCA) and then into UDCA[19]. A recent study conducted in an Italian elderly cohort succeeded to discriminate individuals with a healthier metabolic profile by a microbial community particularly enriched of Christensenellaceae family members. The individuals characterized by Christensenellaceae-enterotype were associated with healthier visceral lipid composition as well as with a trend in lowering serum levels of CDCA[20], whose harmful impact on cholesterol metabolism has been previously demonstrated[21]. On the contrary, the therapeutical benefits of secondary bile acid UDCA in reducing cholesterol solubilization in blood has have been largely demonstrated[22]. In line with these overall evidences, in our study we confirmed a negative correlation between fecal levels of CDCA and *Christensenellaceae R7 group*, but not with its secondary bile acid UDCA, suggesting its major absorption in the gut. In this cluster, we have also identified a positive correlation between circulating levels of SMs species and fecal 13-methylmyristic acid, also known as 13-methytetradecanoic acid (13-MTD). 13-MTD is a saturated iso-fatty acid that derives from soy fermentation and has been extensively studied for its apoptotic properties, especially in certain cancer cells[23] other than being largely proposed as a marker of adipose tissue turnover[24]. Interestingly, An *in vitro* study proposed the 13-MTD as a precursor for microbial production of poly(hydroxyalkanoates) (PHAs)[25]. PHAs are well-known polyesters that are synthetized by a multitude of diverse bacterial strains,

in the presence of unbalanced growth conditions, where carbon source is in excess but other vital nutrients, such as nitrogen, oxygen and phosphorus are missing. In this same cluster, fecal levels of the SCFA propionate were also related to plasma levels of different SMs species (38:2, 41:2) with the microbial intermediation of *Ruminococcaceae UCG-002*. Despite no specific genera from Ruminococcaceae has been addressed to propionate production pathways[26], we could speculate its intermediation in propionate metabolism. Overall, these observations suggest that the complex cross-talk of this cluster between beneficial microbial communities and SMs in plasma metabolome is involved in cholesterol homeostasis via the modulation of bile acids and 13-MTD metabolisms in the gut.

A group of fecal BCAAs and aromatic amino acids were clustered together with plasma LPC and LPI species, with LPI 22:6 negatively correlated with *Subdoligranulum*. The genus *Subdoligranulum*, from the Ruminococcaceae family, is a Gram-negative, strictly anaerobe, non-spore-forming, butyrate-producing organisms[27]. Interestingly, it has been recently proposed as beneficial bacteria for metabolic health and has been positively correlated with HDL cholesterol[28]. The role of serum levels of BCAAs like leucine, isoleucine as well as LPCs species in obesity and type 2 diabetes[29] and their association with LDL and total cholesterol has been previously demonstrated[29]. However, few studies have been conducted examining fecal concentrations of BCAAs and their

relation with cardiometabolic status. As an example, a recent study conducted in a cohort of obese individuals found both plasma and fecal levels of BCAAs associated with BMI and HOMA-IR suggesting a role of circulating BCAA in insulin resistance development. In our study, the positive correlations between fecal BCAA and different lysophospholipids, suggest the intermediate function of *Subdoligranulum* in the metabolic health of the host, potentially by regulation of lipid metabolism. Indeed, lysophospholipids including LPCs species are involved in different physiological functions, including membrane biosynthesis and signaling pathways[30]. Interestingly, certain polyunsaturated LPC species such as LPC22:6 (which we found to be correlated with total cholesterol levels) were found to be anti-inflammatory, neutralizing the in vivo inflammatory effect induced by saturated LPC16:0[31]. Intriguingly, LPC18:3, which showed the same correlation pattern as LPC22:6, has been previously proposed as an accurate predictor of the efficacy of immune suppressor therapies in case of patients suffering from chronic autoimmune diseases, suggesting its major role in the inflammatory processes. Also, serum levels of different LPCs species, among them LPC 22:6, 18:3 and 20:5, were negatively correlated to BMI and C-reactive protein in obese subjects with significant results in case of LPC 18:3 with BMI and both BMI and CRP in case of LPC 22:6[32]. Thus, we could hypothesize the role of this cluster in promoting the demonstrated anti-inflammatory role of the LPC 22:6 and LPC 18:3. in cardiometabolic health, via modulation of the efficiency of

lecithin-cholesterol acyltransferase, which catalyzes the transacylation of a fatty acid from phosphatidylcholine to free cholesterol in lipoprotein particles[33] and whose deficient activity has been observed in cardiovascular disease[34].

Plasma levels of long-chain acylcarnitines were negatively correlated with fecal levels of cadaverine and spermidine, as well as, negatively correlated with members of Ruminococcaceae family (*Acetanaerobacterium* and *Ruminococcaceae UCG-009*). Plasma levels of long (C12, C14) and medium (C8, C10) chain acylcarnitines with a variable number of carbon atoms, between 8 and 14 are associated with β oxidation of fatty acids[35]. A similar cluster of correlations between medium and long-chain acylcarnitines and both spermidine and cadaverine has been positively associated to BMI in 340 subjects from the Northern Finland Birth Cohort, while carnitine metabolism was correlated with an unknown genus from Firmicutes phylum[36]. *Acetanaerobacterium and Ruminococcaceae UCG-009* are both butyrate-producers belonging to Ruminococcaceae family and have been previously recognized for their potential protective role in high-fat diet-induced obesity in mice[37] Different members of Ruminococcaceae family have been previously correlated with lower levels of medium-chain acylcarnitines, in a randomized crossover clinical trial comparing vegetarians against meat eaters[38]. Besides, some studies have demonstrated the ability of some bacteria to ferment cadaverine for butyrate production[39], somehow supporting our observations

about the co-appearance of Ruminococcaceae members with fecal cadaverine. Therefore, the potential involvement of this cluster of butyrate-producers in energy homeostasis by regulation of fatty acids oxidation is here suggested.

Finally, *Anaerotruncus*, from the family of Clostridiaceae, was highly correlated with fecal levels of dodecanoic acid and methyladipic acid as well as with plasma levels of different steroids species of androsterone sulfate, including DHEAS. Previous studies have observed the involvement of *Anaerotruncus* in the biosynthesis of both steroids and terpenoids in human colorectal cancer[40]. Also, even if a recent *in vivo* study also found a relationship between lower plasma concentrations of DHEAS and impaired glucose tolerance[41] we did not find any significant correlation between plasma levels of steroids and cardiometabolic parameters.

A dual-*omics* cluster was also identified in our study. To our knowledge, this study is the first to find negative correlations between plasma phospholipid species and TGs with 46 or 48 carbon atoms and <3 double bonds with bacteria from Desulfovibrioaceae family. Previous evidence suggest that Desulfovibrionaceae family members might have an adverse effect on dyslipidemia[42], whereas *Desulfovibrio* might promote gut inflammation and impaired barrier function, through the production of endotoxins deriving from sulphate reduction[43] and upregulation of CD36[44], a critical regulator of lipid absorption in the gut. Therefore, further investigations are needed to elucidate

the negative correlations we found between plasma levels of phospholipids and long-chain fatty acids and Desulfovibrionaceae family.

The present study has some strengths that deserve to be mentioned, like being one of the few studies analyzing the host-microbial crosstalk by using the combination of 3 omics, the use of a multi-platform metabolomics analysis, which allowed to combine targeted and untargeted analytical approaches, in order to cover a wide range of metabolites. Also, the availability of a pretty uniform population characterized by MetS, allowed us to relate the host-microbial cross-talk with cardiometabolic risk parameters. Regarding limitations, this is a cross-sectional study in a small-sized population, and no causal relationship between the microbiome and the metabolome or between the metabolome and the panel of cardiometabolic risk factors can be inferred from the identified correlations. Furthermore, the use of 16S rRNA sequencing does not allow to unravel of the taxonomical composition of gut microbiota up to the strain level. A further limitation is that we evaluated a sample of individuals mainly consisting of adults with MetS that could limit the generalizability of our results to other populations. Other larger studies would be necessary to confirm our novel findings, ideally, prospectively.

In conclusion, this study identified a network of interactions between microbiota and both circulating and fecal metabolites revealing 5 distinct clusters characterized by a host-microbial cross-talk in adults with MetS. Plasma levels

of SMs with <44 carbon atoms and 1 double bond were correlated with members of Ruminococcaceae and Christencellaceae families as well as negatively with fecal bile acids and microbial-derived metabolites suggesting the involvement of this cross-talk in cholesterol homeostasis. Circulating levels of anti-inflammatory LPCs like LPC 22:6 and 18:3 were correlated with the beneficial bacteria Subdoligranulum and clustered with fecal BCAA and AAAs suggesting the role of this microbiota-metabolomes cluster in lipid metabolism. On the other hand, plasma levels of long-chain acylcarnitines were negatively correlated with Ruminococcaceae members, as well as negatively correlated with a potential metabolic substrate for butyrate producers' bacteria, suggesting the involvement of this cluster in the regulation of energy homeostasis via fatty acids oxidation. These findings contribute to further our understanding of the interplay between the microbiome and the systemic and fecal metabolic environments that could link with cardiometabolic health.

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analysis; SG, CP, MB, drafted the paper; all the authors revised the manuscript for important intellectual content, and read and approved the final version.

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Conflict of interest statement

The authors declare that they have no competing interests.

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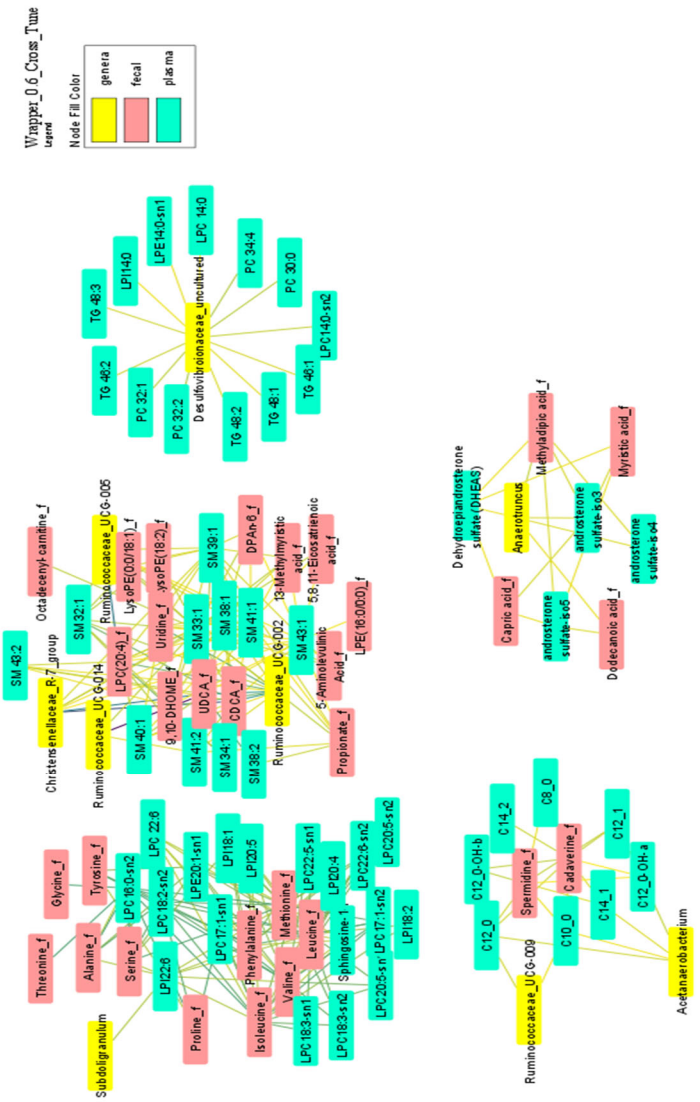
Table 1: Baseline characteristics of study participants

Characteristics	Baseline n=47
Age (years)	50.6 (48.6, 52.6)
Women n (%)	30 (63.8%)
BMI	30.5 (29.9, 31.2)
Waist Circumference (cm)	102.1 (99.3, 104.8)
SBP (mmHg)	135.1 (131.7, 138.6)
DBP (mmHg)	85.0 (82.3, 87.7)
Total Cholesterol (mg/dL)	215.3 (206.1, 224.5)
LDLc (mg/dl)	135.8 (128.1, 143.6)
HDLc (mg/dL)	50.5 (47.6, 53.5)
VLDLc (mg/dL)	28.1 (24.7, 31.5)
Triglycerides (mg/dL)	147.7 (126.3, 169.2)
Glucose (mg/dL)	100.2 (96.5, 103.9)
Insulin (mcUI/mL)	13.4 (11.3, 15.5)
HOMA-IR	3.3 (2.8, 3.9)

All values are given as means (95% CI). Abbreviations: SBP; systolic blood Pressure, DBP; diastolic blood pressure, LDLc; low density lipoprotein cholesterol, HDLc; high density lipoprotein cholesterol, VLDLc; very low-density lipoprotein cholesterol, HOMA-IR; homeostatic model assessment of insulin resistance,

Figure 1: Network analysis of correlation for the tri-omics fingerprint



G. DISCUSSION

1. GENERAL DISCUSSION

This thesis was focused to investigate the effects of MedDiet on gut microbiota composition as well as its potential involvement in metabolic improvement of the host compared to the consumption of a healthy food in the context of a non-MedDiet. To this attempt, we first evaluated the overall microbial diversity and community composition within and between the two nutritional interventions. Secondly, we analyzed the potential associations between the top drivers' taxonomical groups previously identified and the cardiometabolic improvements observed after MedDiet intervention against nuts consumption. Third, we analyzed whether the fecal metabolomics profile could elucidate the cardiometabolic improvements observed after MedDiet and whether their function was related to the most diet-affected microbial genera previously identified. Fourth, we examined the plasma metabolomics fingerprint discriminating the two nutritional interventions and related it with the gut microbial composition at the taxonomical level of genera by a dual-omics approach. Finally, we cross-sectionally explored the host-microbial interaction in our population of adults with metabolic syndrome by analyzing the network of correlations between gut microbial composition, plasma and fecal metabolomics profiles, by using a multi-omics approach.

Our findings, presented in this thesis, demonstrated that, despite the previously demonstrated modulatory effect of nuts consumption on the growth of known beneficial bacteria, a full healthy dietary pattern like MedDiet is promoting a major response on certain microbial genera directly associated to improvements on insulin and glucose metabolisms. Also, the mediation of specific microbial clusters is proposed in the regulation of the circulating metabolomics profile of MedDiet, suggesting

specific key players in the pretty known benefits of MedIDiet on cardiometabolic health.

Different scientific evidences have proposed nuts both as a potential prebiotic food, being able to alter gut microbiota towards the predominance of beneficial probiotic strains[114]-[117] and contemporary attributed them to beneficial properties on cardiovascular and metabolic health[118]. At the same time, MedDiet is a well-known dietary pattern for its properties on reducing cardiovascular risk factors[71] and few evidences are also pointing out its role in guaranteeing gut microbial diversity and selective growth of certain potential probiotic strains, despite most of them derive from observational studies[88],[89],[103],[119]. Conversely, our study firstly succeeded to separately identify, for the first time, the microbiota alterations caused by following a whole dietary healthy pattern like MedDiet compared to the consumption of a single healthy food in the context of a non-MedDiet, thus unraveling the most relevant host-microbial metabolic interactions behind the favorable cardiometabolic changes of MedDiet on the host.

Previous evidences from observational studies based on MedDiet adherence or nuts supplementation in clinical trials failed to find significant differences in global microbial diversity[88][89], even if different potential probiotic strains were consistently found enriched in both of them, similarly to our findings. Importantly, we were able to discriminate two microbial genera that were significantly enriched after MedDiet compared to nuts supplementation, that were *Lachnospiraceae NK4A136 group* and an uncultured genera from Ruminococcaceae family. Despite nuts supplementation was not able to differentially change the microbial abundances with respect to MedDiet, when considering nuts intervention alone, significant increase in the abundances of two

potential probiotic genera like *Roseburia* and *Oxalobacter* were observed. These results confirm some previous findings from different nuts feeding studies and confirm the potential ability of nuts to beneficially alter gut microbiota. However, these alterations were not consistent when compared to MedDiet and, more importantly, were not related to cardiometabolic improvements, which we observed in case of MedDiet against Nuts.

Our study demonstrated a modulatory effect of MedDiet on *Lachnospiraceae NK4A136 group* and the uncultured genus from Ruminococcaceae, both belonging to the phylum of Firmicutes and the class of Clostridia. Lachnospiraceae family belongs to the core of microbiota and, although its controversial function[120], it is mainly composed by SCFAs producers, genetically able to utilize polysaccharides, including starch, inulin and arabinoxylan with substantial variability among species and strains. The potential prebiotic role of *Lachnospiraceae NK4A136 group* was previously investigated both in animals and humans in relation to SCFAs production and on glucose homeostasis[121]-[123]. Recently, a negative correlation has also been evaluated between abundances of *Lachnospiraceae NK4A136 group* and triglycerides levels in the blood[124], suggesting a potential mediator role of this bacteria in lipid homeostasis. Although we did not find any correlation between fecal levels of SCFAs and this genera, we did observe a direct association of this genus with systemic insulin and HOMA-IR, by a down-regulation of fecal levels of cholate. Previous findings, have demonstrated the involvement of Lachnospiraceae and Ruminococcaceae families in the hydroxylation of secondary bile acids from primary bile acid, as source of energy[125]. Therefore, it could be speculated that, in a dynamic situation, with increasing *Lachnospiraceae NK4A136*, the positive association we found between this genus and the

fecal cholate was due to an increased reabsorption of cholate at intestinal level to convert to secondary bile acids. Changes in *Lachnospiraceae NK4A136* were also inversely associated with cadaverine in our study, as well as correlated with increased insulin levels, confirming the suggested role of this metabolite in promoting insulin secretion.

Unravelling the host-microbial interactions which hides behind the cardiometabolic benefits of MedDiet against nuts supplementation in a non-MedDiet context demands the use of a multi-omics approach, which beside the taxonomical composition of gut microbiota could also reveal its systemics consequences[126]. Thus, our approach was to combine the plasma metabolomics profile, discriminating the 2 nutritional interventions, with the overall microbial composition at genus level, in order to untangle the role of gut microbiota in the final end products of host metabolism, as well as their involvement in cardiometabolic risk factors.

Despite the effect of MedDiet on circulating metabolites has been poorly investigated, the observations made in two important clinical trials like PREDIMED[71] and RESMENA[99] coincided with us on its major sequel on lipid metabolism, mainly including PCs, PEs, ChoE and TGs, LPCs and SMs species. Importantly, elevated levels of PCs have been previously associated to increased risk of coronary artery disease and mortality[127], even if this harmful effect seem to be strictly related to the number of instaurations in PCs structure, as was observed in PREDIMED trial, where 5 double bonds were considered the minimum of instaurations to reverse their deleterious effect[128]. Interestingly, PCs are direct substrates for the synthesis of SMs by SM-synthase[129], thus confirming their co-occurrence in our study. At the same time, sphingolipids are known to

promote lipoprotein aggregation, plaque instability and apoptosis, and have been observed in 70-80% of atherosclerotic plaques, together with higher ceramide levels[130]. Consistently, in our study, different SMs species were negatively related to MedDiet intervention. In the same direction, ChoE and TGs with both more than 46 carbons and 3 double bonds, have been previously related to decrease risk of T2D[131] and were positively associated with MedDiet in our study. Interestingly, a set of metabolites including ChoE and TGs with both more than 46 carbons and 3 double bonds, as well as certain PCs and long chain acylcarnitines, that we found positively associated to MedDiet, were forming a connected cluster in the dual-omic network analysis presented in our study. In this connected hub, a cluster of specific microbial genera belonging to Firmicutes phylum and to both Lachnospiraceae and Ruminococcaceae families, as well as some genera from Prevotellaceae family, was showing positive correlations with the mentioned metabolites associated to MedDiet intervention. On the contrary, another cluster of microbial genera including *Oxalobacter*, *Ruminococcaceae UCG009 group* and members from Christencenellaceae family showed negative correlations with the same set of metabolites, suggesting their nuts supplementation-induced enrichment, as was previously observed in case of *Oxalobacter*, significantly enriched after this intervention, irrespectively of MedDiet.

Many other circulating metabolites identified in our study, suggested a strong dietary adherence to interventions, as was in case of α-tocopherol and LPE20:5 and LPC22. Indeed, a higher fish consumption observed after MedDiet in our study, may explain the higher concentration of EPA and DHA related respectively to 20:5(n-3) and 22:6(n-3) fatty acids[132]. In the same line of evidence, derivatives of α-linolenic acid (ALA), an essential diet-derived n-3 fatty acid mainly obtained by nuts, were directly related to nuts supplementation (LPE 18:3, LPC 18:3, 9-OxoODE, 13-HpODE and

its derivative 13-HODE). Finally, confirming the greater beneficial effects of MedDiet, against nuts consumption, on cardiometabolic risk factors, the metabolomic profile related to the dietary interventions in our study, was also positively associated to improvements in insulin and glucose metabolisms.

As we have shown above, the differential effects of the two nutritional interventions on host-microbial interactions could be untangled by the adoption of metabolomics analysis. The evaluation of different source of metabolites, both in feces and in blood, aims to screen the most relevant metabolic axes affected by dietary interventions, thus trying to decipher the specific biological mechanisms behind the potential prebiotic effects of a full healthy dietary pattern like MedDiet.

Finally, to have a better comprehension of the host-microbial crosstalk in our population, we have evaluated the correlation network between fecal and plasma metabolites and gut microbiota. These results showed a network made of 5 separated clusters of highly connected biological components, highlighting 5 different metabolic pathways, in some cases correlated with regulation of cardiometabolic parameters.

As observed in our previous findings, we consistently found a major contribution of different lipid species in the microbial-host interactions identified. However, the contribution of different microbial genera and fecal metabolites was related to each cluster of circulating metabolites. Interestingly, fecal levels of LPCs species and secondary bile acids deriving from cholate, were highly correlated with members from Ruminococcaceae and Christensenellaceae families, which were interconnected with different circulating SMs species, as well Plasma levels of SMs species, seem to particularly affect LDL ability to bind

surface receptors and subsequent internalize cholesterol[133] and their involvement in cholesterol homeostasis and cardiovascular risk has been previously observed. The contemporary mediator role that *Ruminococcaceae UCG-002* and *Christensenellaceae R7 group* have demonstrated in cholesterol metabolism in different observational studies, suggest the involvement of this cluster of microbial-metabolomes in in the metabolic health of the host, by modulation of cholesterol and lipoproteins metabolisms. Interestingly, in this same cluster, we also identified a positive correlation between different SMs species and 13-methylmyristic acid, also known as 13-methyltetradecanoic acid (13-MTD), a saturated fatty acid with the same structure of myristic acid, but with a methyl group on 13th carbon. Normally, this compound constitutes the glycerides of milk and of its derivative dairy products, other than being related to microbial fermentation of soy. 13-MTD is a well-known apoptotic compound[134] with a potential precursor role in microbial production of poly(hydroxyalkanoates) (PHAs), as demonstrated by in an *in vitro* study[135]. PHAs are well-known polyesters that are synthetized by a multitude of diverse bacterial strains, in the presence of unbalanced growth conditions, where carbon source is in excess but other vital nutrients, such as nitrogen, oxygen and phosphorus are missing. Thus, our findings could reflect a loss in the gut metabolic homeostasis due to unfavorable microbial growth conditions. Another cluster was instead composed of different fecal AAAs and BCAAs correlated with LPCs and LPIs species in plasma. In this case, the microbial contribution to host metabolism was demonstrated only in case of *Subdoligranulum*, negatively correlated with LPI 22:6. Previous observations positively associated *Subdoligranulum* with HDL cholesterol levels[136], as well as the contribution of fecal BCAAs in BMI and HOMA-IR in obese people.

Surprisingly, LPC22.6 is a recognized anti-inflammatory marker, with a demonstrate role in prevention of cardiovascular risk, by regulation of lipids metabolism. Therefore, we could hypothesize a major role of this microbial-metabolic cross-talk in the metabolic homeostasis of the host by lipids metabolism modulation. A fourth cluster was instead constituted by medium-long-chain acylcarnitines (C8,10 and C12,14) that were negatively correlated with two members from Ruminococcaceae family (*Acetanaerobacterium* and *Ruminococcaceae UCG-009 group*). As we observed in our previous findings, plasma levels of acylcarnitines with a variable number of carbons, between 8 and 14, have been largely associated to fatty acids oxidation and increased cardiovascular risk factor[137]. The 4th cluster in our network was composed by the presence of a main microbial genera belonging to Firmicutes phylum, *Anaerotruncus*, highly correlated with fecal levels of dodecanoic acid and methiladipic acid as well as with plasma levels of different species of steroids, including (dehydroepiandrosterone) DHEAS. Previous studies have observed the involvement of *Anaerotruncus* in the biosynthesis of both steroids and terpenoids in human colorectal cancer[138]. Scientific evidence suggests the involvement of DHEAS in impaired glucose tolerance[139], thus hypothesizing the intermediation role of the *Anaerotruncus*-steroids communication in glucose homeostasis. To conclude, another dual-omics cluster was constituted by an uncultured genera from Desulfovibrionaceae family constituted a single microbial hub connected with multiple PCs species, LPCs 14:0, LPEs 14:0 and TGs with 48 carbons and various levels of instaurations. Interestingly, the sulfate-reducing bacteria *Desulfovibrio desulfuricans* is a known choline-converter[140],[141], thus suggesting the ability of its belonging family to metabolize compounds containing choline moieties like PCs, LPCs, as we observed in our study

2. STRENGTHS AND LIMITATIONS

The findings presented in this thesis should take into consideration some strengths and limitations that deserve to be mentioned.

This study succeeded to identify the key players of gut microbiota for the beneficial effects of MedDiet on cardiometabolic health against a known beneficial modulator of both gut microbiota diversity and cardiovascular disease. Along this thesis it becames pretty clear that consuming a healthy food like nuts into an overall healthy dietary pattern like MedDiet not only is beneficial for the metabolic status of the host, but it is as well effective in altering the function of gut microbiome towards a beneficial metabolic cross-talk with the host physiological functions. Thus, the major strength of the observations discussed here consists on the contribution of our findings to the growing scientific evidence aiming to unravel the individual and microbial-dependent metabolic mechanisms behind the clinically relevant dietary advices of MedDiet.

To this purpose, the crossover, randomized, controlled design of METADIET study allowed to take into account the intra-individual variability that exists in gut microbiota data, metabolomics profiles and biochemical assessments. Also, the longer time of wash out period was designed in order to avoid or at least to diminish the carryover effect deriving from following two sequential nutritional interventions. The availability of different biological samples and the use of a multiple platform's analysis place this study in the new frontier of clinical nutrition research: the multi-omics generation of precision nutrition.

With regards to limitations, the relatively small sample size and the high inter-individual variability of gut microbiota composition could limit to

reach the statistical significance for some genera. Furthermore, the 16S rRNA sequencing analysis is not so sensitive to describe gut microbiota composition other than the taxonomical grouping at genus level. The multi-omics analysis adopted in these studies consisted on computational challenging machine learning approaches, for which sensitivity, specificity and precision have not been evaluated in reference to either real or theoretical datasets, thus limiting the extrapolation of our results to other populations.

3. FUTURE INSIGHTS

A deeper understanding of the exactly molecular mechanisms that hides behind the associations we have demonstrated between the effects of a specific dietary pattern, like MedDiet, on the host-gut microbiota ecology is needed. The research on gut microbiota is still on its infancy and wheatear more nutritional intervention studies are required to confirm and highlight our observations, a contemporary adoption of synthetic ecology approach is needed to bring gut microbiome research to the next level.

Overcoming the challenging of taking into account the multiple perturbation in computational analysis of gut microbiota data and trying to disentangle the mechanisms behind the great microbial plasticity, there is a urgent need of incorporating culturing and *in vitro* approaches in the design of interventional studies.

H. CONCLUSIONS

The main findings of this thesis consist on the ability of MedDiet to induce greater alteration in gut microbiota as compared to nuts consumption into a non-MedDiet customized diet. The specific conclusions of METADIET are:

1. Following a Mediterranean dietary pattern instead of consuming nuts in the context of a non-MedDiet determine the enrichment of *Lachnospiraceae NK4A136A group* and an uncultured Ruminococcaceae.
2. Higher abundances of *Lachnospiraceae NK4A136A group* are associated to the improvements in glucose, insulin and HOMA-IR levels observed after MedDiet intervention, against nuts supplementation, via the modulation of cholic acid levels in the gut.
3. A specific plasma metabolomic profile made of 65 metabolites is associated to MedDiet intervention against nuts supplementation and this plasma fingerprint is associated to improved cardiometabolic risk factors, in which specific microbial clusters of genera could be acting as intermediates.
4. The cross-sectional multi-omics analysis of fecal and plasma metabolites and gut microbiota composition, offers a picture of the most relevant clusters of correlations which could unravel the host-microbial interplay in the host metabolism of our population of adults affected by MetS.

The findings of this investigation support and complement those of other studies, compelling the importance of following an overall Mediterranean dietary pattern compared to the consumption of a healthy

food in the context of a non-MedDiet to obtain the beneficial contribution of gut microbiota alterations in cardiometabolic health.

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