UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

DIPARTIMENTO DI MEDICINA CLINICA E CHIRURGIA

DOTTORATO DI RICERCA IN
TERAPIE AVANZATE BIOMEDICHE E CHIRURGICHE
XXXIV° CICLO

TESI DI DOTTORATO

EFFECTS OF CHANGING DIETARY HABITS ON THE FECAL MICROBIOME AS AN INNOVATIVE APPROACH FOR THE DEVELOPMENT OF INTEGRATED STRATEGIES OF PREVENTION AND CONTROL OF GLOBAL CARDIOVASCULAR RISK IN THE SALERNO AREA.

Coordinatore del dottorato e Tutor

Ch.mo Prof. Giovanni Di Minno

Dottorando

Veronica Ricci

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EFFETTI DEL CAMBIAMENTO DELLE ABITUDINI ALIMENTARI SUL MICROBIOMA FECALE COME APPROCCIO INNOVATIVO PER LO SVILUPPO DI STRATEGIE INTEGRATE DI PREVENZIONE E CONTROLLO DEL RISCHIO CARDIOVASCOLARE GLOBALE NELL'AREA SALERNITANA.

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ABSTRACT

According to the World Health Organization, coronary artery disease (CAD) is the leading cause of morbidity and mortality in the world with an estimated 17.9 million deaths each year. Several risk factors are involved in the onset and progression of this disease and among them, in recent years, dietary habits and gut microbiota composition have been shown to have a relevant impact. There are many scientific evidences showing the protective role of the Mediterranean diet (MD) in cardiovascular disease especially in CAD primary prevention while less is recognized in secondary prevention. Moreover, alterations in the gut microbiota have been reported as factors responsible for several diseases. Because the mechanisms underlying the protective role of MD have not been fully elucidated, recent attention has focused on the modulation of the gut microbiota by MD in a "protective direction" for the cardiovascular system.

Aim:

The main aim of this study was to improve the knowledge of the mechanisms underlying the beneficial role of MD on the cardiovascular system. In particular, we sought to elucidate the relationship between this dietary pattern and the gut microbiota in CAD patients. Subsequently, in order to carry out a technological transfer from the "Centro Cardiologico Monzino" in Milan to the Check-up Center in Salerno, we conducted the same analysis on a group of healthy subjects or in primary prevention for CVD residing in the Salerno area, who presented a high adherence to DM already at the baseline. Finally, we preliminarily studied the effects of DM on the whole blood transcriptome in CAD patients.

Materials and methods: This study is included in a parallel-group, randomized, open-label, interventional trial to assess the effects of the MD compared to a low-fat diet (LFD) on CAD patients. 103 CAD patients have been enrolled and randomized into the two groups (MD *vs* LFD). Sequencing experiments were performed in order to evaluate diets effects on microbiota composition before (T0), three months after the dietary treatments (T3), and after 1 year (T4) of free diet. Dietary effects have been also evaluated on transcriptome by performing whole blood RNA sequencing on a subgroup of patients at T0 and T3.

Results: The study of the intestinal microbiome of patients included in secondary prevention programs for CAD revealed, already after 3 months of treatment, an increase of several bacterial species producing butyrate, a SCFA with different beneficial effects on the cardiovascular system. In addition, we observed a reduction of bacterial genera such as *Escherichia* and *Klebsiella*, associated with cardiovascular disease and atherosclerotic plaque formation in several studies. These correlations were maintained also after 1 year at the start of study.

The validation analysis between the centers in Milan and Salerno confirmed the efficiency of the techniques used in this work. Furthermore, sequencing of healthy subjects living in a Mediterranean area such as Salerno, again revealed a positive correlation between MD adherence and butyrate-producing bacteria. Preliminary transcriptomic results showed several genes with different expression between T0 and T3 and a correlation of 39 genes with MD-modulated bacteria.

Conclusions and relevance: These data emphasize a positive effect of MD on modulation of the gut microbiome. In particular, finding some beneficial bacterial species positively correlated with MD adherence in both CAD patients and healthy subjects, suggests that, a wider diffusion of the Mediterranean diet should be recommended as a lifestyle change parallel to drug therapy both in primary and in secondary prevention of CAD.

INTRODUCTION

Cardiovascular diseases (CVD) are the main cause of mortality in the world with an estimation of 17.9 million of people each year, according to the World Health Organization. Heart attack and stroke represents 85% of these deaths. CVD include a range of disorders that involve heart and blood vessels damage, such as hypertension, peripheral artery disease, vein disease, stroke and coronary artery disease[1]. In the recent years, the incidence rate of these diseases continues to rise, especially in developing countries, and it has been estimated that CVDs could be the cause of more than 23 million deaths by 2030[2].

This could be due to lifestyle changes, with increased consumption of high-fat foods, high calorie and sugar intake, excessive alcohol consumption, along with a lack of physical activity and increased sedentary lifestyle. These are the main unhealthy habits related to the onset of CVD, responsible for poor quality of life and life expectancy and also a huge cost to the health care system in several countries[3].

As shown in Figure 1, despite primary and secondary prevention initiatives, especially in developed countries, the leading cause of death worldwide is still ischaemic heart disease, which increased the mortality rate in 2019 from 2 million to 8.9 million total deaths.

Ischemic heart disease, also called coronary artery disease (CAD), is a chronic inflammatory disease characterized by remodelling and narrowing of the lumen of the coronary vessels with a consequent reduction in the supply of oxygen and nutrients to the heart. This disease is caused by a hardening of heart vessels, generally defined atherosclerosis.

Clinical manifestations of CAD can include shortening of breath and chest pain of different intensity, called angina. Angina can be classified in stable or unstable based on the symptoms. Unstable angina is characterized by rapid worsening of symptoms with probable development of acute coronary syndrome (ACS), heart failure and sudden cardiac death[4].

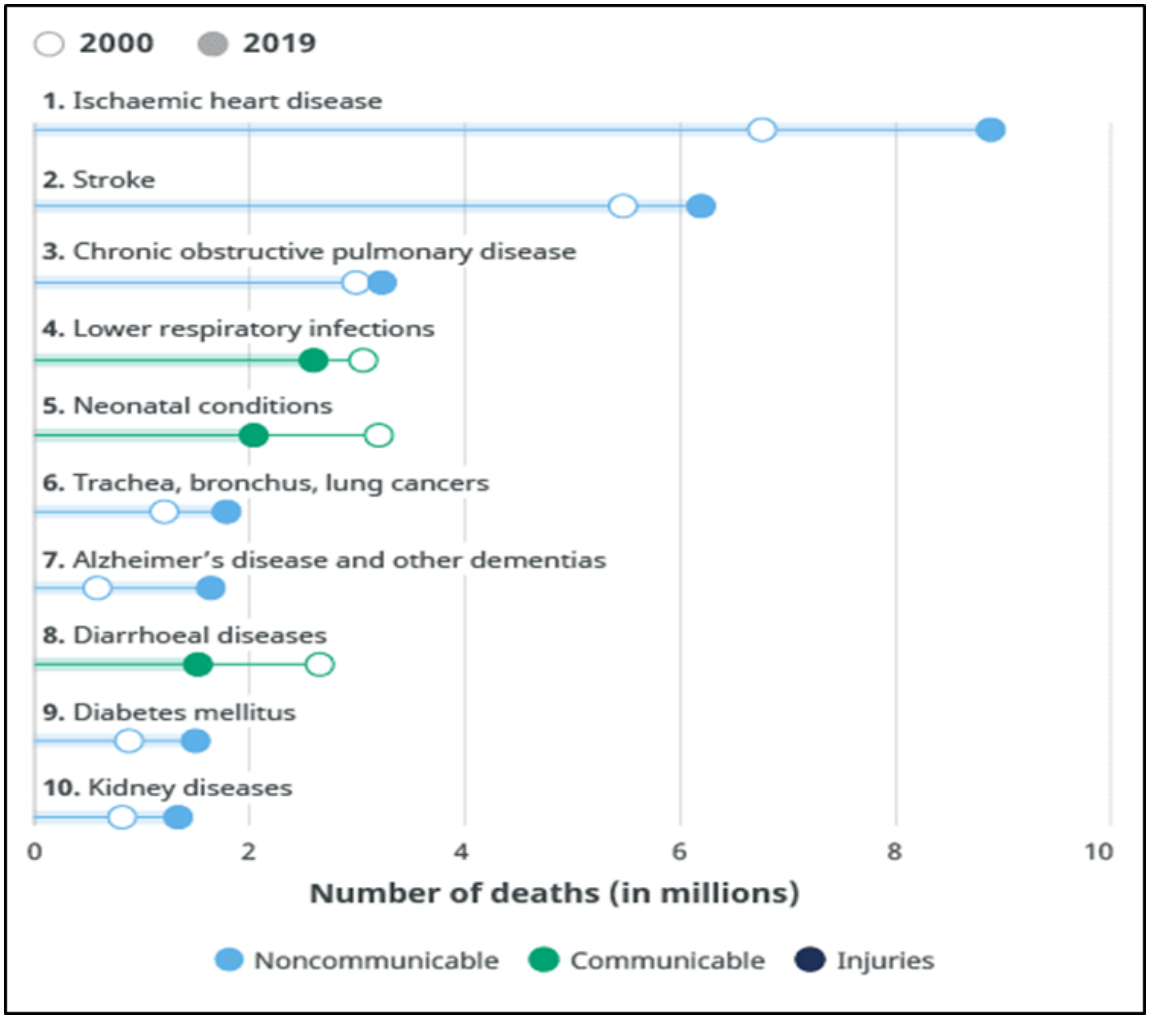


Figure 1: Main causes of death globally. In 2019, the top 10 causes of death accounted for 55% of the 55.4 million deaths worldwide.

Figure from World Health Organization.

DEVELOPMENT OF CORONARY ARTERY DISEASE: THE ATHEROSCLEROTIC PROCESS.

The term atherosclerosis has Greek origins and it consists of the word “athero” meaning gruel and is referred to fatty substance accumulation within the plaque, and the word “skleros” meaning hardening and refers to tissue formation involved in the plaque development.

Atherosclerosis indicates the establishment of an inflammatory process in blood vessels that occurs when calcium deposits settle in the arterial wall leading to the formation of atherosclerotic plaque.

The etiopathogenesis of the atherosclerotic process is complex and multifactorial, and genetic and environmental factors, not yet fully elucidate, are involved[5].

Among the earliest descriptions of atherosclerosis there was the one by Leonardo Da Vinci, who claimed that "the vessels of the elderly restrict the transit of blood through thickening of the tunics."

Towards the end of the 18th century, Rudolf Virchow described the pathogenesis of atherosclerosis, arguing that a pro-inflammatory stimulus causes the initiation of a reparative process with connective cell proliferation and fibrotic fibres deposition leading to fibrous thickening within the vessel wall. This hypothesis was the basis of the Russel Ross injury response hypothesis, which focused on a vascular response to endothelial injury and the subsequent establishment of a chronic inflammatory process[6].

The first sign of atherosclerosis process is the fatty streaks formation in the intimal layer of artery, which is due to a deposition of foam cells in the intimal layer of the arteries. With the interaction of different risk factors, the fatty streak evolves in a fibrous plaque that define the first trigger of atherosclerotic process.

Endothelial dysfunction is the first step in atheroma formation and is characterized by the secretion of several small peptides such as cytokines and growth factors that cause the migration of smooth muscle cells into the vessel lumen and are responsible for the formation of the fibrous cap. Dysfunctional endothelial cells cause retention of lipoproteins in the sub-endothelial space, and this large amount of lipid accumulation can lead to plaque instability with subsequent rupture and thrombotic occlusion [7].

The endothelium has a pivotal role in the regulation of vascular tone and vessel structure. Through a finely regulated process, several constrictor and dilatator elements are responsible of the maintenance of vascular tone. Among the vasodilator substances

released by endothelium there are the nitric oxide (NO), prostacyclin and bradykinin. These endothelial products cooperate synergically to inhibit platelet aggregation and to stimulate fibrinolysis.

The endothelium produces also vasoconstrictor components such as endothelin and angiotensin II, which act also as a pro-oxidant stimulating the production of endothelin [8].

When the endothelium is damaged by external stimuli, the balance between vasodilator and vasoconstrictor molecules is altered resulting in decreased NO production or activity, increased endothelial permeability, platelet aggregation, and production of adhesion molecules that attract inflammatory cells[9].

These activated processes promote the onset of the atherosclerotic event (Figure 2).

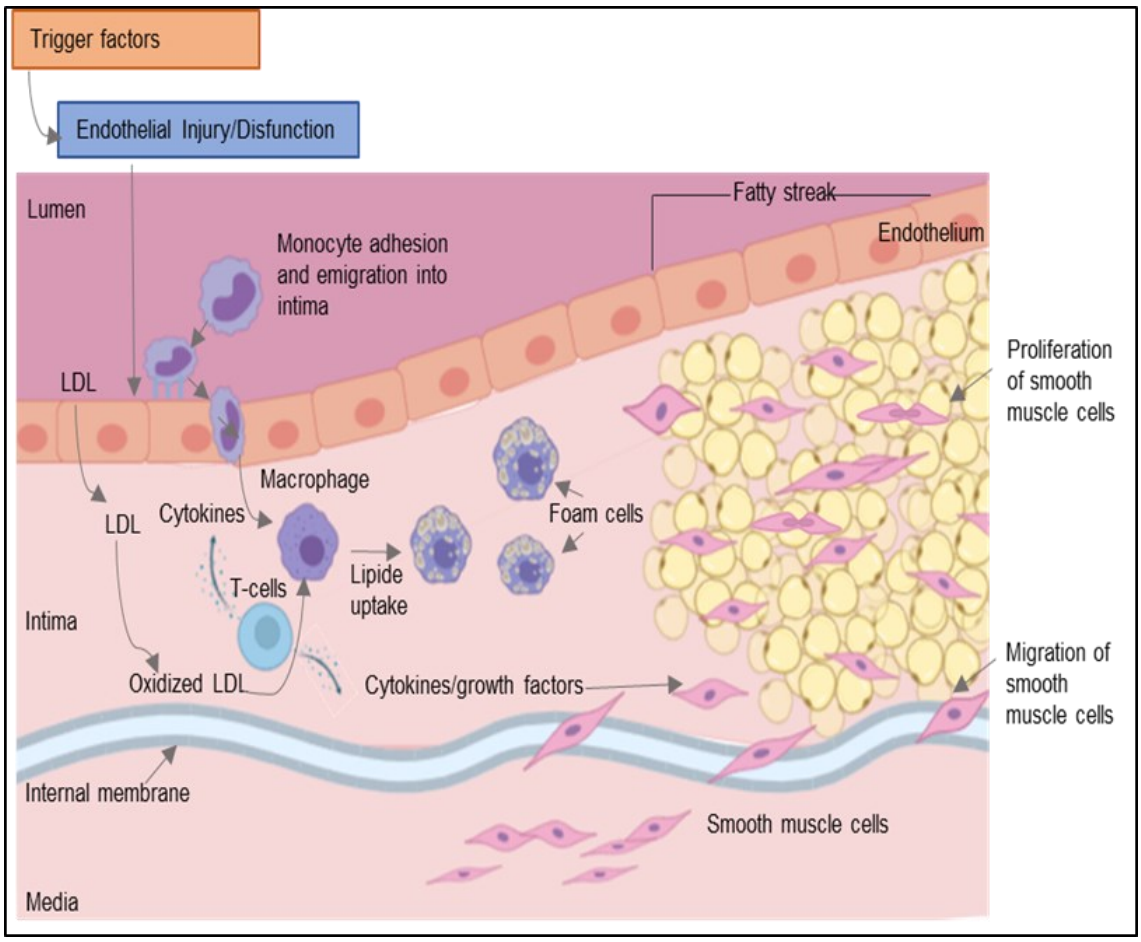


Figure 2: Atherosclerotic plaque formation

A relevant role in atherosclerosis is played by oxidative stress, originated by an altered balance between reactive oxygen species (ROS) production and antioxidant defence mechanisms.

Vascular oxidative stress is known to be a major factor involved in the main atherosclerotic features such as vasoconstriction, vascular remodelling, LDL oxidation and inflammatory events involved in initial steps of atherosclerosis.

The oxidation of LDL to ox-LDL is due to the action of ROS, which is proposed as one of the main causal mechanisms in the atherosclerotic process [10]. Ox-LDL increases endothelial dysfunction by stimulating the secretion of monocyte chemotactic protein (MCP-1), which induces monocyte attraction to the wall. Ox-LDL also acts by inhibiting NO production both through suppression of eNOS activity and by producing a significant amount of superoxide that inactivates NO by forming peroxynitrite [11]. The consequence is the recruitment of circulating monocytes and platelets from the blood to the vessel.

Monocytes differentiating into macrophages, upregulate both toll-like receptors and scavenger receptors activated macrophages and, internalize cell fragments, bacterial endotoxins and OxLDL, leading to lipid accumulation and foam cell formation[11].

The growing factors released from monocytes, induce the proliferation and migration of vascular smooth muscle cells (VSMC) from the medial layer of the artery to subendothelial space. Several monocytes reach the damaged endothelium, and they differentiate in macrophages starting to internalize circulant lipids and lipoprotein, such as LDL, VLDL. Macrophages become lipid-filled cells called foam cells and they aggregate to form lipidic streak, the fist atherosclerotic visible lesion. These formations consisting of monocyte-derived macrophages and T-lymphocytes, are visible and common early in life and may remain so for several years, thickening, or shrinking as they grow[12].

If the triggering stimulus persists along with persistent chronic inflammation, the fatty streak evolves into a more complicated fibro-lipid atherosclerotic plaque because of a continuous migration of smooth muscle cells (SMCs) into the intima that secrete extracellular matrix forming a fibrous lining that covers the plaque forming the fibroatheroma.

A formed plaque contains a lipid core of death foam cells and a coat of deposited collagen fibres and protruding into the vessel lumen represents an obstacle to blood flow[9].

The plaque can be stable with a solid cap formed principally of collagen I and III and this plaque can be static or expands into the lumen of arteria causing a narrowing of vessel lumen called stenosis that compromises blood supply to the myocardium and leads usually to stable angina.

When the fibrous cap is thin and composed of type I collagen, with few SMCs and an abundant lipidic core, the plaque is unstable. This plaque is prone to rupture and exposure of the lipid core to the lumen of the vessel, causes recruitment of platelets to the rupture site with activation of the coagulation process. This ongoing process causes the formation of a thrombus that can completely occlude the vessel resulting in an ischemic attack and stroke [13].

Risk factors involved in coronary artery disease

The exact cause responsible for coronary artery disease is not known, but there is much evidence that certain conditions and habits have a close correlation with the onset of this disease. For several years different epidemiologic studies have tried to clarify the causes responsible of the onset of cardiovascular diseases and in 1957, in a published work of two investigators of Framingham Heart Study, the term 'risk factors' referred to CVD was used for the first time. In this work the authors demonstrated that blood pressure, smoking, high levels of circulant cholesterol and electrocardiographic alterations were associated with an increased risk of incidence of coronary heart disease (CHD) in a Framingham cohort of study in a follow-up period of 6 years[14].

Today, this concept is still valid but new studies and new knowledges have allowed us to discover that many other factors, modifiable or not, are implicated in this complex and multifactorial disease.

The INTERHEART, a case-control study conducted in 52 countries with more than 15000 subjects with a first manifestation of myocardial infarct (MI) and about 14800 healthy controls matched for age and sex, validated the results of the Framingham study. The main finding of this study was to demonstrate that in combination, alcohol consumption, hypertension, dysplasia, diabetes, diet, smoking, lack of physical activity, obesity, and psychosocial factors are responsible for more than 90% of acute myocardial infarctions worldwide[15].

To date, no single factor is indicated as being responsible for the onset of CAD and it has also been demonstrated by the Framingham Heart Study that risk factors tend to group together, affecting morbidity and mortality due to cardiac events[16]

The next paragraphs discuss the major modifiable and non-modifiable risk factors (Figure 3).

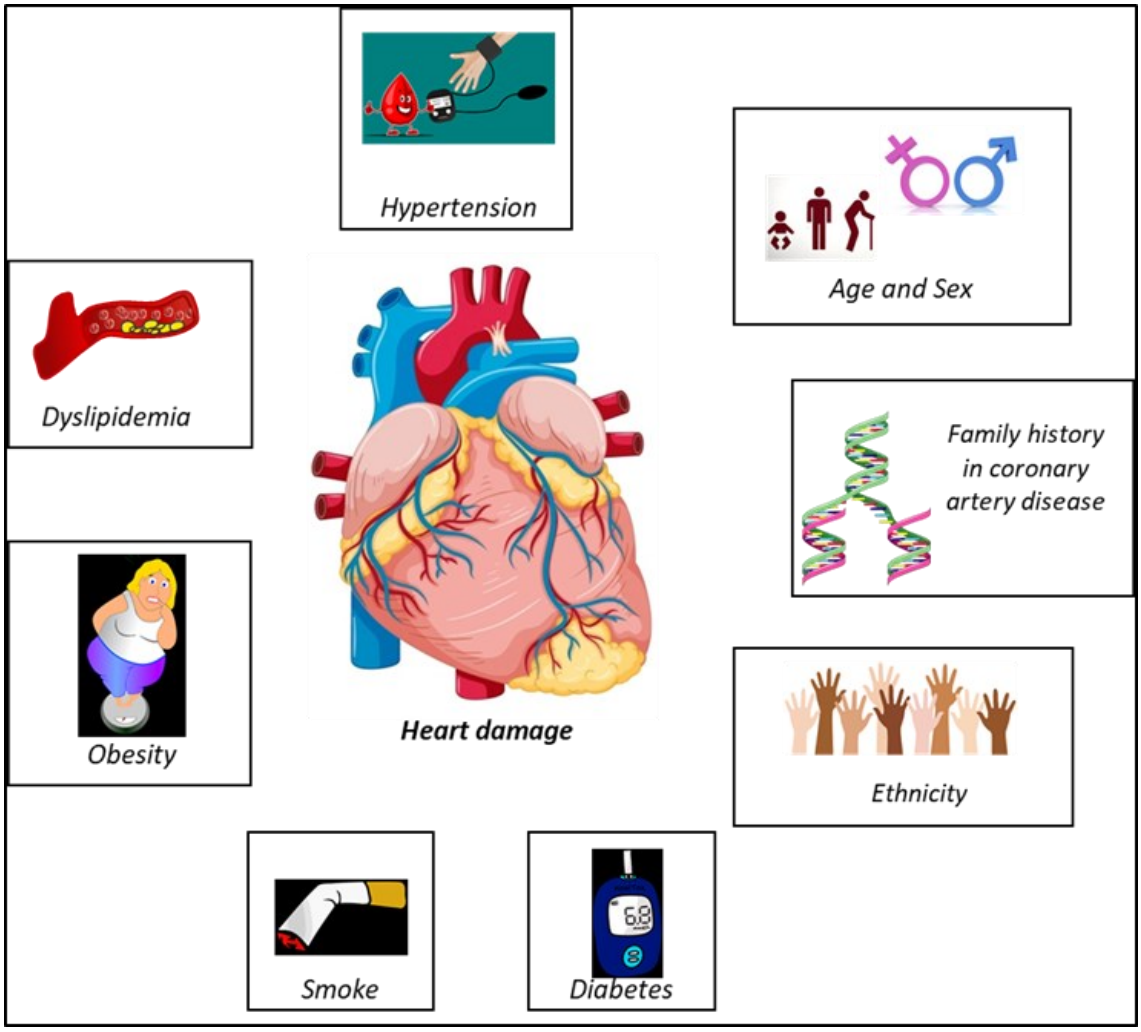


Figure 3: Principal risk factors for CAD

Non-modifiable risk factor for CAD

Age and sex: the risk of onset of CAD increases proportionally with the age and is different between male and female. A study conducted with date collected from Framingham Study cohort after 44 years of follow-up and 20 year of surveillance of their offspring, has allowed to highlight several observations. First, for total coronary events, incidence increases with the age and women have a 10-years delay in the onset compared to men. In contrast, for severe manifestations, such as MI or sudden death, women lag 20 years behind men, but this difference tends to be smaller with increasing age. Moreover, the incidence at 65-94 years compared with 35 to 64 years is more than doubled in men and tripled in women[17].

Another important aspect regards the relation between menopause and CHD. Has been shown that the estrogens production in women during fertile period has a protective effect against the onset of cardiovascular disease increasing vasodilation and angiogenesis and reducing ROS production and fibrosis. The reduced production of estrogens post-menopause might explicate the delay of increased risk of the onset in women compared to men[18].

Ethnicity: Also race and ethnicity have a relevant impact on the onset of CHD, not only for ethnic minority but also for the majority population. For example, CHD incidence is highest among black, Hispanics and American Indian people and lower in Asia or Pacific Islanders. In UK, mortality from ischemic heart disease in south Asian people is higher than in the rest of the population[19]. Levels of glucose intolerance, fasting triglyceride and insulin are more elevated in these people compared to Europeans and this aspect could explain observed differences[20].

Family history in coronary artery disease: Genetic factors substantially contribute on the incidence of CAD. A family history of CAD increases 1.5-2-fold the risk of CAD independently from other risk factors.

A cohort of 2302 male and female participants, composed by the offspring of original Framingham Study cohort, with a paternal or maternal history of premature onset of CVD, was analyzed. The authors found that after 8 years of follow-up, CVD increased 75% in subjects with paternal history and about 60% in those with maternal history of premature CVD[21].

Another study on the same cohort showed that the risk of developing CVD increases of 40% in subject with siblings with CVD[22].

Interesting evidence was also obtained from a cohort study of the INTERHEART Study, which showed that the risk of MI increased if both parents had MI and was higher if they had MI before age 50.

Moreover, this increased risk of CVD was independent of other risk factors, such as age, ratio of total/high-density HDL level, diabetes and smoking status[21].

These types of studies have found genetic mutations implicated in this increased risk. For example, a genetic mutation in the LDL lipoprotein receptor causes

circulating cholesterol levels to be twice as high, predisposing to a higher risk of coronary artery disease[23].

Modifiable risk factor of CAD

Despite the greatest impact on coronary artery disease is ascribed to unmodified risk factors, several modifiable risk factors have a significant role in this pathology. While some of these factors, such as hypertension, are equally distributed among the people, other factors such as diet and pollution, are strongly correlated with country's economic level.

Hypertension: Hypertension is the most prevalent treatable risk factor for CVD that equally affects men and women from all socioeconomic groups. The major issue of this pathology is the long asymptomatic period in which the pathology starts to damage cardiovascular system and kidney. If this pathology is overlooked, it could be the cause of stroke, coronary heart disease and renal disfunction. Diagnosis of hypertension depends on blood pressure measurements and when it is usually above 140/90, is necessary include blood tests, urine analysis and electrocardiographic measures[24, 25].

The correlation between hypertension and coronary heart disease is amply documented.

Blood pressure (BP) is the principal risk factor for the onset of 47% of coronary heart disease[26]. The prevalence of hypertension increases with the age[27] and it has been estimated that 20% of the global population over 60 years old will be affected by this disease in 2030[28].

There is a strong correlation between blood pressure value and CVD. One study showed that systolic blood pressure with a value between 120 and 129 mmHg is associated with lower CVD occurrence and associated mortality risk. In contrast, higher levels of hypertension, with BP >160 mm Hg, increase this risk significantly [29].

There are different mechanisms that explain the role of blood pressure as a risk factor for CHD, such as a physical force on the wall of the vessels and coronary perfusion which are responsible of endothelial damage with increase of its permeability and the consequent LDL cholesterol efflux trough the vessel[30].

Dyslipidaemia: Dyslipidaemia is a metabolic disorder characterized by a consistent increase in plasma levels of cholesterol and triglycerides. More specifically, elevated levels of low-density lipoprotein (LDL) and total cholesterol (LDL-C) or reduced levels of high-density lipoprotein (HDL-C) are observed in this disorder[31].

There are three types of dyslipidaemia, hypercholesterolemia, hypertriglyceridemia and mixed due to both hypercholesterolemia and hypertriglyceridemia. The most common form is hypercholesterolemia defined as a total cholesterol level above 5,0 mmol/L or 190 mg/L and it is responsible for 4.5% deaths in the world [32].

Metabolic disorders are a relevant modifiable risk factor for atherosclerotic disease and their correlation has been widely investigated in several epidemiological studies.

In Cardiovascular Health Study, an epidemiologic study conducted on more than 5000 adults aged 65-98 with an added group of 687 African Americans, showed that levels of HDL-C were inversely associated with the MI risk[33].

In MESA, a Multi-ethnic Study of Atherosclerosis conducted on about 5000 non-diabetic individuals aged 45-84 years, the data showed that combined hyperlipidaemia, hypercholesterolemia and low-HDL levels were associated with multivessel coronary artery disease independently of other risk factors[34].

Moreover a meta-analysis of data obtained from 61 prospective observational studies with more than 55000 vascular deaths showed a positive correlation between total cholesterol levels and mortality for ischemic heart independently from blood pressure levels[35].

Diabetes: There are two main types of diabetes: type 1 diabetes, is an autoimmune disease characterized by progressive and often complete destruction of β cells, that usually causes insulin deficiency and requires treatment with insulin; type 2 diabetes, results from a progressive defect in insulin secretion against a background of insulin resistance and therapy includes lifestyle changes, treatments that increase insulin secretion from the pancreas or increase insulin sensitivity in the peripheral organs, and insulin. More than 90% of diabetic patients have type 2 diabetes[36].

In 1979, data from the Framingham heart study were used to analyse the association between high blood glucose levels and cardiovascular diseases. Based on 20 years of surveillance of the Framingham cohort with cardiovascular event subsequent to diagnosis of diabetes, a two-fold to three-fold increased risk of atherosclerosis was reported. Moreover, the study reported for the first time, a major risk of CVD in women with diabetes compared to men with diabetes[37]. The American Heart Association (AHA) considers diabetes to be one of the top seven modifiable cardiovascular risk factors. On a related note, the AHA reports that at least 68% of people 65 years of age or older with diabetes die with some form of heart disease and 16% die of stroke. In addition, adults with diabetes are two to four times more likely to develop CVD than adults without diabetes[38].
There are multiple mechanisms by which diabetes promote CAD, such as non-enzymatic glycosylation of proteins which interacts with a specific receptor presents on monocyte-derived macrophages, endothelial cells, smooth muscle cells and on all other cells implicates in atherosclerosis process. This interaction causes induction of oxidative stress and proinflammatory response[39].

Obesity: The World Health Organization (WHO) defines obesity as an excessive accumulation of body fat that represents a health risk. The date of WHO shows that 39% of the global population above 18 years of age are overweight and of these, 13% are obese. This risk factor is evaluated by measuring of body mass index (BMI), which is a value obtained from a person's weight divided by the square of his or her height. A person with BMI equal to more 25 is considered overweight while when this value is over 30 is defined obese[40].
Obesity is associated with numerous disease such as CVD, type 2 diabetes, hypertension, and the American Heart Association has reclassified this pathologic condition as a major modifiable risk factor for cardiovascular disease[41]. Among the mechanisms that may explain the link between obesity and CVD, there is the increased production of several cytokines and inflammatory markers due to increased adipose tissue. In fact, this is not only an energy storage organ, but it is also an active endocrine and paracrine organ that releases a variety of reactive substances, such as leptin, adipokine, interleukin-6, and tumor necrosis factor and all of these substances play a role in cardiovascular homeostasis[42]. In addition,

these substances influence insulin resistance, diabetes, fibrinolysis, inflammation, and atherosclerosis[43].

Cigarette smoking: Cigarette smoking is a relevant risk factor in the onset of cardiovascular disease including coronary artery disease, and its damage is related to both active and passive smoking[44]. Has been shown that even smoking 1 cigarette per day, increase the risk to develop myocardial infarct[45].

Moreover, there is growing evidences of adverse effects on circulation system of electronic cigarettes[46].

According to WHO, smoking is responsible for 10% of all CVD cases[47] and it is associated with peripheral vascular disease and atherosclerosis and strongly increased risk of stroke and heart attack. Smoking has interactions with the other major risk factors for CHD, such as serum level lipids, hypertension, and diabetes. Indeed, the simultaneal presence of smoking and another risk factor is estimated to quadruple the risk in comparison to individuals without risk factors[48].

A significantly correlation between smoking and increase of total serum cholesterol, low density lipoproteins and triglyceride serum levels, was showed[49].

The main role of nicotine consumption in the atherosclerotic process involve the endothelial disfunction and the increase in oxidation of pro-atherogenic lipid as well as the reduction of HLD levels promoting the development of a procoagulant environment[50].

CARDIOVASCULAR DISEASE AND DIET

Behind the modifiable factors, lifestyle may have a major impact on the onset of cardiovascular disease. The likelihood of developing CVD is related to an unhealthy diet, smoking habit, alcohol consumption, lack of exercise, and being overweight or obese, whereas healthy eating habits help reduce major risk factors such as hypertension, dysplidemia, and diabetes. Global action plan 2013-2020 proposed by WHO aims to reduce mortality by 25% by 2025, promoting a healthy lifestyle and acting on smoking habits, diet, sedentary and alcohol use[51].

The relationship between diet and coronary artery disease has been deeply investigated.

As mentioned above, oxidative stress plays a key role in the atherosclerotic process, and several epidemiological studies have shown that the intake of antioxidant-rich foods reduces the risk of CVD. In a prospective study by Liu et al, this association was analyzed, and among more than 39000 subjects, the relative risk of CVD in women with higher fruit and vegetable consumption was lower than in women with lower intake. This study suggests a protective effect of these food especially against myocardial infarct[52]. It has been seen that fish consumption also has health benefits and that modest intake of fish, particularly those with higher n-3 fatty acid content, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in particular, reduces the risk of coronary death by 36% and all-cause mortality by 17% [53].

High salt consumption is associated with increased risk of higher blood pressure, and its cardiovascular complications.

A diet principally composed by saturated fats, sodium, high cholesterol levels and sugar, associated with a sedentary life style can increase the possibility to develop atherosclerosis[53].

A higher intake of animal proteins is associated with an increase production of insulin-like grow factor (IGF-1) which is associated with a higher risk of cancer, diabetes and general mortality[54].

The WHO guidelines on cardiovascular disease prevention reported that reducing total fat, saturated fat and salt intake, and increasing fruit and vegetables are likely to have a cardioprotective effect [55].

CARDIOVASCULAR DISEASE AND GUT MICROBIOTA

Although each of the above risk factors has a relevant impact on the incidence of CVD, they cannot explain all cases of CVD.

Several evidences have demonstrated that the composition of the gut microbiota may affect the onset and progression of heart disease.

Gut microbiota: an overview

The human body is colonized by several microorganisms that populate different niches with specific roles in maintenance of human homeostasis. Although the most colonized environment is the gastro-intestinal tract, recent studies have showed the presence of microorganisms also in environments considered “germ-free” for a long time, such as plasma[56].

The human gastrointestinal tract is an anaerobic, nutrient-rich site suitable for hosting a multivariate ecosystem containing approximately 100 trillion microbial cells that make up a well-organized system called the "gut microbiota" which cooperates in close mutual relationship with the host organism.

The gut microbiota includes not only bacteria but also fungi, archaea, viruses and protozoa, and the amount of genomic content in the intestinal tract is referred to as the microbiome and represents a genetic component about 100 times more abundant than the human genome[57].

In the last decade several important studies have set out to explore the host-microbiota interactions and their relation with various disease. The turning point for studies in this field has arrived mainly with the advances in high-throughput sequencing that allowed to overcome the problems correlate with culture technique[58, 59].

Indeed, prior to the development of more advanced technologies, the study of the composition of the human microbiota was limited to traditional bacterial culture techniques with phenotyping of cultured bacteria to assess morphological and biochemical characteristics. However, because most gut microorganisms are strictly anaerobic, it is difficult to study their characteristics through in vitro cultivation. Therefore, this technique is used only for single bacterial groups in particular clinical situations.

For this reason, with the development of new techniques has been revolutionized the approach to this type of study, allowing discovery of the mechanisms in which microbiota is involved[60].

In human studies, the most common method to investigate the gut-microbiota composition is the collection of stool samples, an easy and non-invasive approach to get a sample densely populated with microorganism that well represent the gastric tract. The first method used to classify the microbial population is the amplicon sequencing. The small ribosomal subunit of prokaryotic cells contains RNA molecule, 16S in size with some variation across species therefore the part of DNA most commonly used for taxonomic detection is the 16S rRNA gene [61].

The 16S rRNA gene is approximately 1550 bp long and is composed of nine variable regions interspersed with highly conserved regions. Generally, universal primers, complementary to the conserved regions, are used to sequence the variable regions to allow taxonomic classification.

The obtained reads are clustered into Operational Taxonomic Units (OTUs) according to a predefined level of sequence similarity (generally 97%). This OTU clustering algorithm allows to classify bacterial taxon.

Taxonomically, bacterial are classified in phyla, classes, orders, families, genera and species. [62]

Another approach used to investigate human microbiome is the shotgun sequencing of microbial DNA, in which random fragments of genome are sequenced. This approach is more expensive and required wider data analysis.

According to the findings of the Human Microbiome Project (HMP), the composition of the microbiota is unique to each person and the diversity of the microbiota, which increases during the first years of life and stabilizes with age, can be modulated by several environmental factors, such as dietary habits, host genomics, age, mode of delivery, and antibiotic use. [63] Colonization of the gut microbiota begins with birth and it has been shown that the mode of delivery strongly influences the composition of the microbiota.

In vaginal deliveries (VD), the initial microbial composition was derived from the maternal vaginal environment and an enrichment of health-associated species belonging to the genus *Bifidobacterium* accompanied by a reduction of *Klebsiella* was observed. In contrast, in birth by cesarean section (CS), *Bifidobacterium* colonization is delayed, which seems to suggest that vaginal delivery is essential to acquire these bacteria in early life. Moreover, in CS the neonatal microbiota is enriched by bacteria typically found on

the skin or in hospitals, such as *Staphylococcus* and *Propionibacterium* while higher levels of *Clostridium Difficile* were found in the intestines of these children. [64]

Differences in the microbial composition have been reported also in relation to age and sex.

In a Japanese study of 367 healthy volunteers aged 0-104 years, the authors used high-throughput sequencing to analyse the microbial composition of stool. They reported an opposite trend of association between age and *Actinobacteria* abundance that starts to decrease in the post-weaning phase and continues to decrease with age [65]. Several studies have shown that gut microbial composition is also modulated by sex and this correlation would seem to be involved in the incidence of several diseases such as type 1 diabetes [65] and rheumatoid arthritis. [66]

The figure 4 shows an example of gut microbiota composition in healthy subjects. The dominant gut phyla are *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia*, with *Bacteroidetes* and *Firmicutes* accounting for more than 90% of the total bacterial species[67]. Although the general composition of the gut microbiota is fairly stable there are some spatial differences from the esophagus to the rectum. For example, *Helicobacter* is the dominant genus in the stomach while *Streptococcus* appears to be dominant in the distal esophagus, duodenum and jejunum [68].

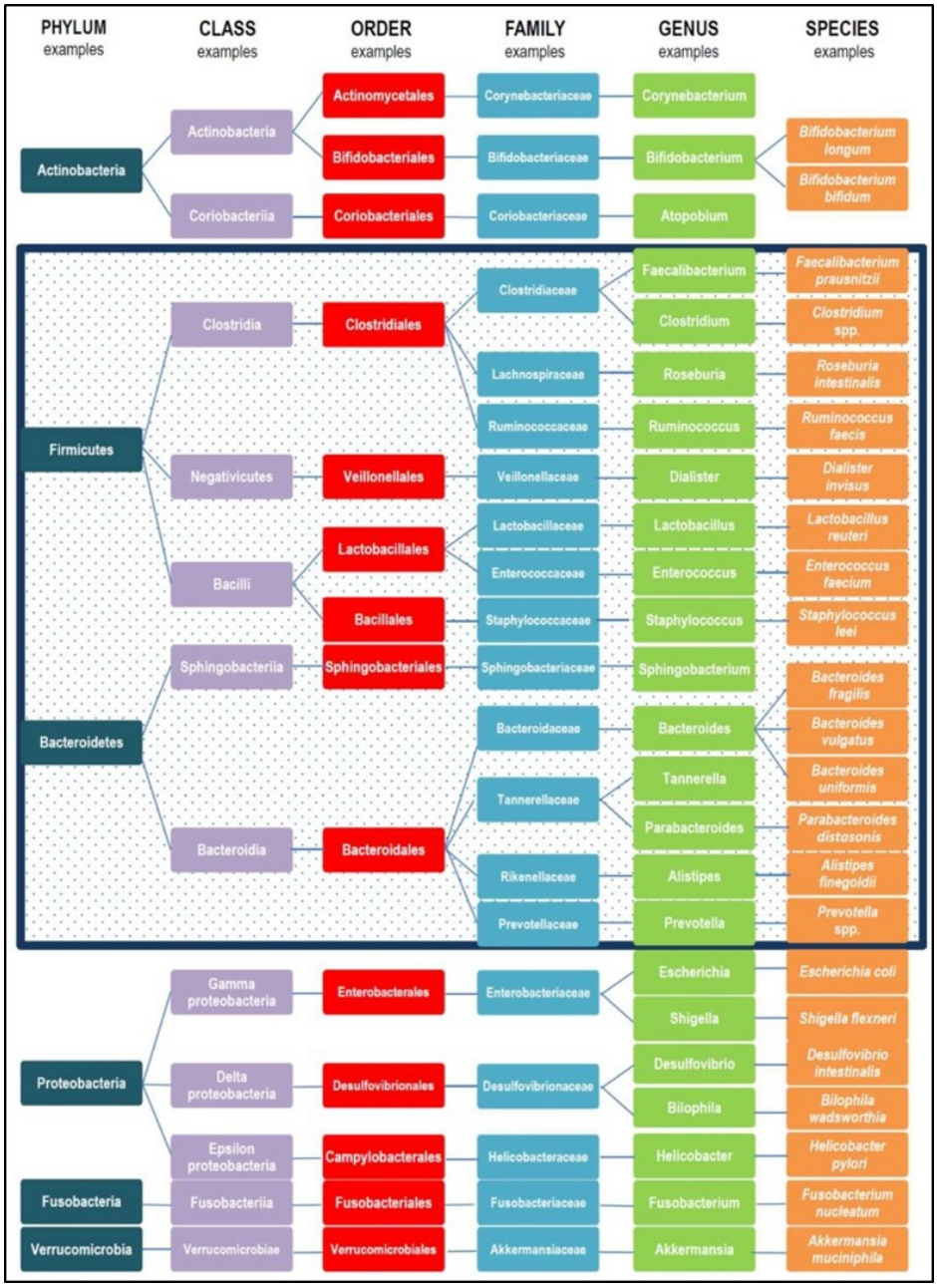


Figure 4: Examples of taxonomic gut microbiota composition. In the box are cited examples of bacteria belonging to Phyla Firmicutes and Bacteroidetes, representing 90% of gut microbiota.
Microorganisms 2019

Gut microbiota can be considered an endocrine organ able to produce several metabolites that can be metabolized by the host enzyme or adsorbed into the systemic circulation with biological activity. The human microbiota, under healthy conditions, has a symbiotic relationship with the host and is involved in a wide range of mechanisms, such as host immunity, metabolism-dependent pathways such as the trimethylamine (TMA)/trimethylamine *N*-oxide (TMAO), short-chain fatty acid (SCFA), bile acid (BA), and uremic toxin pathways, toxin purification, and regulation of gut functions [69].

The next section provides a brief overview of the principal functions of the gut microbiota.

Gut microbiota in nutrient metabolism: Gut microbiota is involved in two principal catabolic pathways, the saccharolytic and the proteolytic pathway. In saccharolytic pathway, the breakdown of complex polysaccharides by organisms such as *Clostridia, Bacteroidia, Bifidobacteriaceae* and *Fecalibacterium* lead to synthesis of short chain fatty acids (SCFAs) such as propionate, acetate and butyrate[70]. These sources of energy for the host are efficiently absorbed into the gut epithelium and they can be used for *de novo* synthesis of lipids and glucose but can also influence host physiological functions[71].

Recent studies showed that SCFAs are also signalling molecules which bind to G-protein coupled to receptors GPR41 and GPR43 expressed in adipose tissue, intestine and immune cells. GPR43 is involved in neutrophils recruitment in the inflammatory response necessary to resolve inflammatory status. Maslowski et al. showed that in both GPR43-deficient mice and germ–free mice there is an increased production of inflammatory mediators[72].

The second catabolic pathway in which the microbiota is involved is the proteolytic fermentation. Several bacterial species are able to degrade proteins such as *Propionibacterium, Clostridium* and *Bacteroides*. This pathway leads to the formation of SCFA but also to the production of metabolites such as ammonia, amines, thiols, phenols and indoles. Ammonia is used by bacteria for amino acid synthesis, so reduced protein absorption can increase ammonia utilization in the gut [73].

Vitamins are essential nutrients for bacterial metabolism and have an essential role as cofactor in these catabolic pathways. It is known that the gut microbiota can synthesize some vitamins, particularly group B and K including biotin, cobalamin, riboflavin and thiamine and also there seems to be difference in molecular structure between vitamins synthesized by bacteria and those from the diet [74].

In addition to SCFAs, another important metabolite of gut microbiota is trimethylamine N-oxide (TMAO). It derived from large quantity of choline, carnitine and phosphorylcholine ingested and converted from a variety of enzymes produced by specific intestinal bacteria, into an intermediate precursor known as Trimethylamine (TMA). TMA with portal circulation arrives in the liver where it is rapidly oxidized by flavin monooxygenase enzymes (FMO) to TMAO and excreted in the urine. This

compound is a waste product of choline metabolism with no function in the human body, but there is some evidence on the association between TMAO and inflammation and risk of developing atherosclerosis[75].

Gut microbiota in immunity and inflammation: The gut microbiota also provides important health benefits by regulating the immune homeostasis of its host, therefor an alteration of this microbial community may cause immune dysregulation and disorders.

Among the mechanisms of dialogue between the host and the microbiota there is the detection of pathogen-associated molecular patterns (PAMPs). These molecules are recognized by toll like receptors (TLRs) and other pattern recognition receptors (PRRs) and there activation is relevant to protection the host from pathogens[76].

Intestinal mucosa is composed of gut-associated lymphoid tissue (GALT), Peyer's patches (PP) and small intestinal lymphoid tissue (SILT) in the small intestine, lymphoid aggregates in the large intestine and immune cells in the lamina propria of gastrointestinal tract. Studies of microbiota lacking animals allowed to investigate and understand the importance of gut microbiota in the development of both intestinal mucosa and systemic immune system. The gut microbiota is required for the generation and maturation of GALTs. GALTs are immune structures abundant in plasma cells responsible of antibody production and these structures play a kay role in antigen presenting cells and in lymphocytes functions and are useful as protective barrier against pathogens. These structures include also PP, crypt patches and isolate lymphoid follicles (ILFs). Germ-free mice shows reduced intestinal lymphatic tissue compared with conventionally bred mice, especially the number of ILFs and of Peyer's patches are reduced, underlining the importance of gut microbiota for lymphoid tissue development and maintenance [77].

Certain bacterial taxa are able to resolve inflammatory status, in particular some members of the *Clostridium cluster XIVa* were inversely correlated with systematic inflammation whereas *F. prausnitzii* has been shown to possess anti-inflammatory effects through the secretion of metabolites capable of blocking NF-κB activation and IL-8 secretion in Caco-2 cells [78, 79].

Gut microbiota in integrity of gastrointestinal tract and antimicrobial protection: There is much evidence on the effects of the gut microbiota on the structure and function of intestinal barrier integrity and epithelial repair processes. Mucus secretion in the lumen of the gastrointestinal tract occurs through the action of specialized cells called goblet cells, and the function of these cells are influenced by the microbiota. *Bacteroides thetaiotaomicron* is implicated in increased expression of the gene encoding the small proline-rich protein II (sprr2A), an essential protein in desmosome integrity [80]. Another relevant role of microbiota in intestinal barrier is represented by *Bifidobacterium* which prevents permeability and bacterial infiltration ensuring a healthy environment of intestinal microvilli [81]. The microbial cell wall peptidoglycan is also responsible for stimulation of tall like receptor signalling (TLR2) implicated in maintenance of tight junctions, promoting integrity of intestinal barrier [82]. Also *Lactobacillus sp.* producing lactic acid, increase antimicrobial protection of host lysozyme and lactic acid has been showed to suppress the gastric pathogen *Helicobacter pylori* [83].

Gut microbiota alterations

Several studies show that qualitative and quantitative alterations in the composition of the gut microbiota are involved in many diseases, such as cancer, inflammation, autism, autoimmune diseases and cardiovascular disease[84]. Different environmental factors, such as diet, toxins, drugs, and pathogens can influence the microbiota with consequent alteration.

In recent years, different authors have explored the relationship between gut microbiota changes and cardiovascular disease. Several works have investigated the role of intestinal microbiota in the onset and progression of atherosclerosis, in fact, it seems to act both on risk factors and on the pathways involved in atherosclerosis.

Modulation in microbial community had been linked to CVD development and metabolic disease, a risk factors for CVD.

As is known, obesity is one of the most relevant risk factors for CVD and gut microbiota composition has been found altered in obese individuals. This pathological condition is characterized by a higher presence of potentially pathogenic organisms (pathobiont). The increased ratio pathobiont/symbiont reduces intestinal barrier integrity promoting

inflammation and pain. [85]. Among different studies on this correlation, a study on mouse model with sibling genetically obese compared to lean and wild type, under the same dietary conditions, showed reduced relative abundance of phylum *Bacteroidetes* in obese individuals [86].

Furthermore, Turnbaugh et al.[87] showed that microbiota alterations are responsible of obese or lean status independently from other genetic factors. Indeed, they transplanted lean and obese microbiome to germ-free recipients and they found that the total body fat was higher in mice transplanted with obese microbiome in comparison to lean microbiome. In a study on the effect of fecal microbiota transplantation from lean subjects to insulin-resistant individuals with metabolic syndrome, an improvement in insulin sensitivity after gut microbiota infusion and an increase in the abundance of rate-producing species were revealed [88]. Interestingly, butyrate functions include the ability to promote satiety and it might have a potential in meliorate obesity and its correlated comorbidities [89]

In the obese subjects, the *Firmicutes/Bacteroidetes* ratio is increased and this is in contrast to the functions of butyrate, since Firmicutes are among the major producers of this metabolite. One explanation is that in obesity, butyrate-producing species decrease but are replaced by other bacteria belonging to the same phylum with a lower capacity to produce butyrate, whose butyrate levels therefore decrease despite the increase in *Firmicutes* [90].

The gut microbiota also influences the risk for type 2 diabetes (TD2) by its contribution in different mechanisms, such as inflammation, the glucose and lipid metabolism and the insulin sensitivity. There is a double action of microbiota on inflammation. Indeed, while some microbial species are involved in a stimulation of low-grade inflammation, other species, such as *Roseburia intestinalis*, promote the expression of anti-inflammatory cytokines and reduced the production of pro-inflammatory cytokines such as IFN-γ[91]. Some microbial species also act by up-regulating the expression of tight junction genes in the colon resulting in reduced intestinal permeability, which instead under TD2 conditions is increased and is associated with metabolic endotoxemia.

As previously reported, dysregulation of cholesterol metabolism is involved in several diseases, especially atherosclerosis and cardiovascular disease. Recent studies have reported an association between some microbial taxa and plasma cholesterol levels. In a study published in 2019, the authors showed that in *Apoe*−/− mice, treated with a mix of antibiotics to deplete the gut microbiota, plasma cholesterol levels were higher than in

normal mice. With depletion of the gut microbiota, they also observed increased intestinal absorption of cholesterol, hepatic cholesterol uptake and bile acids secretion[92].

Bacteria have been suggested as a causative agent of atherosclerosis, due to its implication in different pathways such as lipid accumulation and inflammation.

Different mechanisms have been studied to explain the relationship between the gut microbiota and various inflammatory markers. In obesity and other metabolic disorders, higher levels of lipopolysaccharides (LPS), the principal components of cell wall in gram-negative bacteria, were found[93]. When factors, such as diet or pathogenic bacteria, alter the intestinal epithelial barrier, LPS move into the circulation. These endotoxins, stimulating the macrophage infiltration and inflammatory cytokines productions, cause a local inflammation. Moreover, LPS binds to toll-like receptor present on immune cells, activating pro-inflammatory pathways not only into the intestine but also in other sites[93].

The gut microbiota is also involved in several pathways that lead to the transformation of primary bile acids into secondary bile acids, and through their modulation, it regulates host metabolism. These transformations are manly operated by *Bacteroides, Clostridium, Lactobacillus, Eubacterium* and *Ruminococcus* [94]. Using germ-free and conventionally bred mice, wilt-type and knock-out for FXR (Farnesoid X Receptor, the main sensor of bile acids in the liver and intestine) under a high-fat diet (HFD) for 10 weeks, Parséus et al. demonstrated that by altering the composition of the intestinal microbiota, the onset of obesity could be promoted by modulation of bile acids and through alteration of intestinal FXRs signalling [95].

In addition to these mechanisms through which the microbiota influences the inflammatory processes underlying several cardiac disorders, bacterial DNA has also been found in atherosclerotic plaque and microbiota composition is similar to that found in the gut of the same subjects.

It is possible that this presence influences plaque stability and the development of CVD and may be useful to explore intestinal microbiota composition as potential biomarkers of cardiovascular events[96].

Using shotgun sequencing of the gut microbiota, Karlsson et al. revealed that microbiota composition is modulated in patients with symptomatic atherosclerosis in comparison to controls. They found a higher presence of genus *Collinsella* in patients with stenotic plaques and higher levels of *Roseburia* and *Eubacterium* in healthy controls[97].

In addition to the composition of gut microbiota and its alteration, also metabolic effects of gut microbiota are implicated in CVD development.

Several recent studies have found that TMAO is one of the gut microbiota metabolites involved in the development of atherosclerosis and in other heart diseases as well as inflammation and obesity[98]

Indeed, plasma levels of TMAO and its precursors were found to be elevated in subjects with increased risk of CVD and after antibiotic administration, these levels were found to be significantly reduced, confirming that the production of this metabolite from dietary phosphatidylcholine is dependent on the functions of the gut microbiota [99].

Moreover, in an observational study on patients with chronic heart failure, the authors showed a positive correlation between plasma levels of TMAO, choline and betaine and clinical prognosis of HF. In particular they found higher levels of these metabolites in patients with New York Heart Association (NYHA) classes III and IV, confirming its correlation with clinical disease severity[100].

There are also several studies on murine model in which the authors have demonstrated that elevate levels of TMAO are correlated with expression of proinflammatory cytokines [101], foam cell formations and lipid accumulation [102]. TMAO variability depends on diet and microbiome composition. For example, a diet rich in vegetables, especially some such as cabbage, radish and rocket, can reduce FMO3 activity, with consequent reduced ability to produce TMA. At the same time a high protein uptake seems to have a positive correlation with TMAO levels in urine.

Moreover, in subjects with a higher *Firmicutes/Bacteroidetes* ratio and reduced microbiota diversity, choline was converted to TMAO more efficiently [103, 104].

Diet and gut microbiota

As previously mentioned, diet is a key modifiable factor that influences the composition of the gut microbiota and is responsible for changes in microbial diversity and stability [105]. Several evidences have shown that different dietary habits, influence the gut microbiota and the human health differently (Figure 5).

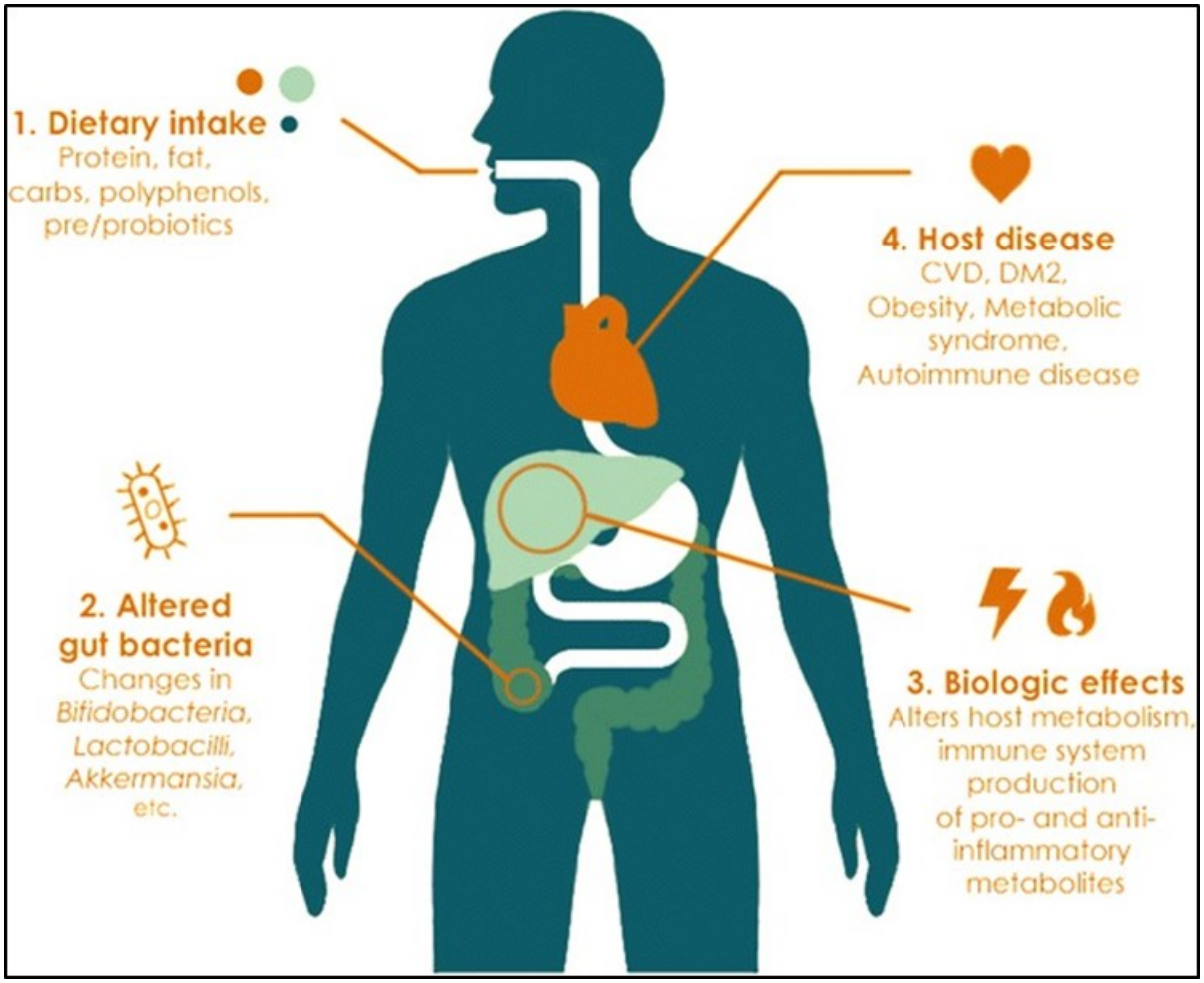


Figure 5: Impact of diet on the gut microbiome and human health.
Journal of Translational Medicine,2017

This relationship can also explain differences in microbiota composition among different geographic location, due to both dietary and genetic parameters. The comparison between gut microbiota of children from rural Africa and from urban Italian zones, showed that dietary habits were radically different and as consequence the microbiota composition appear completely different, with dominance of *Bacteroidetes* in African children and prevalence of *Enterobacteriaceae* in European children[106].

Large variations in gut microbiota as a consequence of dietary habits were observed in different studies. For example, a high-fibre intake is positively correlated with a higher bacterial richness [107] and western diet, principally rich in red meat, animal fat and low fibres intake, is associated with increased amount of *Bacteroides* phylum and *Ruminococcus*[108].

Several studies have suggested that high fat diet increases the anaerobic microflora and the presence of *Bacteroides*. In particular, the high saturated fat diet increases the number

of *Faecalibacterium prausnitzii* while the consumption of monounsaturated fat does not show a relevant change in the relative abundance of the microbiota but is associated with reduction of total- and LDL-cholesterol in plasma [109].

Has been widely demonstrated that dietary carbohydrates are able to modify the gut microbiota composition. Diet rich in glucose, fructose and sucrose leads an increase in relative abundance of *Bifidobacterium* and a reduction of *Bacteroides* [110]. The intake of non-digestible carbohydrates influences the gut microbiota in different ways. A diet rich in whole grain and bran leads to an increase in *Bifidobacterium* and *Lactobacillus* while a feeding with high intake of starch and barley appear to increase the abundance of *Ruminococcus, E. rectale* and *Roseburia* [111-113].

In a more holistic approach, the ability of different types of diets to modulate gut microbiota has been extensively studied in order to understand the best dietary approach to reduce the risk of CVD. Over the years, different dietary patterns have been evaluated, such as diet to stop hypertension (DASH diet)[114], the low-fat and low-carbohydrate diet[115], the vegetarian diet[116] and the Mediterranean Diet (MD). The latter approach is one of the best dietary patterns with highest evidence for protection against CVD onset[117].

THE MEDITERRANEAN DIET

Definition and overview

Ancel Keys, American biologist and physiologist, was the first that in the 60s, after having visited the South of Italy, started a longitudinal, multinational observational study, called Seven Countries Study, to find an association between feed and coronary diseases. His study involved 15 cohorts of people from seven countries (Finland, Greece, Italy, Japan, The Netherland, U.S. and Yugoslavia) with a total of over 11000 men in a period of at least 5-year follow-up. The principal association showed from the study was the reduction of CVD incidence in the population on Mediterranean see, such as Italy, Greece and Yugoslavia [118]. Moreover, it has been proved that the mortality incidence rate increased with increasing distance from the Mediterranean areas [119]. Beginning with these early associations between MD and its components with CVD incidence and CAD mortality, several observational and interventional studies have indicated that MD promotes a cardiovascular health status.

Mediterranean diet composition is similar among different studies, with emphasis on the same kay components. The definition includes high intake of extra virgin olive oil as the main source of fat, cereals, grains, nuts, legumes, fruit and beans, moderate consumption of seafood and red wine, and it usually includes a low intake of meat and dairy food [120]. Other essential components of MD are wheat, grapes and their derivates.

Nowadays, the definition of MD has undergone some modifications because of the continuous changes in eating habits due to social and cultural changes and now is more correct define the MD as a modern dietary approach inspired by the traditional habits of the countries in Mediterranean Area.

The MD composition is usually represented with a pyramidal graphic, realized for the first time in 1993 at the Harvard School of Public Health during an International Conference on MD[121]. Since then other different versions of pyramid have been presented and readapted to different populations. The last version of food pyramid was proposed in 2010 by Mediterranean Diet Foundation Expert Group. This graphic shows in Figure 6 represents the different groups of aliments in relation to the frequency with which the various foods must be consumed, placing at the base the foods that should provide the greatest amount of energy and at the top, foods to be consumed in moderation. In addition, there are also elements at the base of the pyramid that emphasize the

importance of conviviality and a healthy lifestyle, such as physical activity and eating of moderate portions of food in relation to personal energy needs[122].

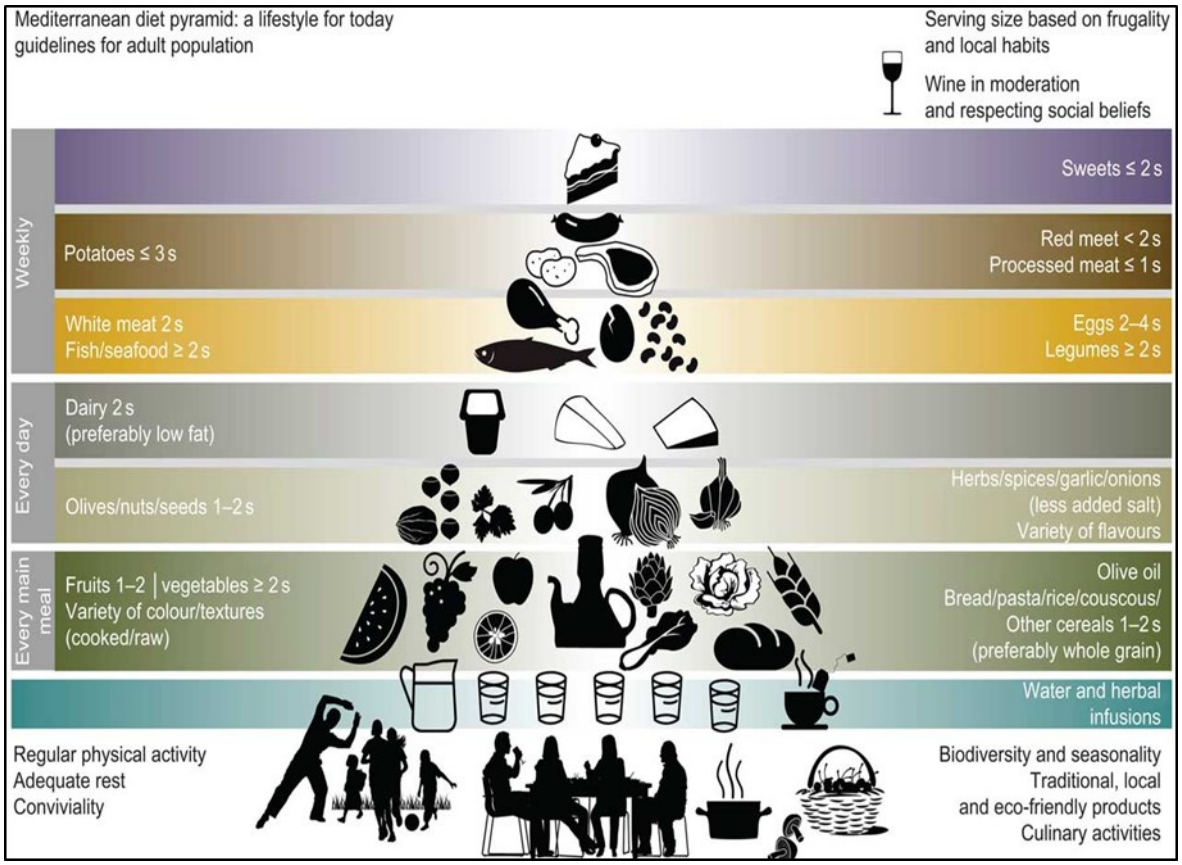


Figure 6: An example of Mediterranean diet pyramid
Public Health Nutr, 2011.

The need to measure dietary intake objectively, leads to the development of food frequency questionnaires (FFQ) as the gold-standard technique to have a low-cost and easy-to-use tool. FFQ are usually composed by 100-200 questions covering food and frequency consumption, portion size and beverage intake[123].

In order to assess an individual's level of adherence to the Mediterranean diet, different index, tools and scales have been developed and used in several epidemiologic studies, but to date, there is no single method of evaluation. One of the most used indexes is the Trichopoulou index, developed in 1995 [124] and modified in 2002 to include fish intake[125]. Based on the consumption of some classes of food (fruits, vegetables, fishes, cereals, meat and dairy products), a score is assigned from 0 (minimal adherence) to 9 (maximum adherence). To valuated this score, a sex-specific median referred to studied population is used. Another very used MD adherence index is the Mediterranean Diet

Adherence Screener (MEDAS), developed and validated in the PREvencion con DIeta MEDiterranea (PREDIMED) study. This is one of the main studies in this field, is a multicenter, randomized, nutritional interventional clinical trial in which the role of MD in the primary prevention of CVD was evaluated. This index assesses adherence to MD and is obtained from the administration of a questionnaire with 14 items. Each answer is given a score of 0 or 1 depending on whether the specific condition is met or not, and the final score, from 0 to 14 points, is obtained by adding up all the answers. A score of 14 points indicates the highest level of dietary adherence [126].

The protective role of Mediterranean Diet in cardiovascular disease

As mentioned above, the first scientific evidences on protective role of DM in cardiovascular disease are those derived from the Seven Countries Study that showed cardioprotective effects of MD. Several types of biochemical, clinical, and epidemiological studies conducted over the past 20 years have helped to further investigate the beneficial properties of this style of eating.

As it is a diet riches of polyphenols, tryptophan, unsaturated fatty acids and natural antioxidants, the Mediterranean Diet has many beneficial effects against different diseases such as cancer, metabolic diseases and cardiovascular diseases[127, 128].

In the recent years there has been an exponential increase in prospective and interventional studies to evidence and support the importance of this diet. Among observational studies, the Northern Manhattan Study in the United States is the first population-based study to establish the association between MD adherence and ischemic stroke, revealing that greater MD adherence is associated with a reduced risk of vascular events and vascular death[129]. Also, a few years later, a study conducted on the same cohort, showed that this type of diet is associated with improved carotid plaque thickness and area on atherosclerotic plaque and this could be the way through which MD could reduce vascular events[130].

In 2011, a large meta-analysis of over 50000 patients from 50 studies, showed that the MD significantly reduced the risk of metabolic syndrome and protected against the increase of risk factors such as waist circumference, lipids, glucose and blood pressure[131].

Others important evidences is this association have been obtained from a large study designed to investigate the relationship between nutrition and cancer, the European Prospective Investigation into Cancer and Nutrition (EPIC) study. The study includes 519978 participants (366 521 women and 153 457 men, 35-70 years) in 23 structures located in 10 European countries, followed for cancer incidence and cause-specific mortality for several decades[132]. Although the study was primarily aimed at oncologic outcomes, it had also the potential to study other diseases, including CAD. For example, some studies were conducted on the Spanish cohort of EPIC study, with a total of 41.438 participants and showed a 40% reduction in the risk of coronary heart disease in subject with high adherence to MD.[133]

On 22.043 adults in Greece enrolled in EPIC study has been demonstrated that a high adherence to the MD was associated with a lower mortality and that there is an inverse association between adherence to the MD and mortality for CAD or cancer[134]. In addition, the association between individual food components and mortality not detected in this first study emerged only after an 8-year follow-up period, showing that moderate ethanol consumption, low meat consumption, and high fruit and vegetable consumption are the main factors influencing the MD effects[135].

On this topic, also some interventional studies are available and among them, the PREDIMED Study is one of the most interesting. The study was carried out in Spain between 2003 and 2011 with the purpose to test the effects of MD on the onset of CVD in subject with age 55-80 years and with presence of diabetes or at least 3 risk factors for CVD. A total of about 7500, were randomized into three groups of diet: MD supplemented with extra-virgin olive oil (EVOO), MD supplemented with nuts and a control low-fat diet (LFD)[136].

The primary outcome was the incidence of CVD and subsequently total mortality, diabetes, metabolic syndrome, peripheral arterial disease, atrial fibrillation, neurodegenerative diseases and major cancers were evaluated.

After a follow-up of 4.8 years, the authors recorded 288 major cardiovascular events: 96 in MD + EVOO, 83 in MD + nuts and 109 in control group observing that MD reduces incidence of major CV events by almost 30% compared to control group[137].

Nevertheless, this study also allowed us to obtain information about the impact of MD on several mechanisms that might be responsible for the beneficial effects of the diet. For example, the effects of this intervention on cellular and serum inflammatory biomarkers[138], on blood pressure[139] and blood glucose or lipidic profile[140].

Since PREDIMED trial evaluated only diet composition, regardless of a healthy lifestyle, such as physical activity, in October 2013 started the PREDIMED PLUS, a new ongoing, randomized, multicentre, primary prevention study with about 7000 participants. Subjects were randomized in 2 groups Mediterranean Diet with EVOO and nuts or hypocaloric Mediterranean Diet with EVOO and nuts, both supported by an intensive physical activity[141].

There are some collateral studies on this cohort, for example recently was published a work conducted on a subsample of 327 individuals of PREDIMED plus cohort, with overweight/obesity and metabolic syndrome. The aim of this work was to assess the association between gut microbiota, body composition and weight loss, after 12-months of lifestyle intervention. The primary outcome was the identification of a faecal microbiota signature potentially related to weight changes in an old population with overweight and obesity[142].

Another relevant study, called Lyon Diet Heart Study, analysed Mediterranean diet in CVD secondary prevention and was conducted at the end of the 1990s. The purpose was to investigate whether a Mediterranean diet enriched with a-linoleic acid (ALA) compared with Western-type diet, may reduce the negative outcome after a first MI. The authors showed a markedly reduction (76%) of cardiac death and non-fatal MI and total mortality[143].

Subsequently, another randomized trial in 1000 patients suffering of angina pectoris, myocardial infarction or with other risk factors for CAD was conducted. The patients were randomized to the MD or a local diet and after 2-years of follow-up, total cardiac endpoint, cardiac death and non-fatal MI were significantly reduced in MD group[144].

In recent years, omics technologies have enabled a more comprehensive study of the influence of dietary components on gene regulation. In the early 2000s, these types of studies led to the birth of the concept of “Nutritional Genomics”. The main goals of this discipline are to study the interaction between dietary components and gene expression in order to understand the molecular mechanisms by which diet acts (nutrigenomics) and to understand how genetic variations in specific genes can influence the effects of diet on disease phenotypes (nutrigenetics)[145]. There are a few studies that have looked at this correlation with MD. For example, the interaction between MD and a polymorphism near the SERPINE1 gene and its effect on triglyceride levels has been reported. In another study, the effects of the interaction between polymorphisms on the melanocortin-4

receptor gene and MD adherence levels on type 2 diabetes and obesity were shown [146, 147].

These gene-diet interaction studies suggested that the effects of MD could be dependent from the genotyping profile.

Mediterranean diet patterns and its components have been found to reduce cardiovascular disease risk principally acting on surrogates of CVD such as blood pressure, lipids, BMI, and glucose levels. However, in recent years attention has been focused on the protective effects of MD mediated by modulation of gut microbiota composition.

Influence of Mediterranean diet on gut microbiota.

The composition of the gut microbiota changes throughout life and diet plays a key role in determining the richness and diversity of the gut microbiota[148]. This Mediterranean dietary pattern is characterized by the intake of vegetables, fruits, nuts, whole grain, fish and unsaturated fats and olive oil, with limited intake of butter, sweets and processed meet.

There are different studies confirming that a rich and balanced diet, such as Mediterranean Diet, is necessary to promote a healthy gut microbiota.

In a transversal study of Gutiérrez-Dìaz et al., the authors have investigated the association between MD and its components with gut microbiota composition in 31 healthy subjects. They found a significant increase in phyla *Bacteroidetes* and a reduction in *Firmicutes* in individuals with greater adherence to MD, and in their feces they measured a higher concentration of butyrate and propionate that could corroborate the beneficial effects of this diet[149]. In another observational study, 153 healthy italian individuals divided into three groups, vegetarians, vegans and omnivores, were followed for 7 days and their dietary habits were assessed. Most of vegetarians and vegans showed a higher adherence to MD and *Lachnospira* and *Prevotella* were both associated to plant-based diet while *L-Ruminococcus* and *Streptococcus* resulted significantly associated with nutrients of animal origin[150].

The CARDIVEG study, aimed to evaluate how the low-calorie MD diet and vegetarian diet (VD) affected gut microbiota composition and SCFA production after a 3-month dietary intervention. They found variation only at the genus level with a significant change in the abundance of *Enterorhabdus, Lachnoclostridium* and *Parabacteroides* in the MD diet and higher SCFA levels in MD compared to VD[151].

Studies that involve intake of specific dietary components have demonstrated how certain bacteria are influenced by specific nutrients.

The intake of dietary polyphenols, naturally occurring in fruits, vegetables, cereals, coffee, tea and wine, can modify the gut microbial composition, enhancing the grow of specific taxa able to metabolized these components into substrates useful for the host. In addition, there is more epidemiological evidence on the health benefits associated with polyphenols, which are also related to a reduction in the risk of chronic diseases[152].

Tzounis et al. in 2011 conducted a human intervention study, in which they analysed the effects of high intake of flavonoids on gut microbiota. The comparison between two groups, those with low and those with high intake of cocoa flavonoids allowed to observe a microbiota modulation in highest intake group, with an increased presence of *Lactobacillus* spp. and *Bifidobacterium* spp., bacterial groups known to be associated with positive effects in the intestine [153].

Among the effects of each MD compound, it has been showed that drinking red wine leads to a decrease in the *Clostridium perfringens*, a pathogen associated with obesity and other diseases[154]. In addition, with red wine intake, an increase in *Bifidobacterium* has been reported resulting in reduced levels of C-reactive protein, a protein involved in inflammation and a marker of cardiovascular risk in healthy subjects [102].

These types of studies have also been conducted in populations affected by disease in which it was seen that the adoption of Mediterranean style causes a modulation of the intestinal microbiota, promoting species with beneficial health effects. However, the results obtained are strongly influenced by the disease and other different variables, such as the number of subjects, the methods of assessment of MD adherence or the type of dietary components[155-157].

In this scenario, more in-depth studies on the interaction between the adherence to the Mediterranean diet, the modification of the intestinal microbiota and the protective role of this dietary regimen could allow the development of new therapies based on targeted dietary and nutraceutical interventions.

OBJECTIVE

The beneficial role of Mediterranean Diet on cardiovascular disease is clarified since the first results of Seven Countries Study [118], where the protective role of this diet on mortality and morbidity for this disease was defined. In recent years, the mechanisms underlying this protective action have been deeply explored. Several epidemiological studies have revealed the role of the MD in modulating plasma composition with improvements in the blood lipid profile, reduction of oxidative processes, decreased risk of thrombosis with a general improvement in endothelial functions. Recently, the ability of this diet to modulate the inflammatory processes that underlie atherosclerotic disease, acting on risk factors such as blood pressure, insulin resistance and BMI, has also been recognized. In addition, several other processes and pathways have been studied and suggested as key factors in MD outcome. One of the latest factors analysed is the gut microbiota. Presumably, it is necessary to analyse all these factors synergistically instead of associating the effects of MD with only a single factor.

Up to now several observational and interventional studies have been focused on the effects of MD in primary prevention for CVD, but the role of this food pattern in secondary prevention remains to be well explored.

Our study is included in the Randomised Interventional Study on Mediterranean Diet (RISMeD), a parallel-group, randomized, open-label interventional study conducted at Centro Cardiologico Monzino, IRCCS, Milan, Italy.

The primary objective of this study was to perform next generation sequencing analysis to evaluate how, in patients with coronary artery disease, a 3-month dietary intervention affects the composition of the gut microbiota. In addition, the same analyses were also conducted after 1 year from randomization to understand whether the changes observed after 3 months were maintained.

A secondary end-point of our study was to assess whether, during our observation period, dietary adherence might be able to modify major risk factors for CAD in our population.

Among the goals of this work there was the technology transfer from Centro Cardiologico Monzino in Milan to Check-up in Salerno. Geographic differences are known to play a relevant role in the onset of CVD, and MD has been indicated as a major factor involved in these differences. That being said, we collaborated with the Check-up diagnostic center of Salerno to analyse the composition of the gut microbiota in healthy Salerno subjects

or patients in primary prevention for CAD with a basal high level of adherence to MD, as expected for people living in the Mediterranean area.

Finally, we conducted preliminary transcriptomic analysis on whole blood in a subgroup of CAD patients, to assess whether there was a correlation between the level of MD adherence, gut microbiota and, gene expression.

MATERIALS AND METHODS

STUDY DESIGN

Study population

The RISMeD study is a parallel-group, randomized, open-label, interventional trial conducted at Centro Cardiologico Monzino, IRCCS, Milan, Italy.

The aim of this study was to evaluate the effects of MD on CAD patient, in comparison to a classic low-fat diet (LFD). The study, approved by CCM Institutional Ethics Committee, was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent to participate was obtained from all subjects.

From 2015 to 2018, 130 Caucasian male and female patients, of Italian origin, aged between 30 and 75 years, with a recent history of cardiovascular revascularization (angina pectoris, coronary bypass or percutaneous coronary intervention) were enrolled. Exclusion criteria were a history of diabetes, food intolerance, BMI <19 or > 33, previous cancer or autoimmune diseases, use of drugs or food supplementation with probiotics, omega-3 fatty acids or antioxidants and high adherence to Mediterranean Diet (MEDAS score >10). After a period of clinical stabilization, at least 60 days after the end of surgery (T0), patients were randomized into two group, MD or LFD with the software "Food Therapy" by DS Medica Milan.

Diet interventions

Patients assigned to the LFD diet were given a standard cardiac patient diet, with a reduced intake of sugar and saturated fats, customized in terms of total calories without additional recommendations, whereas patients assigned to the MD group received a more controlled intervention.

MD was personalized in terms of total calories, total lipids, saturated/unsaturated fatty acids. The diet included: legumes 2-3 times a week; fish at least 3 times a week; raw or cooked vegetables (preferably rich in antioxidants), twice a day; 30 to 45 g olive oil per day; fresh fruits twice a day; not more than 150 g of red meat per week. It has been suggested to consume red wine in moderation (men 1-2 and woman 1 glass per day). Cold cuts, butter, cakes and fatty cheeses were discouraged. Table 1 shows the composition of the two assigned diets; on average the assigned MDs are significantly higher in terms of total energy, plant protein, simple carbohydrates, total fat, unsaturated and monounsaturated fat, fiber food (both soluble and insoluble), and most vitamins and

minerals. There are no, however, differences between the two dietary interventions in terms of total saturated fat.

For the first three months, all patients were monitored monthly with a reinforcement of dietary recommendations to ensure the maintenance of adequate adherence to the assigned diet.

Characteristics	MD	LFD	p-value (MD vs LFD)
Total calorie intake (kcal)	1706.2±150.3	1560.2±220.4	***<0.001***
Protein (g)	72.9±7.2	74.6±6.8	0.181
Carbohydrate (g)	217.7±22.0	213.8±24.0	0.354
Total fat (g)	59.1±5.5	46.1±5.7	***<0.001***
Saturated fat (g)	10.6±1.5	10.4±1.8	0.6
Monounsaturated (g)	8.7±1.4	6.3±1.4	***<0.001***
Polyunsaturated (g)	33.8±3.2	23.1±3.6	***<0.001***
Cholesterol (mg)	134.8±27.5	164.4±39.4	***<0.001***
Alimentary fibers (g)	35.3±5.2	29.0±5.1	***<0.001***
Soluble fibers (g)	7.8±1.6	6.3±1.4	***<0.001***
Insoluble fibers (g)	22.0±3.5	17.0±3.2	***<0.001***
Sodium (mg)	1095.7±238.37	1373.6±414.5	***<0.001***
Potassium (mg)	3999.3±365.0	3406.6±372.2	***<0.001***
Calcium (mg)	614.6±137.3	547.2±147.4	***0.011***
Phosphorus (mg)	1323.4±166.3	1196.9±172.2	***<0.001***
Iron (mg)	14.6±1.9	12.9±1.9	***<0.001***
Magnesium (mg)	321.5±36.1	277.4±32.1	***<0.001***
Glucose (g)	17.0±3.7	13.6±4.1	***<0.001***
Tocopherol (mg)	17.2±1.4	12.4±2.4	***<0.001***
Ascorbic acid (mg)	270.6±65.0	206.2±66.3	***<0.001***
Retinol (µg)	1895.1±316.5	1449.4±303.6	***<0.001***

Table 1: Mean daily values of energy and nutrients delivered to patients throughout the study. Variables are expressed as mean±SD. *p*-values are shown for comparison of MD *vs* LFD. Paired t-test was used.

Dietary assessment

The first dietary assessment was performed at the enrollment with the administration of the EPIC Food Frequency Questionnaire validated on the Italian population, to assess the nutritional habits of patients related to the year preceding recruitment. This questionnaire consists of 248 questions on foods and beverages commonly used in Italy.

For each food the FFQ includes the consumption frequency (daily, weekly, monthly, annual, never or almost never), the portion usually consumed and for many foods the type of seasoning used and the type of cooking. The data were processed using a specially software at the National Institute of Tumors.

Furthermore, to assess the degree of adherence to the traditional Mediterranean diet, at baseline (T0), after 3 months (T3) and after 1 year (T4), MEDAS score was used. Obtained from a 14-item questionnaire developed and validated by the Spanish PREDIMED study, the MEDAS score range from 0 (no MD adherence) to 14 (complete MD adherence). The PREDIMED questionnaire is reported in Table 2.

Questions	Criteria for 1 point
1. Do you use olive oil as main culinary fat?	Yes
2. How much olive oil do you consume in a given day (including oil used for frying,salads,out-of-house meals,etc)?	≥4 tbsp
3. How many vegetable serving do you consume per day? (1 serving : 200g [consider side dishes as half a serving])	≥2 (≥1 portion raw or as a salad)
4. How many fruit units (including natural fruit juices) do you consume per day?	≥3
5. How many serving of red meat, hamburger, or meat products (ham, sausage, etc.) do you consume per day? (1 serving:100-150g)	<1
6. How many serving of butter, margarine, or cream do you consume per day? (1 serving: 12g)	<1
7. How many sweet or carbonated beverages do you drink per day?	<1
8. how much wine do you drink per week?	≥7 glasses
9. How many serving of legumes do you consume per week? (1 serving: 150g)	≥3
10. How many serving of fish or shellfish do you consume per week? (1 serving 100-150 g of fish or 4-5 units or 200 g of shellfish)	≥3
11. How many times per week do you consume commercial sweets or pastries (not homemade), such as cakes, cookies, biscuits, or custard?	<3
12. How many serving of nuts (including peanuts) do you consume per week? (1 serving: 30 g)	≥3
13. Do you preferentially consume chicken, turkey, or rabbit meat instead of veal, pork, hamburger, or sausage?	Yes
14. How many times per week do you consume vegetables, pasta, rice, or other dishes seasoned with sofrito (sauce made with tomato and onion, leek, or garlic and simmered with olive oil)?	≥2

Table 2– 14-item PREDIMED questionnaire. If the answer meets the predefined criteria, it is assigned a score of 1, or vice versa 0. Higher score reflects better adherence to a traditional MD pattern. *PLoS One, 2012 [126]*..

Samples collection

For each subject, a stool sample and a whole blood sample were collected at T0, T3, and T4. The stool samples were collected in Stool Nucleic Acid Collection and Preservation Tubes *(Norgen)* and stored at -20°C to preserve nucleic acid stability.

Whole blood was collected using Tempus™ Blood RNA Tubes *(ThermoFisher)* for stabilization and isolation of total RNA. The tubes contained 6 mL of stabilizing reagent and when 3 mL of blood was added and mixed with the reagent the cellular RNases were

inactivated and the RNA precipitated. Tempus tubes were stored at -80°C until sample processing.

STOOL DNA EXTRACTION

Microbial DNA from stool samples was extracted using Stool DNA Isolation Kit *(Norgen)* following the manufactured protocol.

Briefly, 200 mg of stool were added to a Bead Tube with 1 ml of Lysis Buffer and mixed together. Then 100 µl of Lysis Additive was added and the mixture was mechanically lysed using TissueLyser II *(Qiagen)* at 20 Hz for 3 minutes. After centrifugation for 2 minutes at 20000 x g, up to 600 µl of supernatant were transferred into a new DNAse free tube and 100 µl of Binding buffer was added. The samples were mixed by inverting the tube and incubated for 10 minutes on ice. The lysate was centrifugated for 2 minutes at 20000 x g to pellet any debris and 500 µl of supernatant were transferred into a new tube. An equal volume of 70% ethanol for molecular biology was added to the lysate and the mixture was vortexed.

To isolate DNA, the lysate was applied to a spin column and centrifugated for 1 minute at 20000 x g. The flowthrough was discarded and the column was washed with 500 µl of binding buffer C and centrifugated for 1 minute at 10000 x g. Then, 500 µl of wash solution A were added to the column twice, each time followed by centrifugation for 1 minute at 10000 x g. The column was spinned for 2 minutes at 10000 x g in order to completely dry the resin.

DNA was eluted directly by adding 100 µl of Elution Buffer to the column and centrifugated for 1 minute at 10000 x g after 1 minute of incubation at room temperature. The sample concentration was measured using NanoQuant Plate™ *(Tecan)*. DNA was stored at -20° C for downstream applications.

WHOLE BLOOD RNA EXTRACTION

The Tempus™ Spin RNA Isolation Kit *(ThermoFisher)* was used for the extraction of total RNA from blood collected in Tempus Blood RNA Tube *(ThermoFisher)*. The frozen samples were thawed at room temperature and then the content was transferred into a clean 50 mL conical tube. To the initial volume of 9 mL, (6 mL of stabilizing reagent + 3 ml of whole blood) 3 mL of 1X PBS were added, to bring the total volume at 12 ml.

After centrifugation at 4°C for 30 minutes at 3000 x g, the supernatant was discarded by inverting the tube taking care that the pellet did not detach from the bottom of the tube, and left upside down on absorbent paper for 2 minutes to clean the tube of blood droplets. Then, 400 µl of RNA purification Resuspension Solution were added into the tube in order to resuspend RNA pellet.

RNA purification filter was used to purify RNA. First, the membrane was prewetted by adding 100 µl of RNA Purification Wash Solution 1 into the filter followed by 400 µl of resuspended RNA. The column was centrifugated for 30 seconds at 16000 x g. The flowthrough was discarded and 500 µl of Wash solution 1 were added into the filter, and the sample was centrifugated for 30 seconds at 16000 x g. A second ad a third purifications were performed with 500 µl of Wash solution 2, followed by another centrifugation for 30 seconds at 16000 x g. To dry the membrane, the column was centrifugated for 30 seconds at 16000 x g. The purification filter was transferred into a new tube and 100 µl of Nucleic Acid purification Elution Solution were added into the filter. The tube was incubated for 2 minutes at 70 °C and then it was centrifugated for 30 seconds at 16000 x g in order to collect the eluate. The collected eluate was re-added into the filter and then centrifugated for 2 minutes at 18000 x g. Approximately 90 µl of RNA eluate were transferred in a new tube.

Next, a DNase reaction was performed using TURBO DNA-free™ Kit *(Ambion)* to remove trace to moderate amounts of contaminating DNA from the purified RNA. Briefly, 0.1 volume of 10X TURBO DNase Buffer and 1 µl of TURBO DNase were added to the RNA, and the mixture was incubated at 37°C for 20-30 min. The reaction was stopped by adding 0.1 volume of DNase inactivation reagent, incubating the sample for 5 min at room temperature, and centrifuging at 10000 x g for 1.5 min. The obtained RNA was transferred to a new tube.

The samples were quantified using NanoQuant Plate™ *(Tecan)* and stored at – 80°C for followed applications. RNA integrity was evaluated using Agilent 2100 Bioanalyzer *(Agilent Technologies, Santa Clara, CA, United States)*

SAMPLE PREPARATION FOR 16S rRNA GENE SEQUENCING

16S amplification and amplify products purification

In order to evaluate the gut microbiota composition, 1.5 ng of microbial DNA were used as starting genomic material. For the amplification of 16S rRNA, The Ion 16S™ Metagenomics Kit *(ThermoFisher)* was used. The kit contains two pool of primer which target seven hypervariable regions of bacteria 16S rRNA (V2, V3, V4, V6, V7, V8, V9) and an Environmental Master Mix v2.0 to amplify targeted regions. Two PCR (1 for each set of primers) were conducted for each sample:

Component	Sample or positive control volume	Negative control volume
2X Environmental Master Mix	7.5 µl	7.5 µl
16S Primer Set (10X) [1]	1.5 µl	1.5 µl
DNA (sample or diluted E. coli DNA control)	1.5 µg sample or 2 µl diluted control [2]	N/A
Negative Control (water)	To 15 µl	To 15 µl

[1] V2-4-8 or V3-6, 7–9

[2] Dilute the E. coli DNA control stock 1:20 (1.5 ng/µL) with DNA Dilution Buffer. Use 2 µl of the diluted DNA control (3 ng DNA input) in the positive control reaction.

Amplification was carried out using ProFlex PCR System *(Applied Biosystems)* using the followed program:

Stage	Temperature	Time
Hold	95°C	10 min
Cycle 18 cycles	95°C	30 sec
	58°C	30 sec
	72°C	20 sec
Hold	72°C	7 min
Hold	4°C	∞

After PCR, an equal volume of each amplification reaction was combined, and an amount of 1.8 X Agencourt® AMPure® XP *(Beckman Coulter)* microspheres were added to purify the amplification reaction. The mixture was incubated 5 minutes at room temperature, and then the tube was place on a magnetic rack for 3 minutes to separate the beads from

solution. The supernatant was discarded and after two washes with 300 µl of 70% ethanol, the beads were air-dried at room temperature for 4 min and resuspended with RNAse/DNAse free water. The supernatant containing the eluate was transferred to new clear 1.5-mL tube.

The amplicons were analysed using Agilent 2100 Bioanalyzer High Sensitivity DNA Kit *(Agilent Technologies, Santa Clara, CA, United States)*

Library preparation

Library preparation was performed using Ion Plus Fragment Library Kit *(ThermoFisher)*. A range of 20-50 ng of pooled short amplicons input were used for end repair reaction with 10ul of 5X End Repair Buffer and 0.5 µl of End Repair Enzyme. The samples were incubated at room temperature for 20 minutes, and then another purification step was performed using 1.8 × sample volume of Agencourt® AMPure® XP *(Beckman Coulter)* beads. After 2 washes with 70% Ethanol, the samples were eluted adding 12.5 µl of Low TE directly to the beads pellet.

Adapter and barcode ligation and nick repair were performed in a 0.2-mL PCR tube combining the reagents as indicated:

Component	volume
DNA	12.5 µl
10X Ligase Buffer	5 µl
Ion P1 Adapter	1 µl
Ion Xpress™ Barcode X	1 µl
dNTP Mix	1 µl
Nuclease-free Water	24.5 µl
DNA Ligase	1 µl
Nick Repair Polymerase	4 µl
TOTAL	**50 µl**

The following thermal protocol was followed:

Stage	Temperature	Time
Hold	25°C	15 min
Hold	72°C	5 min
Hold	4°C	∞

The purification step was performed with 1.4 × sample volume of Agencourt® AMPure® XP *(Beckman Coulter)* beads, and the sample was washed twice with 500 µl of 70% ethanol. The library was eluted in 12.5 µl of low TE.

Library amplification

The library was amplified following the below reaction and thermal protocol:

Component	Volume
Platinum™ PCR SuperMix High Fidelity	50 µl
Library Amplification Primer Mix	2.5 µl
Unamplified library	12.5 µl
TOTAL	**65 µl**

Stage	Temperature	Time
Hold	95°C	5 min
5 cycles	95°C	15 sec
	58°C	15 sec
	70°C	1 min
Hold	4°C	Hold for up to 1 hour

The library was purified by adding 1.5 × the sample volume of Agencourt® AMPure® XP *(Beckman Coulter)* beads to each sample followed by two washes with 70% ethanol. The final barcoded and amplified library was eluted in 20 µl of Low TE.

Library quantification

Library quantification was performed using the Ion Universal Library Quantitation Kit *(ThermoFisher)* to perform a relative qPCR. Ten-fold dilutions from the E. coli DH10B Ion Control Library (68 pM) were prepared as shown in the table:

Standard Control	Library	Nuclease-free Water	Dilution factor	Concentration
1	5 µl undiluted Control Library	45 µl	1:10	6.8 pM
2	5 µl Std 1	45 µl	1:100	0.68 pM
3	5 µl Std 2	45 µl	1:1000	0.068 pM
4	5 µl Std 3	45 µl	1:10000	0.0068 pM
5	5 µl Std 4	45 µl	1:100000	0.00068 pM

Libraries were diluted with serial dilutions in order to target a concentration within the serial dilutions of control library. For each sample, 3 technical replicates qPCR reactions and 3 independent dilution were prepared as shown in the table:

Dilution	Library input	Nuclease-free Water
1:10	2 µL of sample library stock	18 µl
1:100	5 µl of 1:10	45 µl
1:1000	5 µl of 1:100	45 µl
1:10000	5 µl of 1:1000	45 µl

DNA SEQUENCING

Sequencing was performed by Ion GeneStudio S5 Prime System *(Life Technologies)* using the Ion 510 & Ion 520 & Ion 530 Kit-Chef and 510 chip which allows to obtained up to 3 million reads per chip. For each run, a Planned Run was created within the Torrent Suite Software setting the run parameters. Fifty pM diluted libraries were randomly pooled (16 samples per pool). Templated Ion sphere particles preparation and chip loading were performed by the automated Ion Chef System and Ion 510 & Ion 520 & Ion 530 Kit-Chef reagents and disposables. Loaded Ion 510 Chips were run on Ion GeneStudio S5 Prime System (all kits and instruments for sequencing were provided by Thermo Fisher Scientific).

SAMPLE PREPARATION FOR WHOLE BLOOD RNA-SEQUENCING

RNA Precipitation

In order to have input RNA concentration > 70ng/ul for use in Globin Clear procedure, total RNA was precipitated with the following reaction:

Component	Volume
5M Ammonium acetate	0.1 volume
Glycogen	5 ug
100% Ethanol	2.5 volumes

The mixture was placed at -20°C for 2 hours, and centrifuged at 12000 x g for 30 minutes at 4°C to recover the RNA.
The supernatant was carefully removed and 200 mL of ice cold 70% ethanol was added. The mixture was centrifuged at 12000 x g for 15 minutes at 4°C.
After careful removal of the supernatant, RNA was resuspended in 20 µl of nuclease-free water.

Globin clear procedure

Whole blood mRNA is composed of a large portion of globin mRNA transcripts that can interfere with the detection of less abundant mRNAs in whole blood.

To remove the effects of globin mRNA, the samples were treated with GLOBINclear-Human *(Ambion)*.

The following reagents were combined in a 1.5 tube:

Component	Volume
Total RNA	1-2 ug in 14 µl of Nuclease-free Water
Capture Oligo Mix	1 µl
100% Ethanol	2.5 volumes
2X Hybridization Buffer (previously warms to 50°C in hybridization oven)	15 µl

The mixture was placed in a prewarmed incubator at 50°C for 15 minutes to allow the biotinylated oligonucleotide to hybridize with the globin mRNA.

After this incubation, 30 µl of prepared Streptavidin Magnetic Beads were added to each sample and the RNA bead mixture was placed at 50°C for 30 minutes; during this incubation, streptavidin binds to the biotinylated oligonucleotide that captures globin mRNA on the beads.

Purified globin mRNA was transferred to a new tube and purified by adding 100 µl RNA Binding Buffer to the enriched RNA sample, then 20 µl of Bead resuspension Mix was added to each sample.

After magnetic capture, the sample was washed with 200 µl of Wash Solution and finally eluted in 30 µl warm (58°C) Elution Buffer.

PolyA isolation from Purified Total RNA

In order to isolate polyadenylated mRNA, the samples were treated with Dynabeads® mRNA DIRECT™ Micro Kit *(ThermoFisher)* following the protocol provided.

Specifically, RNA after treatment with Globin clear was heated at 70°C for 2 min for thermal denaturation and then 50 µl of Lysis/binding buffer was added to the sample. The mixture was transferred in a new tube contained 20 µl of washed Dynabeads Oligo (dT) and incubated 5 minutes at room temperature to allow the binding of polyA mRNA to dynabeads. The RNA was washed twice with two provided buffer and was eluted with 25 µl of the pre-heated (80°C) nuclease-free water.

A re-bind of polyA mRNA to the beads was performed by adding 25 µl of Lysis/Binding Buffer to each sample. The sample was washed again and finally the beads was resuspended in 10 µl of the pre-warmed (80°C) nuclease-free water. The tube was placed on magnetic stand and the supernatant containing poly A mRNA was transferred into a new tube.

Prepare whole transcriptome libraries

Barcoded libraries were prepared using Ion Total RNA-Seq Kit v2.0 and Ion Express RNA-Seq Barcode kit *(ThermoFisher)*. The RNA fragmentation reaction was performed as indicated:

Component	Volume
RNA sample and nuclease-free water	8 µl
10X RNase III Reaction Buffer	1 µl
RNase III	1 µl

The mixture was incubated in a thermal cycler at 37°C for 3 min and 20 µl of nuclease-free water was added immediately.

The fragmented RNA was purified with Nucleic Acid Binding Beads resuspended in Binding Solution Concentrate and then it was washed with 100% ethanol and with Wash Solution provided by the kit. Sample was eluted adding 12 µl of pre-warmed (37°C) Nuclease-free Water. After evaluating the size distribution of fragmented RNA using Agilent™ 2100 Bioanalyzer™ instrument with the RNA 6000 Pico Kit *(Agilent*

Technologies, Santa Clara, CA, United States), RNA was dried in the centrifugal vacuum concentrator SpeedVac™ at low heat up to a final volume of 3 µl.
Subsequently RNA was hybridized with 5 µl of hybridization master mix and incubated at 65°C for 10 min followed by another incubation at 30°C for 5 min.
The ligation reaction was prepared as follows:

Component	Volume
Hybridization reaction	8 µl
2X Ligation Buffer	10 µl
Ligation Enzyme Mix	2 µl

The mixture was incubated at 30°C for 1h
The obtained sample was than reverse-transcribed with an incubation at 70°C for 10 min in the following reaction:

Component	Volume
Nuclease-free Water	2 µl
10X RT Buffer	4 µl
2.5 mM dNTP Mix	2 µl
Ion RT Primer v2	8 µl
Ligated RNA sample	20 µl

Finally, 4 µl of 10X SuperScript III Enzyme Mix was added to legated RNA and the mixture was incubated at 42°C for 30 min.
cDNA was purified as previously described and eluted in 12 µl of pre-warmed (37°C) Nuclease-free Water.
cDNA fragments of 200 bp of each sample were amplified by 16 cycles of PCR using the specific "Barcode BC primers" for library demultiplexing following the reaction:

Component	Volume
Platinum™ PCR SuperMix High Fidelity	45 µl
Ion Xpress™ RNA 3' Barcode Primer	1 µl

Reaction was performed in a thermocycler:

Stage	Temperature	Time
Hold	94°C	2 min
Cycle (2 cycles)	94°C	30 sec
	50°C	30 sec
	68°C	30 sec
Cycle (16 cycles)	94°C	30 sec
	62°C	30 sec
	68°C	30 sec
Hold	68°C	5 min

Amplified cDNA was purified as previously described and eluted in 15 µl of pre-warmed (37°C) Nuclease-free Water.

1 µl of the final library was analysed and quantified using the Agilent™ 2100 Bioanalyzer™ instrument with the High Sensitivity DNA Kit *(Agilent Technologies, Santa Clara, CA, United States).*

RNA SEQUENCING

Sequencing was performed by Ion GeneStudio S5 Prime System *(Life Technologies)* using the Ion 550 Kit-Chef. For each run, a Planned Run was created within the Torrent Suite Software setting the run parameters. One-hundred pM diluted libraries were randomly pooled (6 samples per pool). Templated Ion sphere particles preparation and chip loading were performed by the automated Ion Chef System and Ion 550 Kit-Chef reagents and disposables. Loaded Ion 550 Chips were run on Ion GeneStudio S5 Prime System (all kits and instruments for sequencing were provided by Thermo Fisher Scientific).

STATISTICAL ANALYSIS

Bioinformatic analysis: microbiome

We chose the NIH Human Microbiome Project (https://www.hmpdacc.org/hmp/HMRGD/) as reference database for our analysis. This repository collects the nucleotide sequence of more than 15000 bacterial genomes, present in human organs or tissue. We selected the 445 bacteria annotated in the human gut. Sequenced reads were aligned against the bacterial genome references by the minimap2 sequencer[158]; then, we processed the resulting BAM files to assess the bacterial abundance. We exploited the 'DaMiRseq' Bioconductor/R package [159] 1) to discard species with less than 5 mapped reads in less than 10% of samples; 2) to normalize data (counts per million in logarithmic scale – log2CPM); and, 3) to perform the exploratory analysis, which allows identifying confounding factors and outliers. Finally, we evaluating the correlation between microbiome and several clinical and demographical variables, exploiting the 'limma' R package[160]. Specifically, we assessed the correlation at the baseline (T0), after three months (T3) and after 1 year (T4) and comparing the differences between the three time points (Δ). Each plot has been generated by the 'ggplot2' R package.

Bioinformatic analysis: transcriptome

A sequential alignment procedure was performed to map raw reads against the GRCh38 Human Genome reference (release 99). First, all reads were aligned by 'STAR'[161]; then, using 'Bowtie2'[162], we locally mapped those reads discarded by STAR. Gene expression quantification was computed by 'featureCounts'[162]. The 'DaMiRseq' Bioconductor/R package was used to filter out genes (less than 10 counts in more than 50% of samples), perform the normalization (variance stabilizing transformation), and search for confounding factors [159]. Correlation analysis was performed by the 'limma' R package [160]. A gene was deemed significant whether the p-value was < 0.05.

RESULTS

STUDY POPULATION

Patients enrollment

The randomization process and completion of study appointments are shown in Figure 7. From a total of 286 eligible patients, with a recent history of coronary revascularization, selected for randomization, only 130 decided to participate in the study. Participants were divided into two groups, 64 to the Mediterranean Diet intervention and 66 to the Low-Fat Diet.

Finally, inconclusive treatment, antibiotic therapy, and technical problems caused the exit of 14 MD and 15 LFD from the study in the first three months. Thus, 103 patients completed analyses of microbiome at three months after dietary intervention (50 MD and 53 LFD).

Among these, only 30 MD and 35 LFD completed analyses at 1 year after randomization.

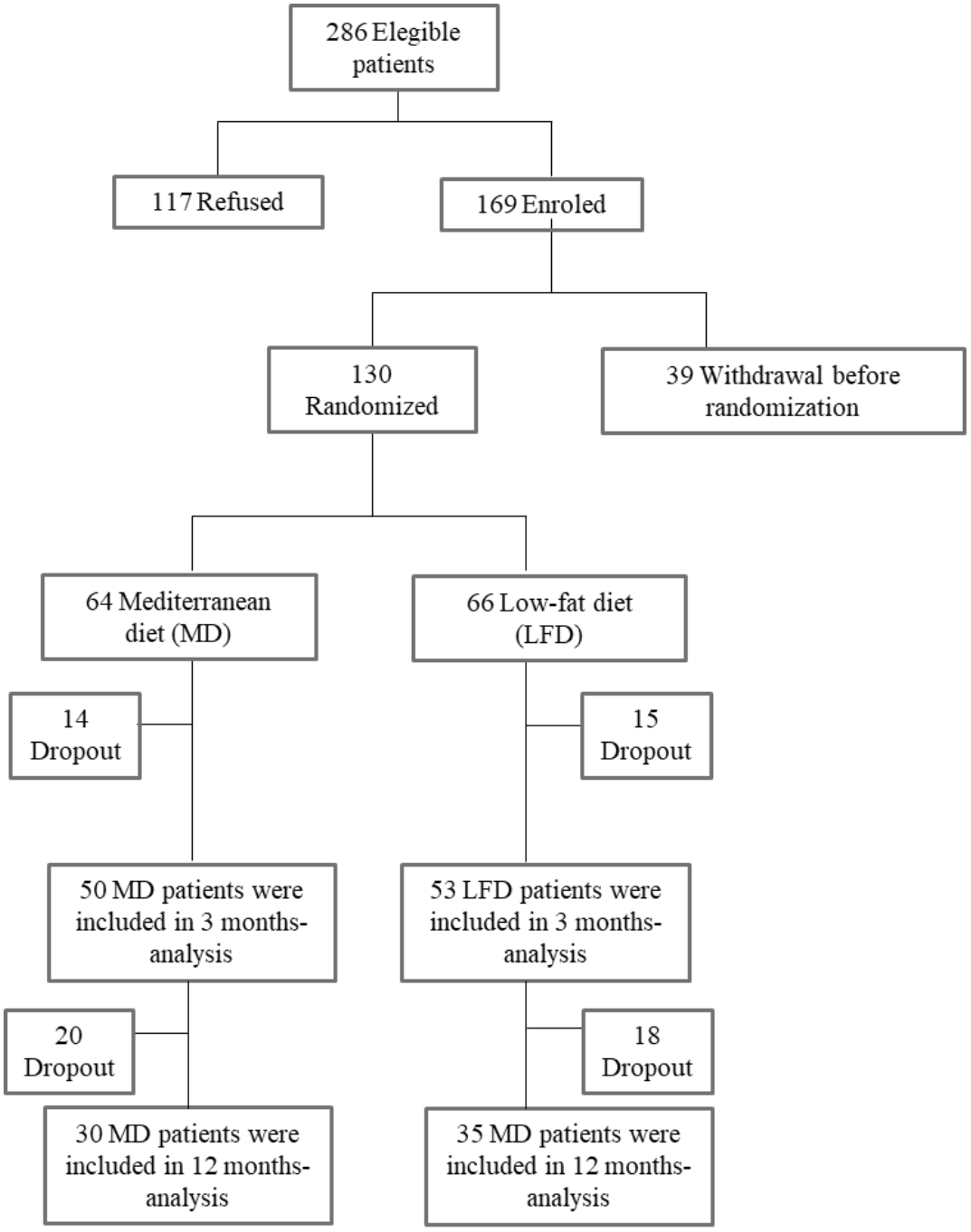


Figure 7: Study flow diagram of patients from enrollment to the end of diet intervention: Mediterranean diet (MD) or low-fat diet (LFD).

Patients baseline characteristics

Table 3 shows the baseline demographic characteristics and clinical parameters of the study participants, stratified by groups.

Variable	All population (n=103)	MD (n=50) T0	LFD (n=53) T0	p value (MD vs LFD)
Age - years	61.5 ± 8.1	61.7 ± 7.3	61.4 ± 8.9	0.940
Male gender - no. (%)	89 (86.4)	41 (82)	48 (90.6)	0.260
Weight (Kg)	79 ± 12.9	77.5 ± 12.7	80.4 ± 13.1	0.360
Height (m)	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	0.300
BMI (Kg/m2)	27.5 ± 3.5	27.4 ± 3.4	27.6 ± 3.7	0.990
Waist circumference (cm)	98.1 ± 9.9	97.7 ± 9.7	98.5 ± 10.1	0.900
Total cholesterol (mg/dl)	166.3 ± 28.4	172.8 ± 29	160.2 ± 26.7	**0.030**
HDL (mg/dl)	51.4 ± 13.5	50.3 ± 12.6	52.5 ± 14.4	0.560
LDL (mg/dl)	93.3 ± 25.7	100.4 ± 26.7	86.6 ± 23	**0.008**
Triglyceride Levels (mg/dl)	107.8 ± 47.3	110.3 ± 52.7	105.4 ± 42	0.980
hs-CRP (mg/dl)	1.6 ± 2.1	1.5 ± 1.7	1.6 ± 2.4	0.640
Fasting glycaemia (mg/dl)	102.3 ± 10.5	100.7 ± 9.2	103.8 ± 11.4	0.320
SBP (mmHg)	133.4 ± 17.5	133.6 ± 14.6	133.3 ± 20	0.780
DBP (mmHg)	79.7 ± 9.8	79.1 ± 10.1	80.3 ± 9.5	0.780
Smoke				
smoker - no. (%)	12 (11.65)	3 (6)	9 (16.98)	0.080
ex-smoker - no. (%)	63 (61.16)	32 (64)	31 (58.49)	0.570
non-smoker - no. (%)	28 (27.18)	15 (30)	13 (24.52)	0.530
Hypertension - no. (%)	72 (69.90)	36 (72)	36 (67.92)	0.673
Dysplidemia - no. (%)	81 (78.64)	37 (74)	44 (83.02)	0.337
Hyperglycemia - no. (%)	27 (26.21)	7 (14)	20 (37.73)	**0.007**
MEDAS - no.	5.9 ± 1.7	5.9 ± 1.7	5.9 ± 1.8	0.750

Table 3– Baseline characteristics of the whole population (n=103). Quantitative variables are expressed as mean±SD and categorical variables as number (percentage). p-values are shown for comparison of baseline characteristics of patients (MD vs LFD). For continuos variables the t-test and for binary variables the Fisher's exact test were used. Body mass index (BMI), high-density lipoprotein (HDL), low-density lipoprotein (LDL), high sensitive-C reactive protein (hs-CRP), systolic blood pressure (SBP), diastolic blood pressure (DBP), Mediterranean diet adherence score (MEDAS).

In summary, the population consisted of middle to older-aged adult, mainly male patients, as expected in CAD. Clinical parameters were included within the normal ranges except

for fasting glycaemia which exceed the limit usually recognize for normoglycemia (100 mg/dl), but no diabetic patient was enrolled.

Most of the population was hypertensive (69.9%) and dyslipidemic (78.64%) and averagely overweight (average BMI: 27.5 ± 3.5). Few participants (11.65%) were current smokers, but most of the population was ex-smoker (61.16%).

There were no significant differences between the study groups for any of the reported characteristics at baseline except for total cholesterol and LDL cholesterol level, which were higher in the MD than LFD group (172.8 ± 29 vs 160.2 ± 26.7, p=0.03 and 100.4 ± 26.7 vs 86.6 ± 23, p=0.01, respectively) and percentage of hyperglycaemic subjects higher in LFD group (14% in MD vs 37.73% in LFD, p =0.007).

In both groups, MEDAS index at baseline denotes a low adherence to Mediterranean diet (MEDAS score < 7).

The distribution of MEDAS score at baseline is reported in Figure 8.

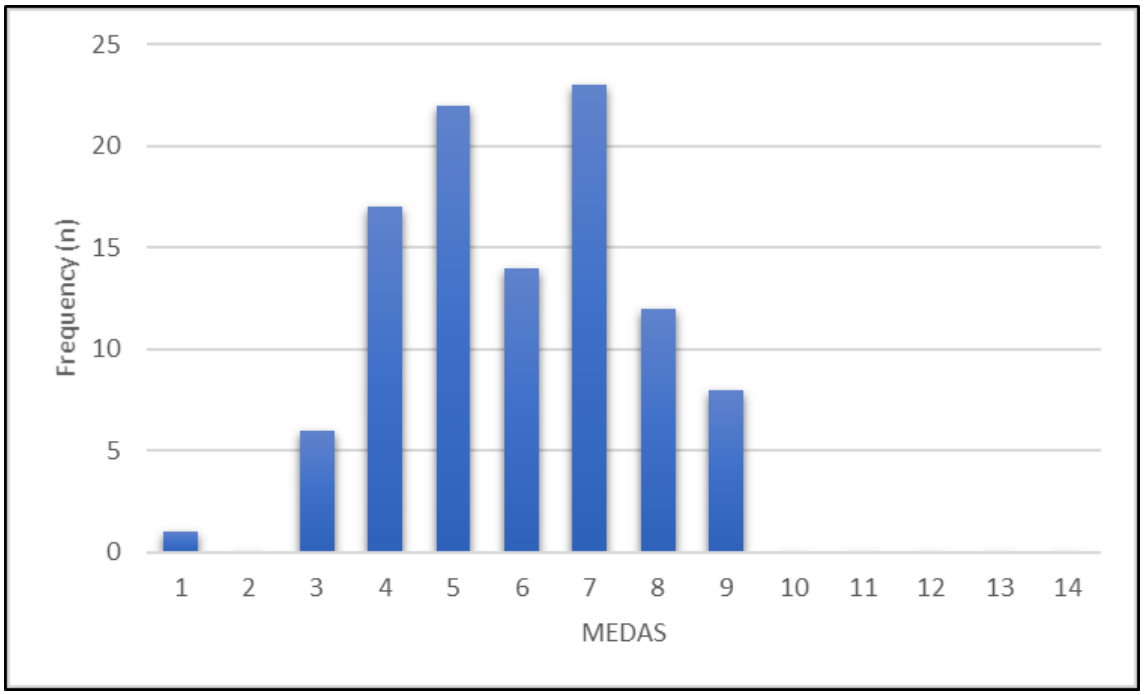


Figure 8 – Distribution of the Mediterranean diet adherence screener score (MEDAS) in the whole population (n=103) at baseline.

As shown in Table 4, there were no significant differences in baseline eating habits between the two intervention groups.

Nutrients	MD (n=50)	LFD (n=53)	p-value (MD vs LFD)
Energy Kcal	2246.9 ± 636.3	2298.6 ± 735.3	0.70
Total carbohydrate (g)	278.7 ± 91.4	282.0 ± 103.1	0.87
Complex carbohydrate (g)	165.1 ± 61	166.6 ± 66.9	0.91
Simple carbohydrate (g)	113.3 ± 46.7	114.9 ± 59.5	0.87
Total protein (g)	81.2 ± 25.2	81.7 ± 29.6	0.93
Animal protein (g)	51.8 ± 19.5	52.0 ± 24.0	0.97
Plant protein (g)	29.3 ± 9.2	29.6 ± 9.4	0.89
Total fat (g)	83.0 ± 23.5	84.7 ± 31.4	0.76
Saturated fatty acids (g)	25.7 ± 9.3	26.8 ± 11.5	0.57
Monounsaturated fatty acids (g)	39.3 ± 10.9	39.2 ± 14.3	0.96
Polyunsaturated fatty acids (g)	11.1 ± 3.3	11.4 ± 4.8	0.72
Fibers (g)	22.5 ± 7.3	23.2 ± 7.8	0.67
Iron (mg)	11.2 ± 3.1	11.7 ± 4.2	0.49
Calcium (mg)	832.5 ± 327.4	833.8 ± 330.8	0.98
Sodium (mg)	2255.8 ± 864.2	2423.8 ± 938.3	0.35
Potassium (mg)	3254.0 ± 803.1	3264.2 ± 1000.6	0.95

Table 4 - Dietary habits of patients relative to the year prior to randomization

Monitoring of classical risk factors after a 3-months treatment

Table 5 shows anthropometric and clinical data of the entire population stratified by group, measured at T3, and the T0-T3 change.

Variable	All population T3 (n=103)	p value T0 vs T3	MD (n=50) T3	p value (MD T0 vs T3)	LFD (n=53) T3	p value (LFD T0 vs T3)	p value (MD T3 vs LFD T3)
Weight (Kg)	75.6 ± 12.3	**< 0.0001**	74.7 ± 12.6	**< 0.0001**	76.6 ± 12	**< 0.0001**	0.442
BMI (Kg/m2)	26.4 ± 3.4	**< 0.0001**	26.4 ± 3.4	**< 0.0001**	26.3 ± 3.4	**< 0.0001**	0.792
Waist circumference (cm)	94.8 ± 9.4	**< 0.0001**	94.3 ± 9.4	**< 0.0001**	95.3 ± 9.4	**< 0.0001**	0.707
Total cholesterol (mg/dl)	156.9 ± 34.3	**0.0003**	162.6 ± 36.9	**0.012**	151.5 ± 31	**0.012**	0.150
HDL (mg/dl)	51.0 ± 13.5	0.477	50.1 ± 11.8	0.786	51.8 ± 15	0.476	0.690
LDL (mg/dl)	86.5 ± 28.2	**0,001**	92.4 ± 31	**0.017**	80.9 ± 24.4	**0.043**	0.080
Triglyceride Levels (mg/dl)	97.4 ± 45.3	**0,005**	100.9 ± 49.7	0.078	94 ± 40.9	**0.033**	0.690
hs-CRP (mg/dl)	1.7 ± 3.9	0.756	1.6 ± 2.7	0.895	1.8 ± 4.8	0.780	0.540
Fasting glycaemia (mg/dl)	101.7 ± 10.7	0.467	100.1 ± 8.8	0.612	103.3 ± 12	0.610	0.240
SBP (mmHg)	125.9 ± 16.9	**< 0.0001**	124.8 ± 18	**< 0.0001**	126.9 ± 15.9	**0.007**	0.380
DBP (mmHg)	75.7 ± 10.4	**< 0.0001**	75 ± 11.7	**0.0004**	76.3 ± 9	**0.0004**	0.470
MEDAS - no.	9 ± 1.90	**< 0.0001**	9.8 ± 1.7	**< 0.0001**	8.2 ± 1.7	**< 0.0001**	**< 0.0001**

Table 5 – Variation clinical parameters between T0 (baseline) and T3 (three months after diet) in the whole population (n=103), in MD and in LFD groups. Comparison of clinical parameters between the two intervention groups at T3. Variables are expressed as mean±SD. p-values are shown for comparison of T3 vs T0. Paired t-test was used. Body mass index (BMI), high-density lipoprotein (HDL), low-density lipoprotein (LDL), high sensitive-C reactive protein (hs-CRP), systolic blood pressure (SBP), diastolic blood pressure (DBP), Mediterranean diet adherence screener score (MEDAS).

The adherence to MD, according to MEDAS score, increased in both MD and LFD groups from T0 to T3 (5.9 > 9.8, $p<0.001$ and 5.9 > 8.2, $p<0.001$, respectively).

Both diet interventions were able to considerably reduce weight, BMI, waist circumference, total cholesterol, LDL cholesterol and blood pressure. A reduction in triglycerides levels was observed only for the LFD group.

Notably, except for the MEDAS score, there were no significant differences between the intervention groups at T3.

The analysis was also made considering the whole sample, evaluating how much the patients have modified their eating habits towards a Mediterranean style, regardless of the treatment group.

The distribution of MEDAS score of whole population at T3 is reported in Figure 9.

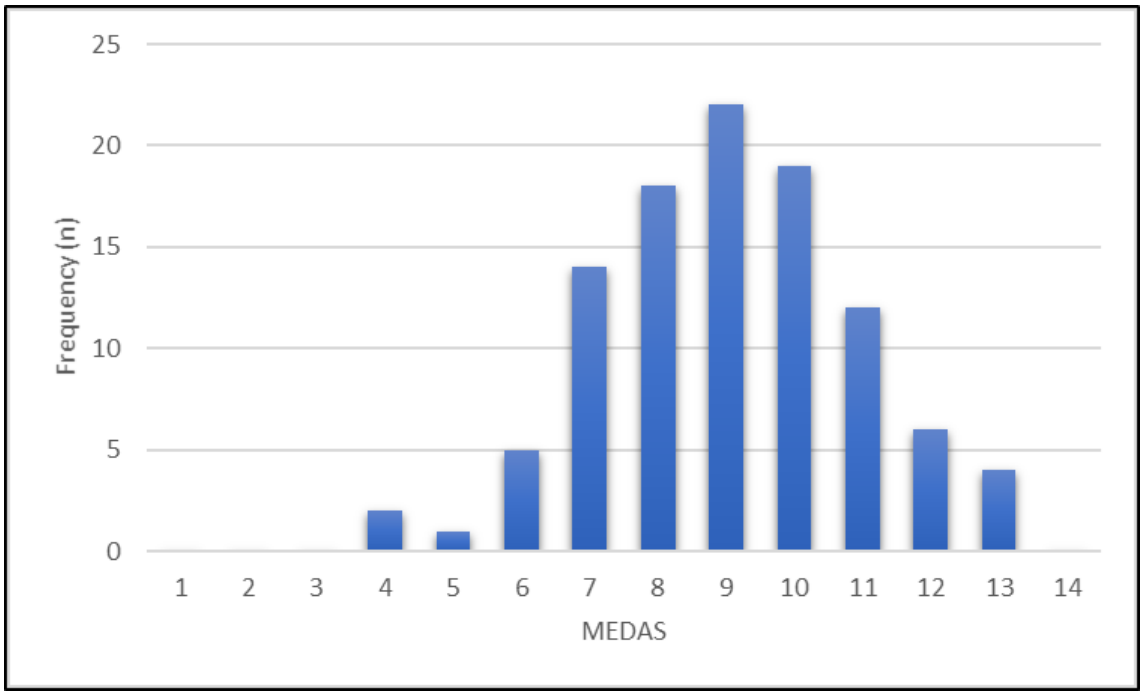


Figure 9 – Distribution of the Mediterranean diet adherence score (MEDAS) in the whole population (n=103) at T3.

Table 6 shows the correlation coefficients between the variation of MEDAS score and classic risk factors (T3-T0). A significant negative relationship was observed between the change in MEDAS score and the change in BMI, waist circumference, total cholesterol, LDL cholesterol, and hs-CRP.

Variable	Score MeDAS (delta T3-T0)	p-value
BMI (Kg/m2)	-0.21	**0.03**
Waist circumference (cm)	-0.25	**0.01**
Total cholesterol (mg/dl)	-0.25	**0.01**
HDL (mg/dl)	-0.05	0.64
LDL (mg/dl)	-0.21	**0.04**
Triglyceride Levels (mg/dl)	-0.17	0.10
hs-CRP (mg/dl)	-0.25	**0.01**
Fasting glycaemia (mg/dl)	-0.16	0.10
SBP (mmHg)	-0.13	0.19
DBP (mmHg)	-0.07	0.49

Table 6-Correlation coefficients between changes in anthropometric and biochemical parameters (T3-T0) and change in MeDAS score (T3-T0), in the whole sample.

Body mass index (BMI), high-density lipoprotein (HDL), low-density lipoprotein (LDL), high sensitive-C reactive protein (hs-CRP), systolic blood pressure (SBP), diastolic blood pressure (DBP), Mediterranean diet adherence score (MEDAS).

EXPLORATIVE ANALYSIS OF GUT MICROBIOTA COMPOSITION BY 16S rRNA GENE SEQUENCING ON WHOLE POPULATION AT BASELINE.

To investigate the effects of MD on microbiota composition, in order to better understand the protective role of this diet on the cardiovascular system, we performed 16S rRNA gene amplicon sequencing in 103 subjects in secondary prevention for cardiovascular disease.

We first analysed the baseline microbiota profile in terms of more abundant taxa. Most reads aligned were bacterial belonged to the phyla Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Figure 10).

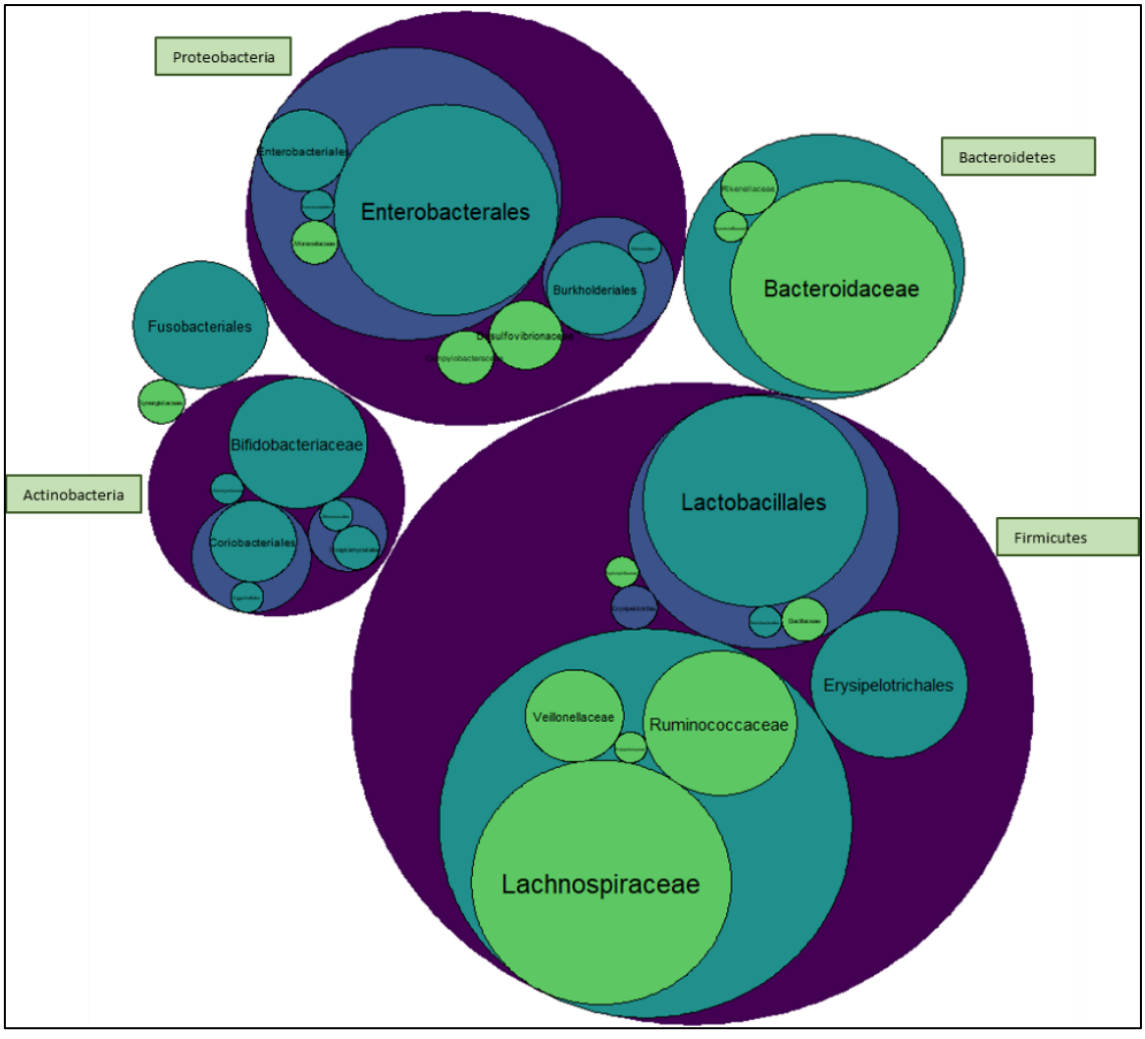


Figure 10: Proportional circle plot showing the abundant taxa found in our population at T0. Purple circles indicate phylum, blue circles indicate class, dark green circles indicate order, and light green circles indicate family.

The graph in Figure 11 shows that the most abundant microbial families in our population are also those present in a greater percentage of subjects.

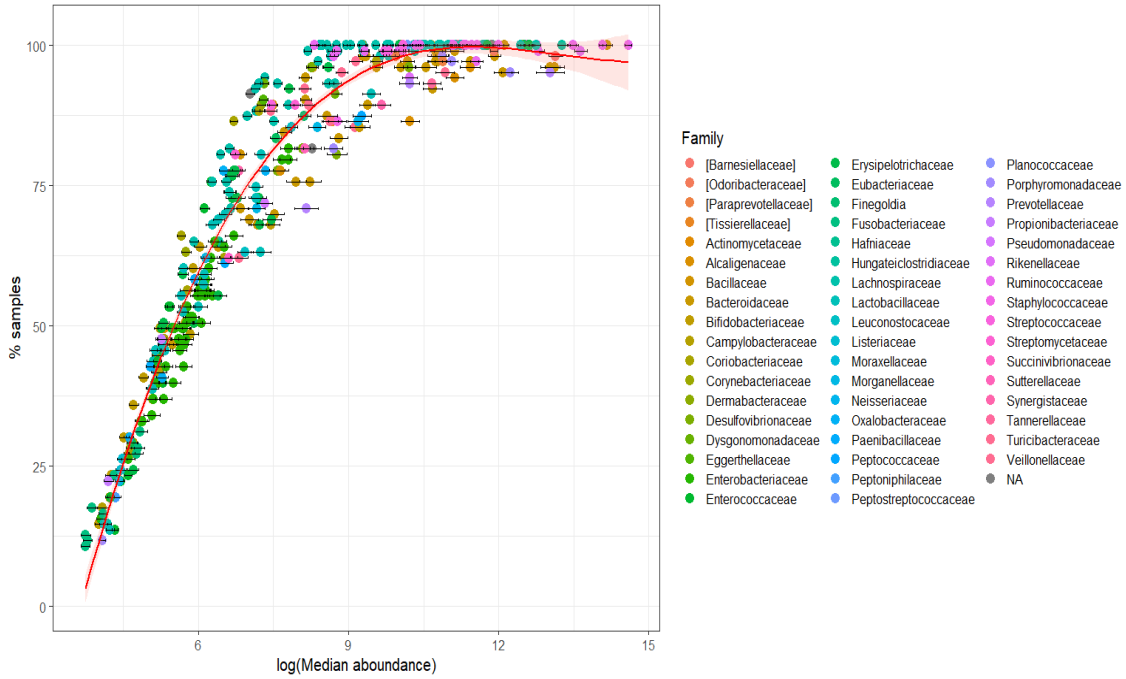
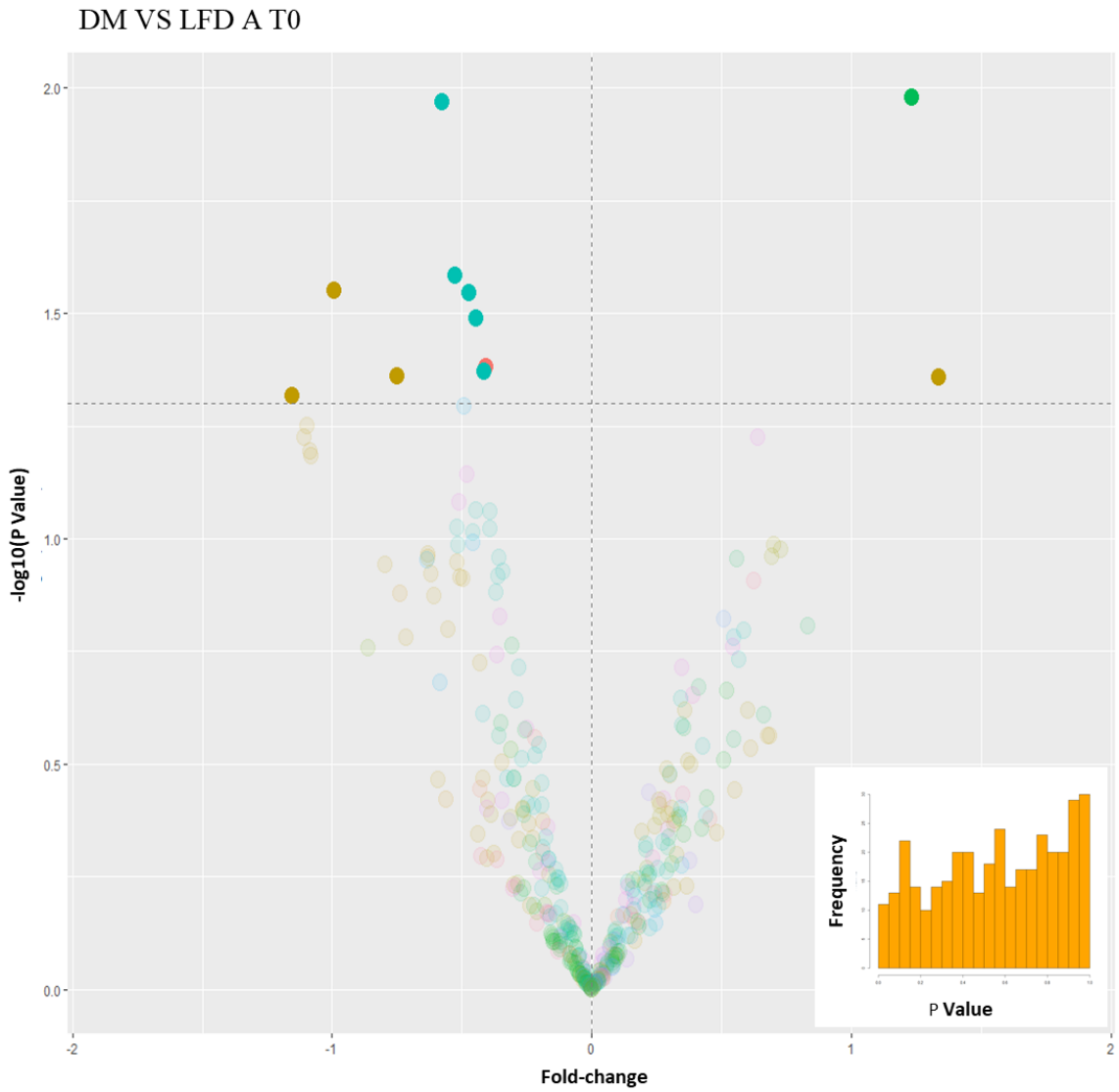


Figure 11: Dot plot analysis that reports the correlation between family abundance and percentage of samples. A legend of the bacterial families is reported.

EFFECTS OF DIETARY INTERVENTION ON GUT MICROBIOTA COMPOSITION

Comparison analysis of GM composition between MD and LFD at T0 and T3

To explore the effects of each diet on gut microbiota composition, a differential analysis was performed. As Figure 12 shows, although there are few significant differences between the two groups at T0 and T3, when looking at the histogram of p-values these differences cannot be considered reliable.



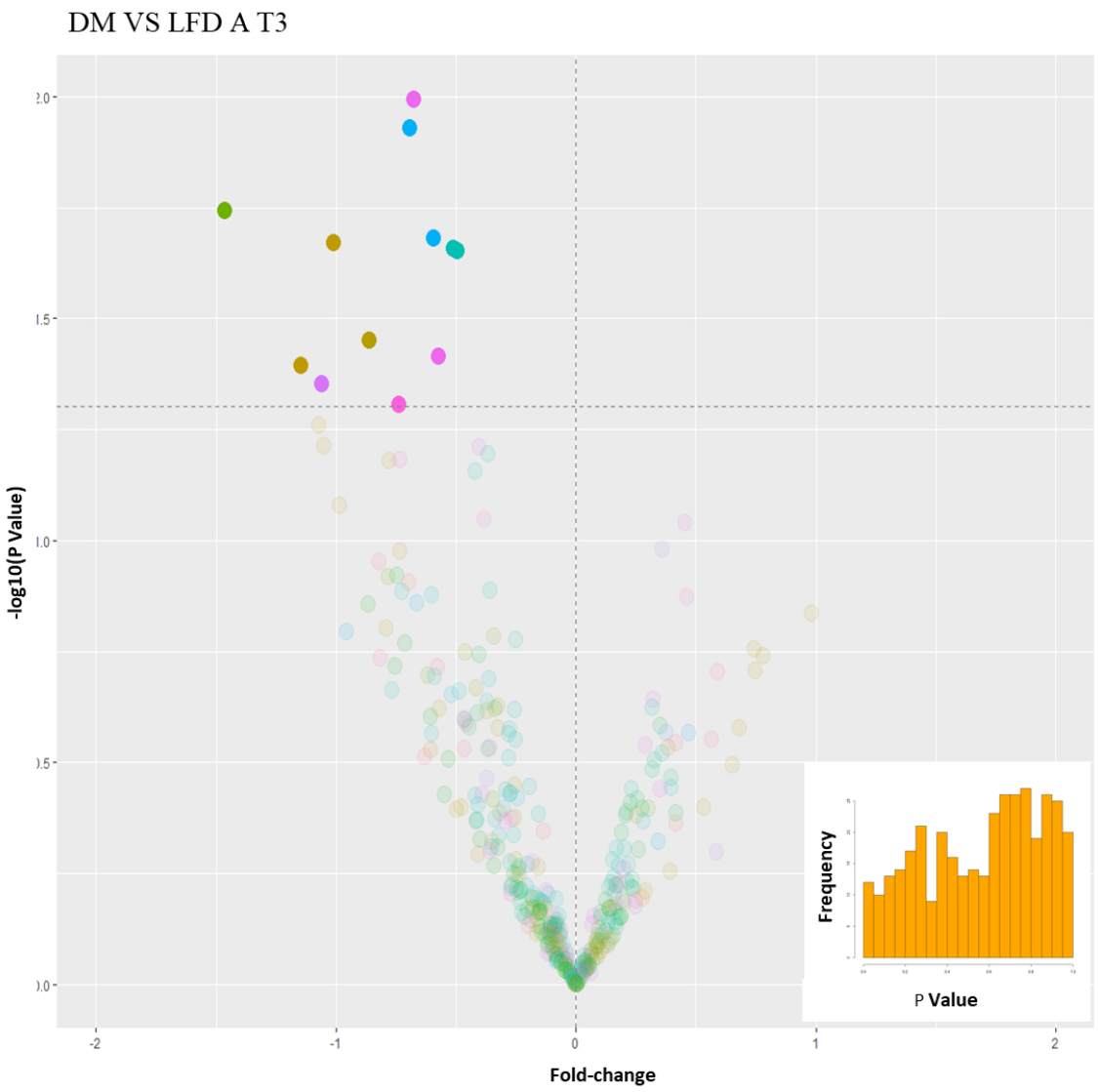


Figure 12: Differential analysis showing differences between LFD and MD at T0 and T3. The horizontal dashed line indicates the significance threshold level (p=0.05). For each analysis, p-value histogram was shown. A legend of the bacterial families is shown in Figure 11.

Analysis of GM composition in the whole population

The results obtained from "the intention to treat" analysis could be explained by the fact that no significant clinical and anthropometric differences were present between the two groups at T3. Moreover, although in the LFD group the mean MEDAS score was significantly lower than in the DM group, it was still indicative of a high adherence to a Mediterranean diet (MEDAS >7). It is likely, therefore, that the entire population has changed its dietary habits towards a greater adherence to MD regardless of the group to which the population belonged.

Based on this premise, subsequent analyses were conducted considering the whole population, as a single intervention group. As showed in Figure 13, the analysis of diversity metrics at species level revealed that the Mediterranean diet had no significant impact on alpha-diversity as expressed by the Shannon index between T0 and T3.

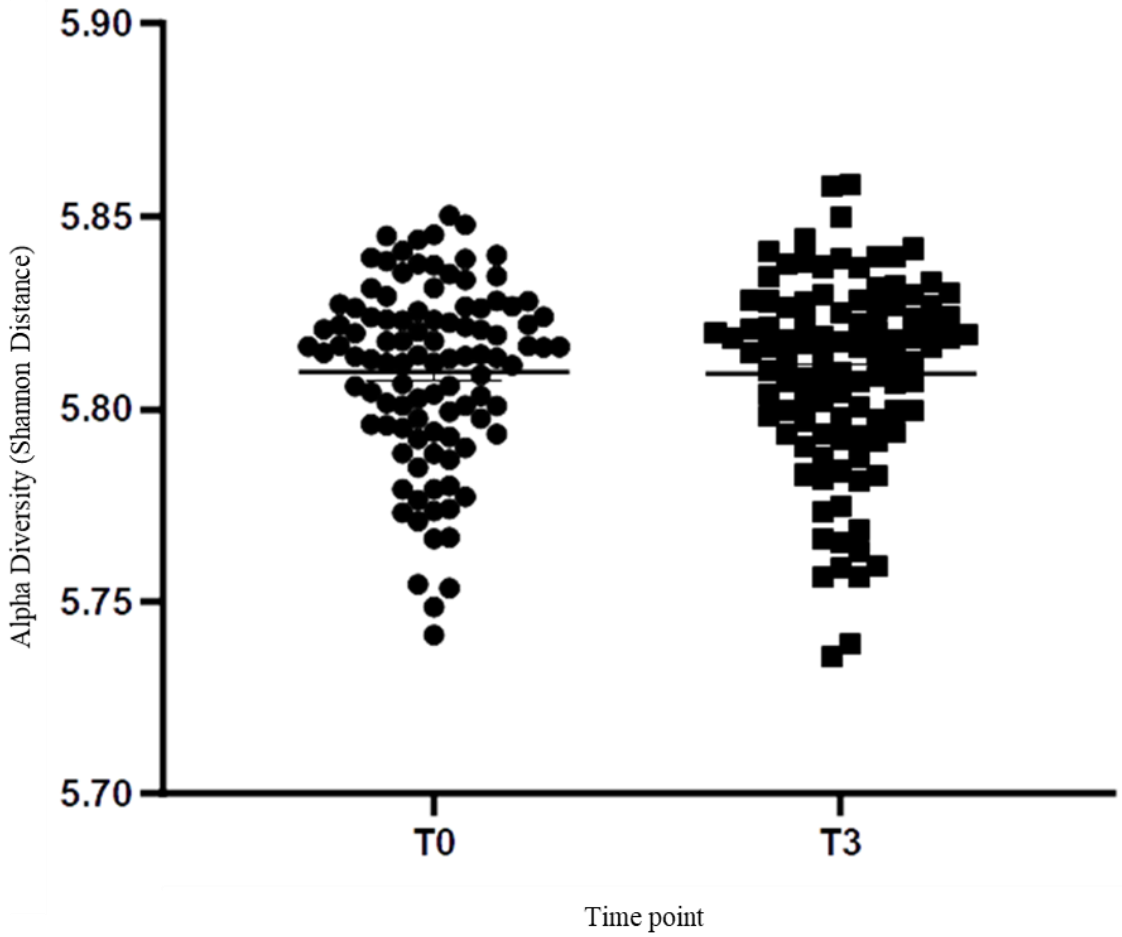


Figure 13: Shannon diversity Index is plotted for patients at T0 and T3.

Next, we performed a correlation analysis between MEDAS score and genera of bacteria found in our population both at T0 and T3 time point. Tables 7A and 6B report the genera positively and negatively associated with the level of MD adherence at baseline and 3 months after randomization, respectively.

A

Genera	r	p-value
Acinetobacter	0.25	0.014
Paenibacillus	0.23	0.021
Anaerococcus	0.22	0.024
Bacillus	0.22	0.029
Dermabacter	0.22	0.032
Coprobacillus	0.22	0.033
Actinomyces	0.21	0.033
Enterococcus	0.21	0.033
Anaerostipes	0.21	0.035
Bacteroides	0.21	0.035
Dorea	0.21	0.041
Fusobacterium	0.20	0.042
Bifidobacterium	-0.20	0.045
Coprococcus	0.20	0.047
Holdemania	0.20	0.048
Escherichia	-0.20	0.049

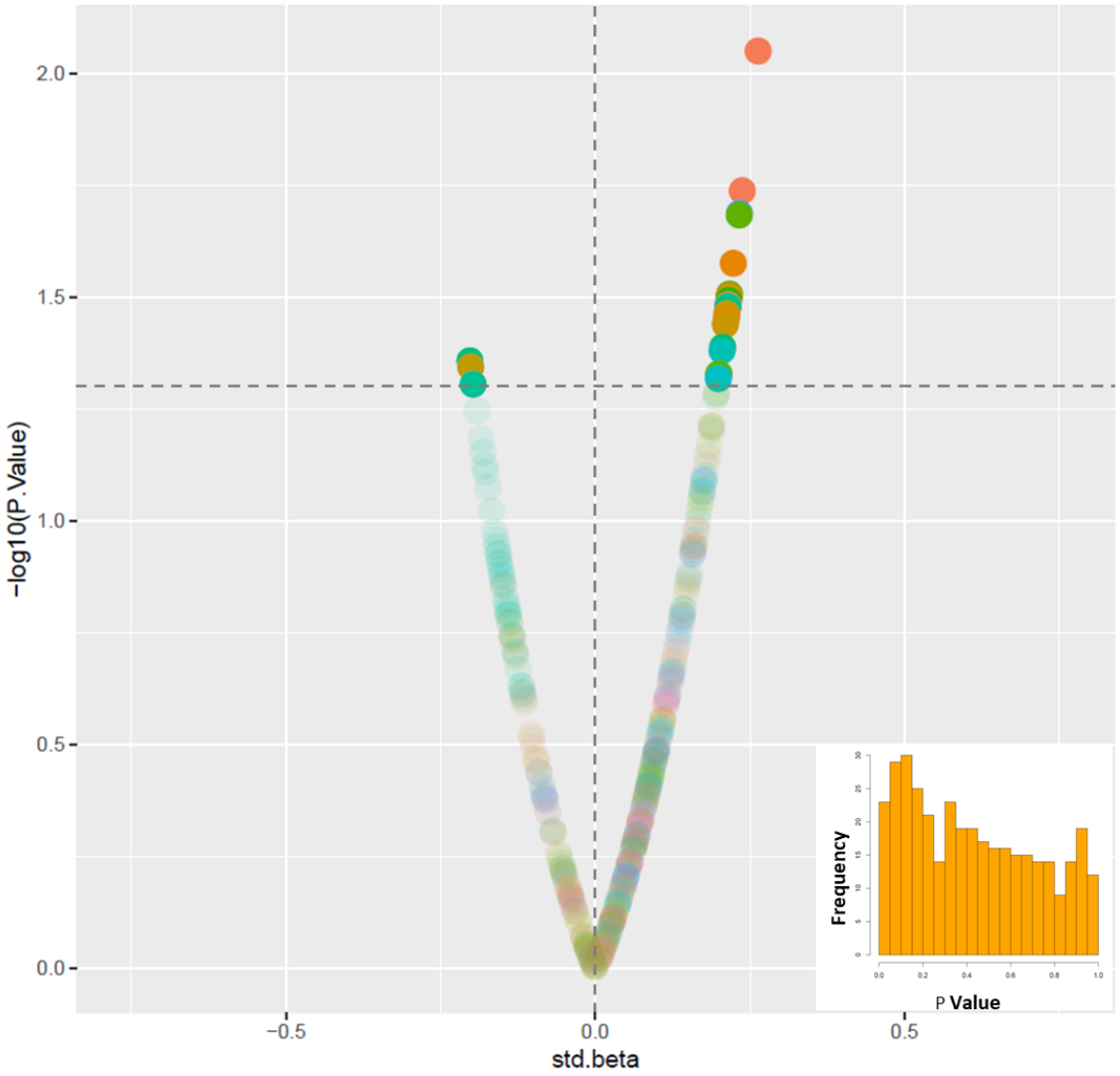
B

Genera	r	p-value
Bacteroides	0.38	< 0.001
Eubacterium	0.34	0.001
Coprococcus	0.34	0.002
Acidaminococcus	-0.30	0.003
Butyrivibrio	0.27	0.01
Ruminococcus	0.27	0.01
Escherichia	-0.28	0.01
Enterobacter	-0.26	0.01
Yokenella	-0.26	0.01
Blautia	0.25	0.01
Faecalibacterium	0.24	0.01
Citrobacter	-0.25	0.02
Clostridium	0.29	0.02
Paenibacillus	0.24	0.02
Listeria	0.23	0.02
Tyzzerella	0.22	0.03
Klebsiella	-0.22	0.03
Holdemania	0.20	0.04

Table 7: Pearson's coefficient correlation and p-value between genera abundance and adherence to MD at T0 (A) and T3 (B) of significant associated genera.

As shown also in Figure 14 multiple species belonging to different genera are significantly correlated with MEDAS at T3 compared to T0. These different correlations can be explained by the fact that after 3 months from dietary recommendations, the composition of the intestinal microbiota changes due to healthier dietary habits adopted by patients, with emergence of different species or different expression of those present at baseline. We must also consider that the same MEDAS score can be the result of a different food intake, for example 3 portions of legumes per week give the same score as 3 portions of fish per week, but they are different foods that may differently affect the gut microbiota.

T0



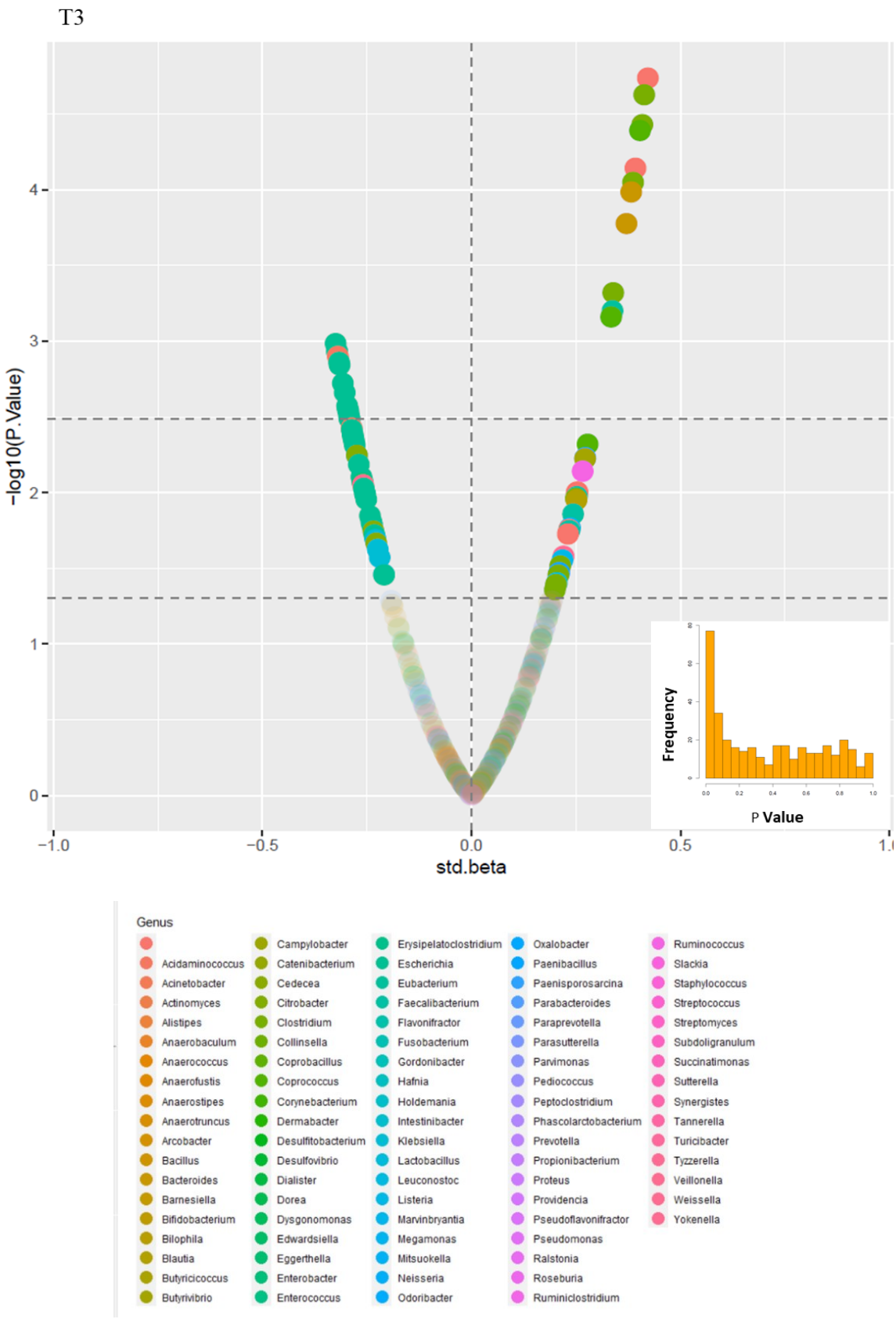


Figure 14: volcano plot showing the correlation indexes of all genera comparing MEDAS at T0 and T3. The horizontal dashed line indicates the significance threshold level (p=0.05). At T3, the upper horizontal dashed line indicates the

significance threshold adjusted by multiple comparisons. For each analysis, p-value histogram was shown. A legend of the bacterial genera is reported.

We decided to investigate the association between increased MD adherence at T3 (Δ MEDAS T3-T0) and changes in GM composition. As showed in Table 8 and in Figure 15, the analysis revealed significant positive and negative associations between different bacterial genera and the change in MEDAS score from T0 to T3. The Delta MEDAS score is normalized to the score at baseline for each patient.

Δ T3-T0

Genera	r	p-value
Dorea	-0.44	< 0.001
Bifidobacterium	-0.34	0.002
Butyrivibrio	-0.30	0.005
Paenibacillus	-0.31	0.005
Unclassified Lachnospiraceae	-0.29	0.008
Clostridium	-0.29	0.012
Holdemania	-0.27	0.012
Enterococcus	-0.30	0.014
Ruminococcus	-0.29	0.016
Coprococcus	-0.26	0.017
Bacillus	-0.25	0.018
Intestinibacter	-0.25	0.020
Erysipelatoclostridium	-0.25	0.021
Parvimonas	-0.24	0.025
Listeria	-0.24	0.027
Eubacterium	0.23	0.032
Pediococcus	-0.23	0.033
Anaerofustis	-0.23	0.034
Paraprevotella	-0.22	0.038
Paenisporosarcina	-0.22	0.038
Bacteroides	-0.22	0.039
Escherichia	-0.22	0.041
Oxalobacter	-0.22	0.044

Table 8: Pearson's coefficient correlation and p-value between changing in genera abundance and variation in adherence to MD from T0 to T3 of significant associated genera.

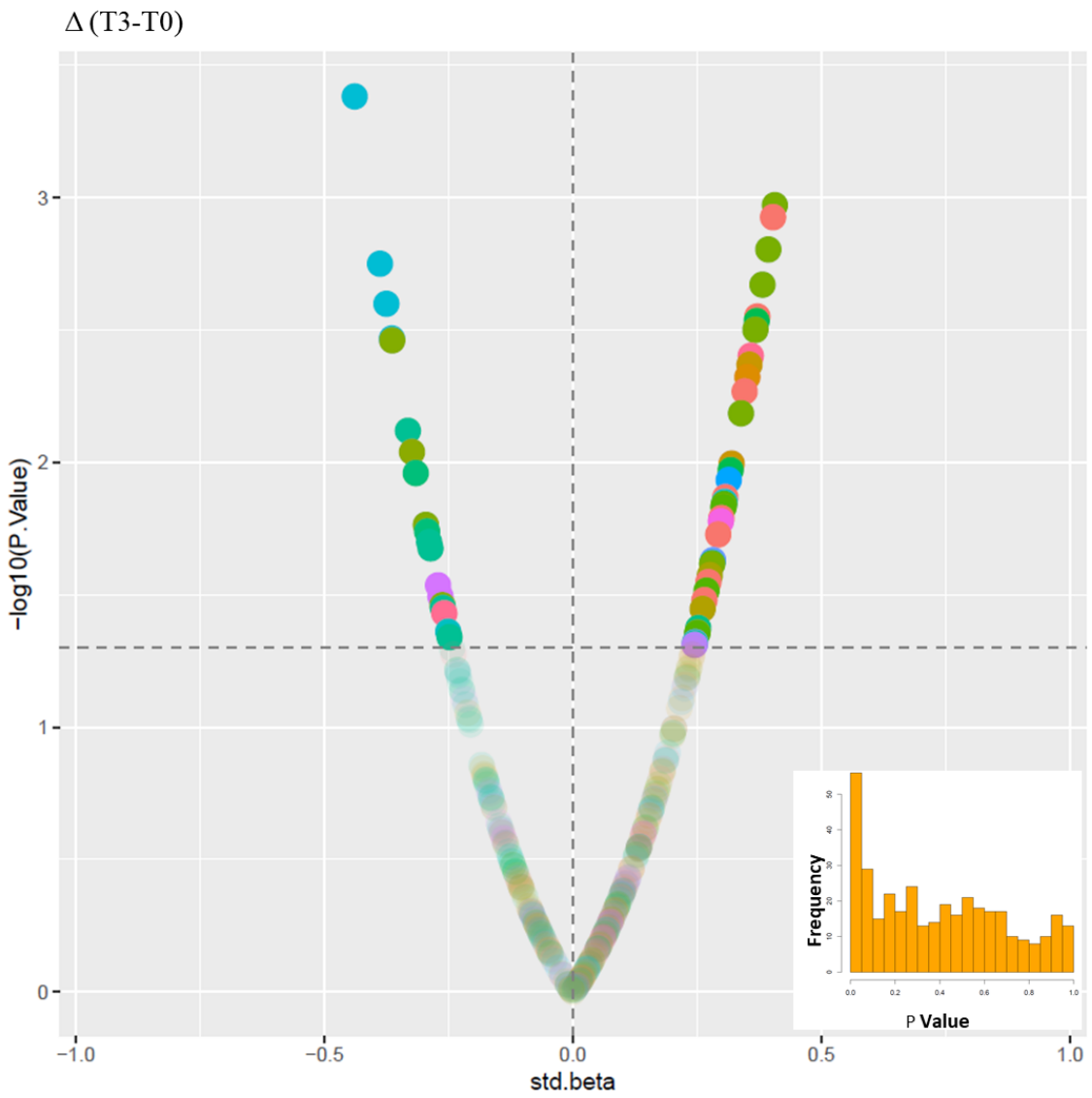


Figure 15: Volcano plot showing the correlation indexes between all genera changing and Δ MEDAS from T0 to T3. The horizontal dashed line indicates the significance threshold level (p=0.05*). p*-value histogram was shown. A legend of the bacterial genera is shown in figure 14.

Since after 3 months from enrollment we observed a significant reduction of some risk factors for CVD, we wanted to assess whether there were microbial changes related to this reduction.

Significant correlations were found between changes in microbiota composition and changes in BMI and waist circumference from T0 to T3 as showed in Table 9.

BMI

Genera	r	p-value
Coprococcus	-0.30	0.006
Erysipelatoclostridium	-0.28	0.008
Blautia	-0.27	0.009
Bifidobacterium	-0.27	0.010
Clostridium	-0.31	0.010
Dorea	-0.28	0.012
Bacteroides	0.27	0.014
Unclassified Lachnospiraceae	-0.28	0.015
Ruminococcus	-0.26	0.015
Streptomyces	-0.25	0.017
Phascolarctobacterium	-0.25	0.018
Eubacterium	-0.24	0.023
Lactobacillus	-0.23	0.030
Enterococcus	-0.22	0.038
Escherichia	-0.21	0.040
Paenibacillus	-0.21	0.043

WAIST CIRCUMFERENCE

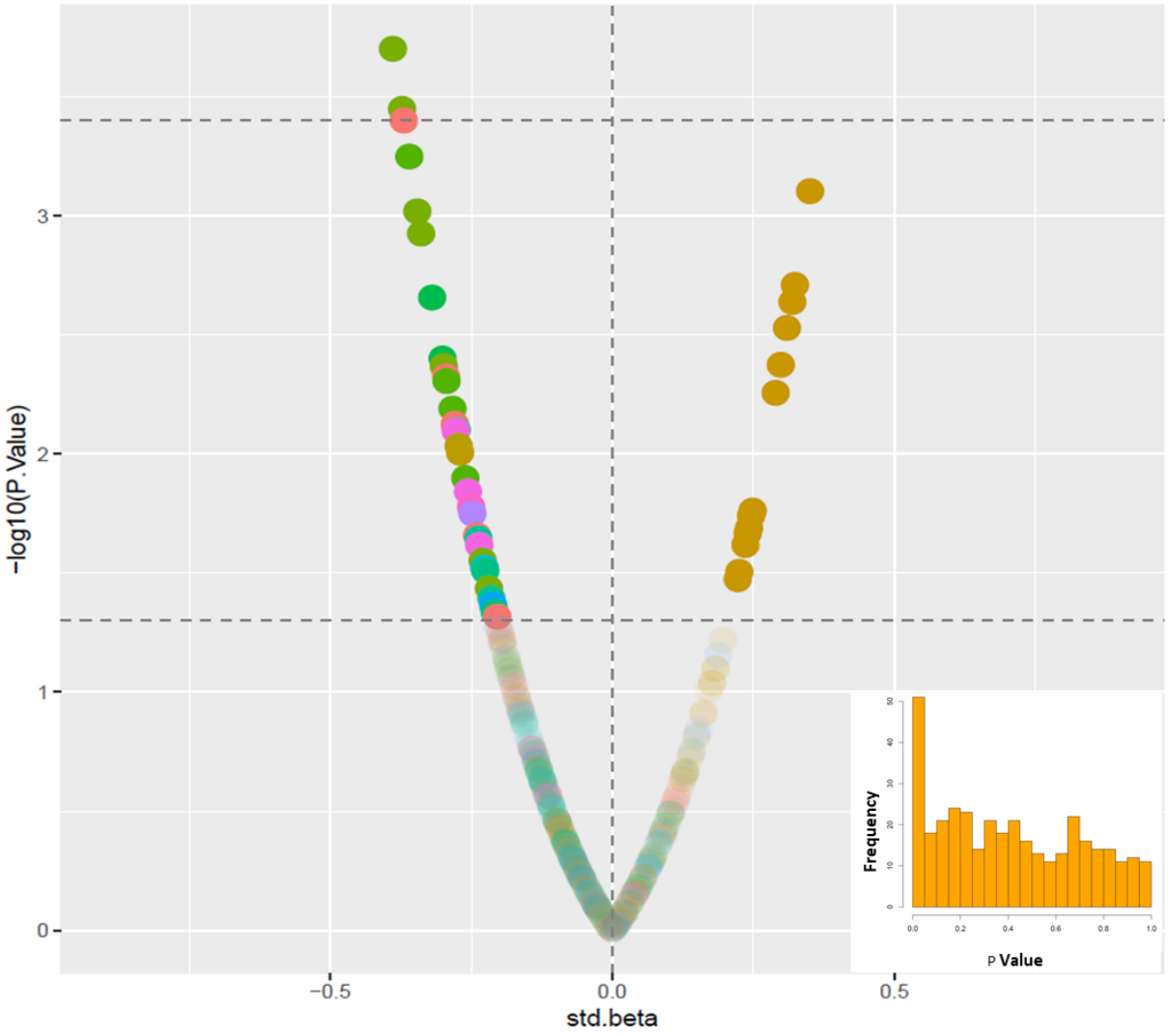
Genera	r	p-value
Dorea	-0.44	< 0.001
Bifidobacterium	-0.34	0.002
Butyrivibrio	-0.30	0.005
Paenibacillus	-0.31	0.005
Unclassified Lachnospiraceae	-0.29	0.008
Clostridium	-0.29	0.012
Holdemania	-0.27	0.012
Enterococcus	-0.30	0.014
Ruminococcus	-0.29	0.016
Coprococcus	-0.26	0.017
Bacillus	-0.25	0.018
Intestinibacter	-0.25	0.020
Erysipelatoclostridium	-0.25	0.021
Parvimonas	-0.24	0.025
Listeria	-0.24	0.027
Eubacterium	0.23	0.032
Pediococcus	-0.23	0.033
Anaerofustis	-0.23	0.034
Paraprevotella	-0.22	0.038
Paenisporosarcina	-0.22	0.038
Bacteroides	-0.22	0.039
Escherichia	-0.22	0.041
Oxalobacter	-0.22	0.044

Table 9: Pearson's coefficient correlation and p-value between changing in genus abundance and variation in BMI and waist circumference from T0 to T3 of significant associated genera.

Interesting to note that, bacteria belonging to the genera *Clostridium*, *Dorea, Coprococcus, Blautia and Ruminococcus,* which were positively correlated with higher MD adherence, were found to be negatively associated with reduced BMI and waist circumference.

Notably, we also observed a positive correlation between several species of *Bacteroides* and BMI reduction (Figure 16).

BMI



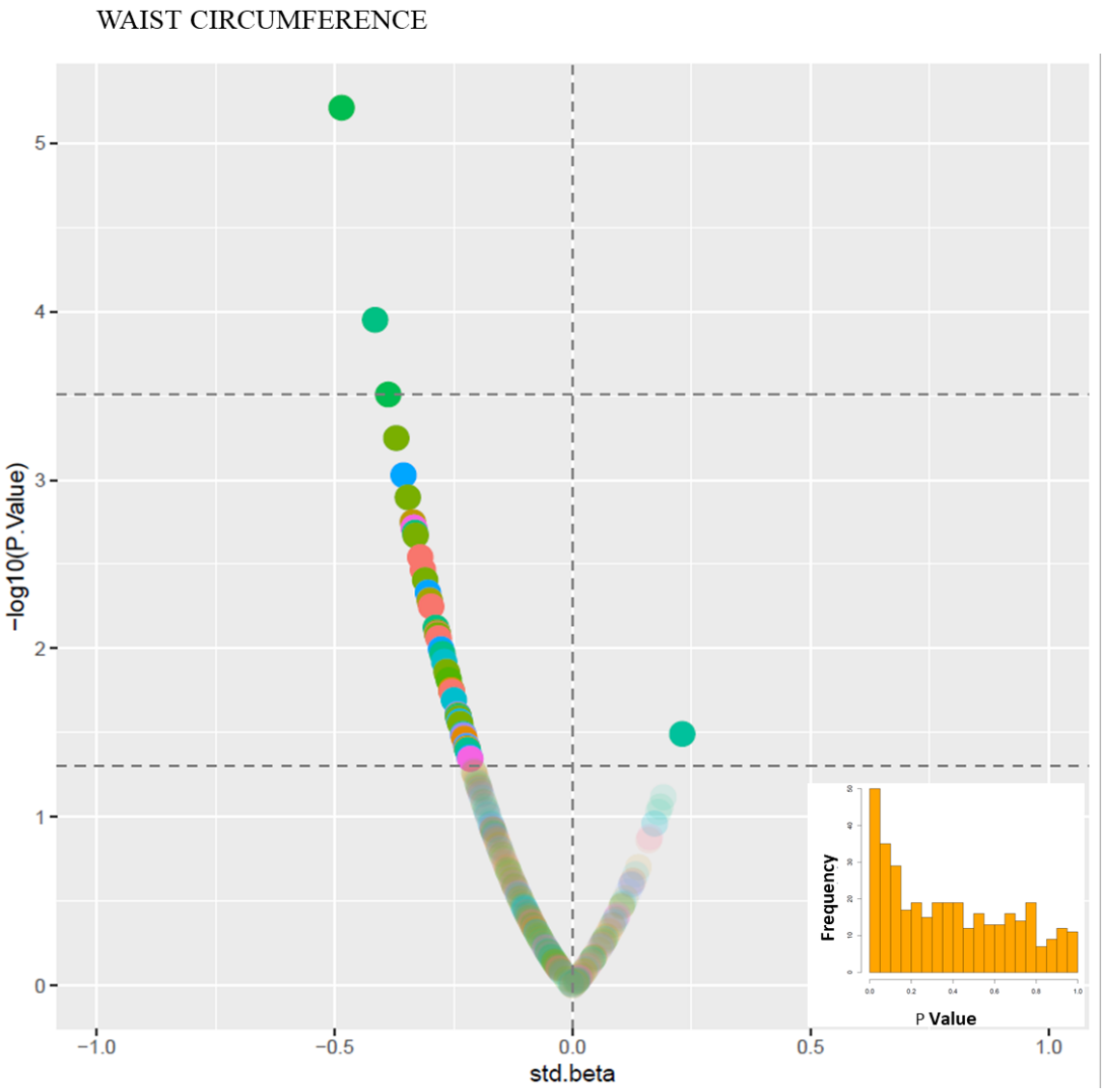


Figure 16: Volcano plot showing correlation indices between all changing genders and changes in BMI and waist circumference from T0 to T3. The horizontal dashed line indicates the significance threshold level (p=0.05). The upper horizontal dashed line indicates the significance threshold adjusted by multiple comparisons. *p*-value histogram was shown. A legend of the bacterial genera is shown in figure 14

Change in GM composition in the whole population after a 12-months treatment.

To assess whether the associations between MD and gut microbiota observed after three months of dietary intervention were maintained even after 1 year from randomization, microbiota sequencing analyses were conducted on samples collected 12 months after enrollment. After 1 year, data from only 63 patients were available. Table 10 shows clinical parameters comparison between the 3 time point in this subgroup of patients. After 12 months, patients keep a high level of adherence to MD and a significative

reduction of total cholesterol level were observed between T3 and T4. Interestingly, hs-CRP and fasting blood glucose levels were significantly lower at T4 than at baseline.

Variable	Subgroup T0 (n=63)	Subgroup T3 (n=63)	Subgroup T4 (n=63)	p-value T0 vs T4	p-value T3 vs T4
Weight (Kg)	79.5 ± 11.3	75.5 ± 10.6	76.5 ± 10.9	**< 0.0001**	0.080
BMI (Kg/m2)	27.6 ± 3.0	26.3 ± 2.9	26.6 ± 3.0	**< 0.0001**	0.062
Waist circumference (cm)	97.9 ± 8.2	94.5 ± 8.3	95.1 ± 8.2	**< 0.0001**	0.083
Total cholesterol (mg/dl)	167.4 ± 28.0	154.2 ± 34.4	149.7 ± 28.8	**< 0.0001**	**0.0002**
HDL (mg/dl)	52.1 ± 14.4	51.3 ± 14.3	52.2 ± 13.5	0.940	0.340
LDL (mg/dl)	94.1 ± 25.6	84.6 ± 27.7	79.4 ± 22.9	**< 0.0001**	0.083
Triglyceride Levels (mg/dl)	106.3 ± 47.8	91.4 ± 38.7	90.7 ± 42.4	**< 0.0001**	0.859
hs-CRP (mg/dl)	1.7 ± 2.4	1.8 ± 4.4	1.1 ± 1.2	**0.021**	0.420
Fasting glycaemia (mg/dl)	102.2 ± 10.5	101.5 ± 11.1	100 ± 9.9	**0.032**	0.154
SBP (mmHg)	131.6 ± 17.3	125.3 ± 16.7	125.3 ± 17.5	**0.004**	0.964
DBP (mmHg)	78.2 ± 9.5	74.8 ± 11.4	77.3 ± 10.9	0.451	0.073
MEDAS - no.	6.0 ± 1.8	9.2 ± 2.0	8.6 ± 2.2	**< 0.0001**	**0.009**

Table 10 – Variation clinical parameters between T0 (baseline), T3 (three months after diet) and T4 (1 year after randomization) in a subgroup of patients (n=63). Variables are expressed as mean±SD. Paired t-test was used. Body mass index (BMI), high-density lipoprotein (HDL), low-density lipoprotein (LDL), high sensitive-C reactive protein (hs-CRP), systolic blood pressure (SBP), diastolic blood pressure (DBP), Mediterranean diet adherence score (MEDAS).

As reported in Table 11 and in Figure 17, comparative analysis between T3 and T4, revealed that 1 year after medical recommendation, accompanied by maintenance of high MD adherence, a positive correlation is still observed between MD adherence and several bacteria, such as *Clostridium, Blautia, Dorea*, and *Coprococcus*.

Δ T4-T3

Genera	r	p-value
Pediococcus	0.35	0.008
Paenisporosarcina	0.33	0.015
Clostridium	0.33	0.020
Blautia	0.31	0.022
Parvimonas	0.31	0.024
Bacteroides	0.31	0.026
Desulfitobacterium	0.29	0.034
Dorea	0.28	0.042
Intestinibacter	0.27	0.043
Coprococcus	0.28	0.043

Table 11: Pearson's coefficient correlation and p-value between changing in genus abundance and variation in MEDAS from T3 to T4 of significant associated genera.

Δ (T4-T3)

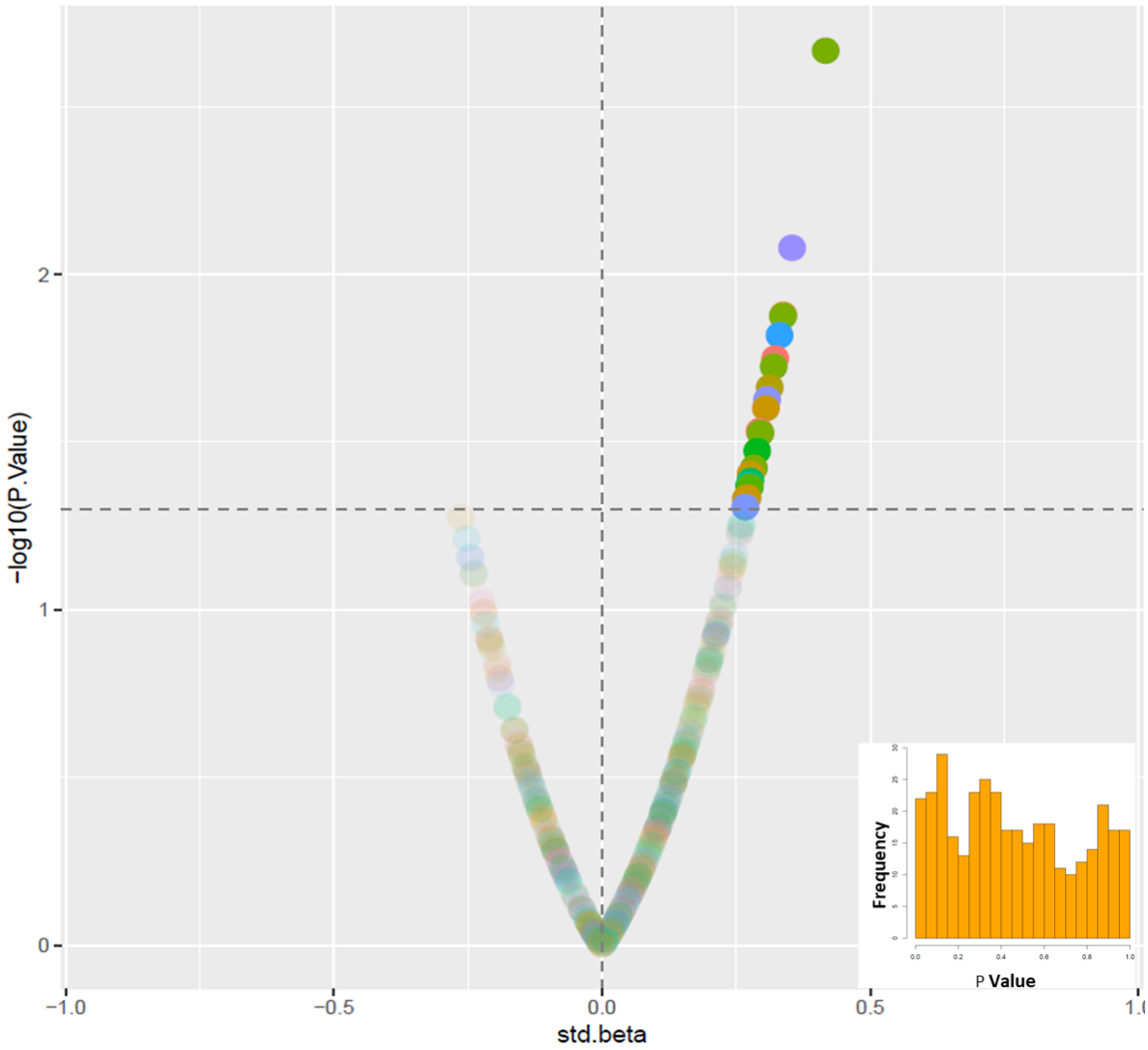


Figure 17: Volcano plot showing the correlation indexes between all bacteria genera changing and Δ MEDAS from T3 to T4. The horizontal dashed line indicates the significance threshold level (p=0.05). p-value histogram was shown. A legend of the bacterial genera is shown in figure 14.

EXPLORATIVE ANALYSIS OF GUT MICROBIOTA COMPOSITION BY 16S rRNA SEQUENCING ON SALERNO SUBJECTS

The goals of this work also included a technology transfer between two research center, the Centro Cardiologico Monzino in Milan, where all experiments on CAD patients were performed, and the Diagnostic Center Check-up in Salerno.

The main purpose of this collaboration will be to perform same experiments conducted in Milan, on patients living in the Mediterranean area and included in different prevention programs,

Up to now, we conducted a technical validation analysis by sending a subset of our fecal samples to Salerno Center and compared the sequencing results obtained in the two laboratories. 395 between genera and species were identified by both centers and for each sample, the species abundances were correlated. For each sample compared, the correlation index is shown in Table 12.

Sample	r	R2	p-value
RM026.T0	0.88	0.77	P<<0.001
RM026.T3	0.83	0.69	P<<0.001
RM037.T0	0.83	0.69	P<<0.001
RM037.T3	0.85	0.72	P<<0.001
RM064.T0	0.88	0.77	P<<0.001
RM064.T3	0.88	0.77	P<<0.001
RM084.T0	0.85	0.72	P<<0.001
RM084.T3	0.88	0.77	P<<0.001
RM097.T0	0.79	0.63	P<<0.001
RM097.T3	0.81	0.66	P<<0.001
RM117.T0	0.83	0.69	P<<0.001
RM117.T3	0.81	0.66	P<<0.001
RM120.T0	0.83	0.69	P<<0.001
RM120.T3	0.86	0.74	P<<0.001
RM123.T0	0.86	0.74	P<<0.001
RM123.T3	0.85	0.72	P<<0.001

Table 12: Person's correlation indexes (r), corresponding R2 and p-value show the reliability between species identified at CCM and Salerno Center.

Until now, 63 subjects, health or in primary prevention for CAD, have been enrolled at Check-up. The administration of FFQ was used to investigate dietary habits and it has revealed an already high adherence to MD as expected for this population (Table 13).

Variable	Salerno subjects (n=63)
Age - years	49.8 ± 12.6
Male gender - no. (%)	29 (46)
Weight (Kg)	78.2 ± 15.7
Height (m)	1.7 ± 0.1
BMI (Kg/m2)	27.0 ± 4.4
Waist circumference (cm)	101.3 ± 15.3
Total cholesterol (mg/dl)	196.1 ± 43.4
HDL (mg/dl)	56.8 ± 14.9
LDL (mg/dl)	117.9 ± 36.6
Triglyceride Levels (mg/dl)	99.9 ± 45.3
SBP (mmHg)	122.7 ± 12.6
DBP (mmHg)	80.5 ± 7.7
Hypertension - no. (%)	4 (6)
Dysplidemia - no. (%)	1 (1.5)
Hypercolesterolemia -no. (%)	2 (3)
Diabetes - no (%)	2 (3)
MEDAS - no.	8.5 ± 1.4

Table 13: Demographic characteristics and clinical parameters of Salerno subjects (n=63). Quantitative variables are expressed as mean±SD and categorical variables as number (percentage). Body mass index (BMI), high-density lipoprotein (HDL), low-density lipoprotein (LDL), systolic blood pressure (SBP), diastolic blood pressure (DBP), Mediterranean diet adherence score (MEDAS).

On Salerno subjects we performed correlation analysis between MEDAS score and gut microbiome composition. As shown in Table 14 and in Figure 18 several genera showed a significant association with MEDAS score.

Among these we found genera such as *Clostridium, Eubacterium* and *Ruminococcus* positively correlated also with high MEDAS at T3 in CAD patients.

Genera	r	p-value
Erysipelatoclostridium	0.35	0.008
Clostridium	0.30	0.021
Providencia	0.30	0.022
Prevotella	0.30	0.025
Lactobacillus	0.29	0.028
Bifidobacterium	-0.28	0.032
Ruminococcus	0.28	0.032
Anaerobaculum	0.27	0.040
Eubacterium	0.26	0.043

Table 14: Pearson's coefficient correlation and p-value between genera abundance and adherence to MD in Salerno subjects of significant associated genera.

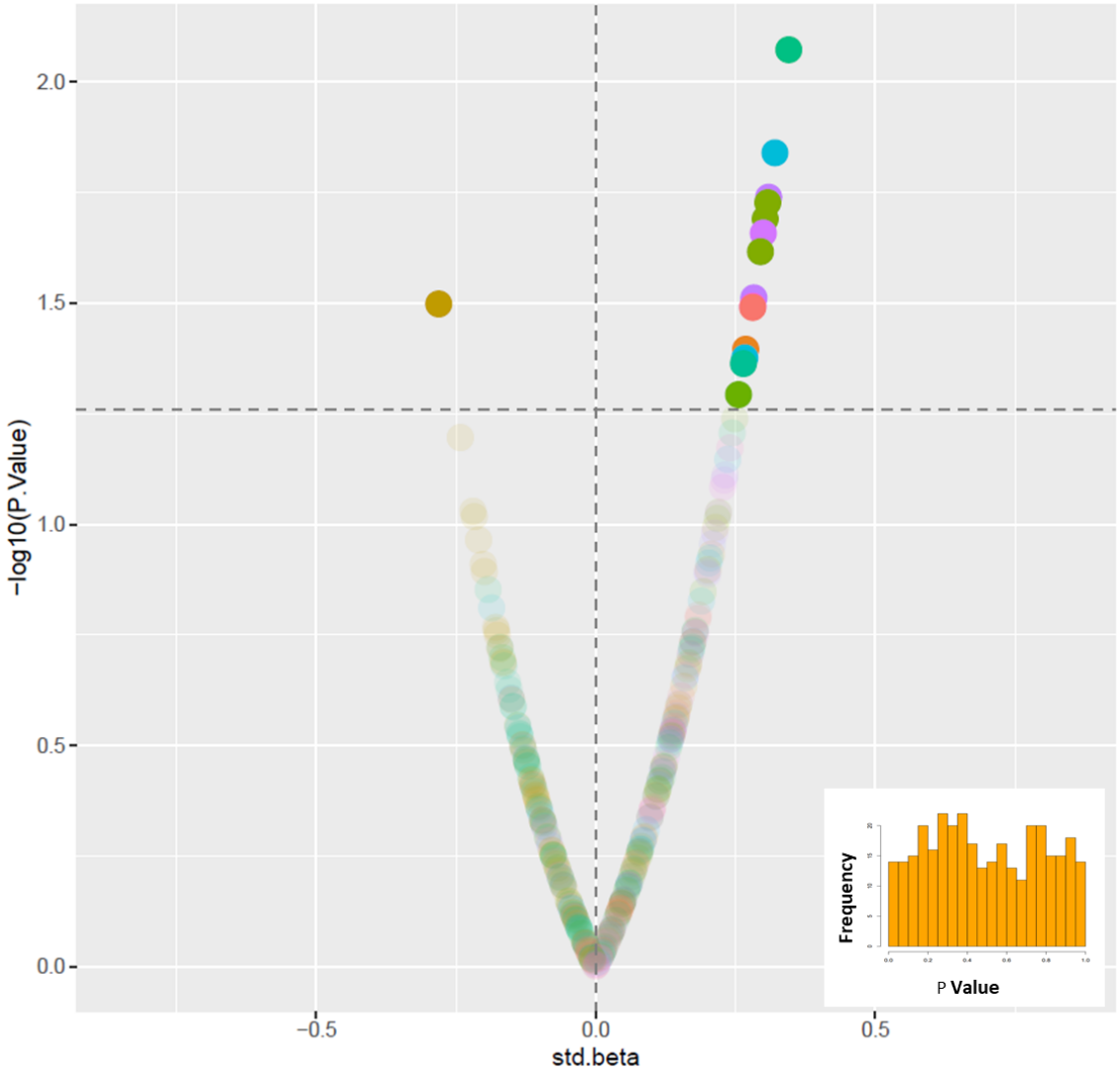


Figure 18: Volcano plot showing the correlation indexes between bacteria genera and MEDAS in Salerno subjects. The horizontal dashed line indicates the significance threshold level (p=0.05). *p*-value histogram was shown. A legend of the bacterial genera is shown in figure 14

TRANSCRIPTOME ANALYSIS

A total of 14.5 ± 5.7 million reads per sample mapped on the reference genome, allowing the identification of 16048 expressed genes out of 60583 annotated. We detected 487 genes, whose expression difference between T3 and T0 was significantly associated with the MEDAS variation. Specifically, 271 genes showed a positive correlation with MEDAS variation, while 216 genes had a negative relationship with MEDAS change over time.

To find a potential association between taxa and genes, the Spearman correlation was calculated between genes and bacteria correlated with MEDAS variation.

Heat map in Figure 19 showed that different bacteria correlate with different genes.

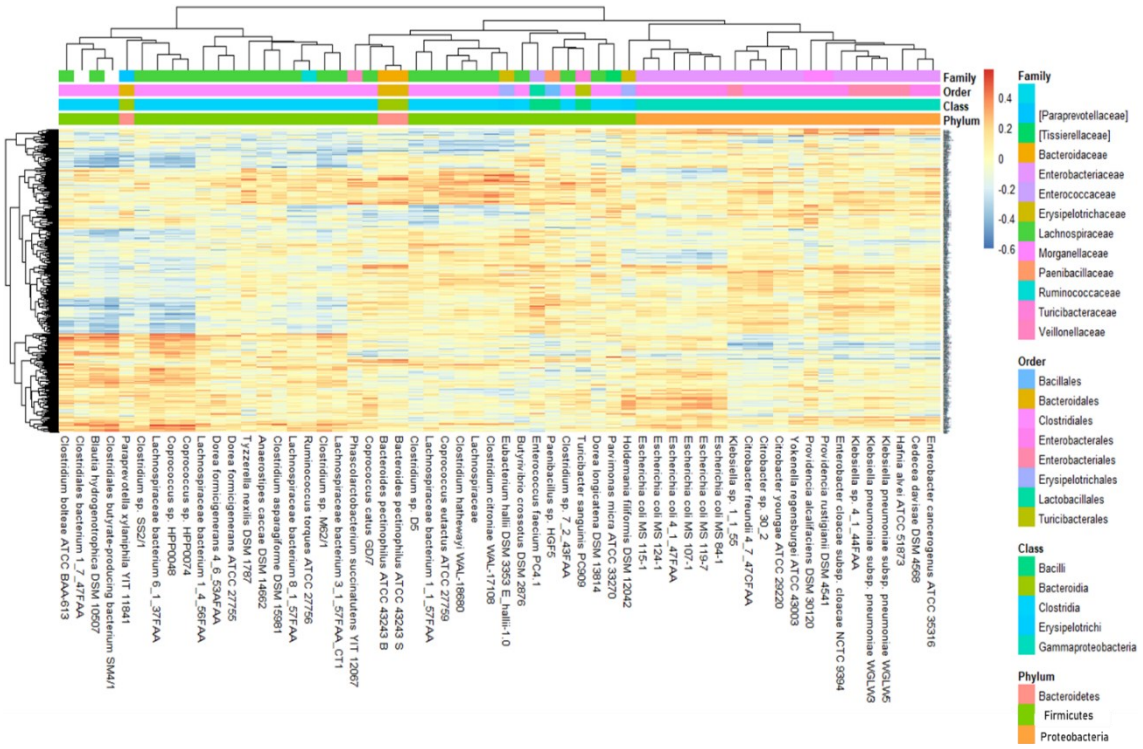


Figure 19: Heatmap of the microbial species (*x*-axis) that were found to be associated with different genes (*y*-axis). Each taxonomic rank is colored according to the reported legend. The direction of association is indicated by color.

We found 39 annotated genes significantly ($p<0.005$) correlated with several bacteria species showed in Figure 20.

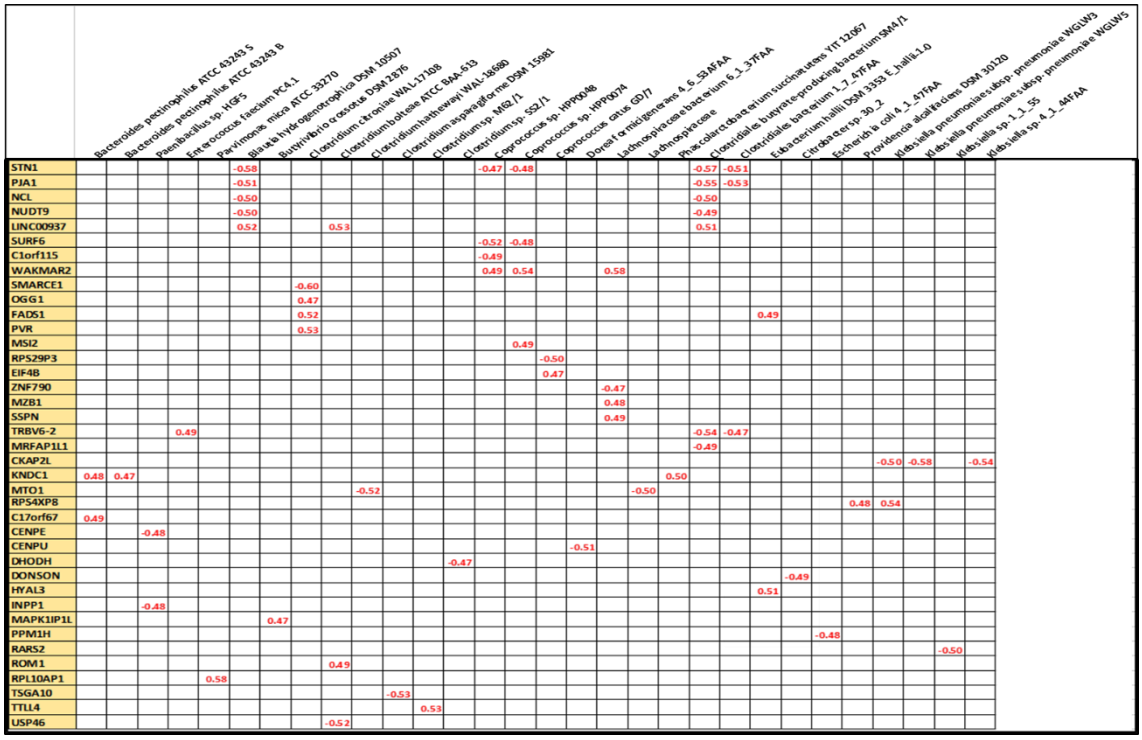


Figure 20: List of genes significantly correlated with bacterial species. In the squares are indicated the coefficient of correlations. ($p < 0.0005$)

DISCUSSION AND CONCLUSIONS

MODIFIABLE RISK FACTORS FOR CAD AND MEDITERRANEAN DIET

Several risk factors could lead to the onset of cardiovascular diseases, but only some of them are modifiable through diet intervention. Obesity, displydemia, hypertension, and hyperglycemia are clinical disorder often associated with increased risk of atherosclerosis. In our population, after three months of diet intervention, it was possible to observe statistically significant reductions in weight, BMI, waist circumference, total cholesterol, LDL, triglyceride levels, and blood pressure. On the contrary, no modifications have been observed in glycaemia, hs-CRP, and HDL. Interestingly, these reductions were confirmed after a not monitored period of 1 year also accompanied by a reduction in levels of glycaemia and hs-CRP. This data suggests that maintaining a healthy diet continues to modify cardiovascular risk factors over time. These results seem to reflect the high adherence to MD of patients and are also in line with the negative correlations found between Δ MEDAS score and Δ risk factors.

Our results are in accordance with a study performed by M. C.D Thomazella et al. on patients in secondary prevention for CVD. After three months of monitored dietary habits (MD and LFD), a significant reduction in BMI, waist circumference, and blood pressure was registered in the entire population, while no changes were found in HDL and glycaemic levels[163].

Moreover, some studies have reported that MD style, rich in nuts, olive oil, fruits, and vegetables, promotes a reduction of blood pressure levels[131].

Despite the recognized MD benefits, this diet is often incorrectly associated with weight gain because of its high intake of fatty foods such as olive oil and nuts. Previous studies and our study have confirmed that MD does not induce weight gain, but on the contrary leads to a reduction in waistline and BMI. Likely, a high intake of vegetables, olive oil, and nuts induces thermogenesis, increases satiety, and decreases energy intake from other food sources[164].

GUT MICROBIOTA AND MEDITERRANEAN DIET

Although there are several epidemiological and few interventional studies that established the relationship between MD and CVD, there is not a fully knowledge about the mechanisms underlying this association. Since MD influences the gut microbiota, it has been hypothesized that this relationship also drives the beneficial effects of this dietary pattern. The main objective of our study was to analyse the gut microbiome in secondary prevention patients after a randomized dietary intervention in MD and LFD. The first analysis, conducted by keeping the two intervention groups separated, did not yield significant results. Several considerations could be made to explain these results. First, considering the two groups after three months of intervention with MD and LFD, both groups, showed a significant increase in adherence to MD, based on the MEDAS score achieved (MEDAS > 7)[126].This could be explained both by the fact that for ethical reasons it was necessary to provide all patients with generic indications for healthy eating, and by the fact that probably having received recommendations from physicians made patients more aware of their eating style, hiding the differences between groups.

Moreover, although randomized controlled trials like this are usually considered the most informative studies, the dietary trials reserve many challenges, such as inadequate adherence to the diet dependent on the patient's preference. In addition, there are few quantitative methods to control the diet adhesion[165].

Therefore, we conducted the analysis by considering the entire population and using the MEDAS score as an indicator of dietary adherence, independently of the group classification.

Four main phyla, *Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria* characterize our population at baseline and this distribution is in line with previous observations[67].

At 3 months after enrollment, increased adherence to MD correlates positively with several butyrate-producing bacterial genera such as *Clostridium*, the pioneer of butyrate production, but also *Coproccoccus*, *Ruminococcus, Blautia*, and *Lachnospiraceae spp*[166]. Butyrate has multiple beneficial effects on cardiovascular system. Indeed, this SCFA is responsible to gut barrier integrity necessary to prevent the translocation of microbial cell wall component, such as lipopolysaccharides, known to be strongly pro-inflammatory[167]. In addition, butyrate is also involved in the inhibition of oxidative stress and has an anti-inflammatory role by modulating immune cells such as dendritic

cells, macrophages, and T-lymphocytes[168]. The protective role of butyrate in atherosclerosis has also been well documented. Sodium butyrate supplementation has been observed to slow atherosclerotic plaque progression by reducing macrophage migration and increasing plaque stability[169]. Reduced levels of butyrate-producing bacteria in the gut have been associated with different disorders, such as inflammatory bowel disease, obesity, diabetes mellitus, and hypertension[170-173]. Moreover, in a study by Qi Zhu et al. 16S rRNA sequencing of stool samples from patients with coronary artery disease and healthy controls, revealed a higher abundance of Clostridia in the control group than in patients[174].

Among genera positively associated with higher adherence to MD in our patients, we also found *Blautia* and *Anaerostipes*. Bacteria belonging to the former are often associated with a healthy state and are involved in the control of gut inflammatory processes, atherosclerosis, and maturation of the immune system[175]. Instead, the latter was found depleted in patients with acute coronary syndrome (ACS) and enriched in ACS patients treated with oral statins. This genus has been negatively correlated with indicators of disease severity, probably due to its ability to produce butyric acid[176].

Alongside the increase in heart-healthy genera, we also observed a negative correlation between the adherence to MD and genera belonging to *Enterobacteriaceae* family such as *Klebsiella, Enterobacter, Citrobacter, Escherichia, Yokenella* and *Hafnia*.

Microbial species belonging to this family were found associated with CAD in some previous studies[177]. *Klebsiella* microbes colonize atherosclerotic plaque and could contribute to aetiopathogenesis of cardiovascular diseases onset.

In addition, these types of bacteria have been shown to require carbohydrate sources for growth and this may explain their decrease in our population after reduced sugar intake[178].

Enterobacteriaceae resulted increased in individuals with atherosclerotic cardiovascular disease in a study of Jie et al. in 2017[179]. It may be explained by the fact that these bacteria contain molecular components that directly increase the inflammatory response[180].

Since overgrowth of the *Enterobacteriaceae* family has been found in several inflammatory conditions, it is reasonable to think that in our population adherence to MD leads to a reduced inflammatory state confirmed by the reduced levels of these bacteria [181].

Another key factor in the onset and progression of atherosclerosis is the oxidative stress. *Escherichia Coli* reduction can decrease the uric acid production with subsequent reduction of vascular endothelial disfunction and inflammation[182, 183].

Correlation analyses between variation in gut microbiota and risk factors after 3 months of dieting showed a negative association between the previously mentioned butyrate-producing bacteria and reduced BMI and waist circumference.

The anti-obesity properties of butyrate are well documented. Indeed, it may play a role in this context by increasing energy expenditure through activation of β3 adrenergic receptors, mediators of lipolysis in white adipose tissue[184].

Moreover, a study by B.A Peters et al. demonstrated lower *Clostridiaceae* abundance in overweight compared with healthy-weight American adult participants[185].

Probably, the Mediterranean diet directly influences the modulation of the gut microbiota in a positive way and at the same time the intake of fiber, nuts and fruits and a lower intake of processed foods result in a reduction of some risk factors for cardiovascular disease.

Although it was possible to conduct sequencing analyses 1 year after enrollment on only a subset of subjects, the correlations were consistent with those observed at T3. This result confirms the effects of MD on the gut microbiota over time.

As previously specified, another objective of this work was to transfer our analysis to the study of subjects residing in southern Italy, specifically in the city of Salerno, where the greatest adherence to a Mediterranean-type diet is reported. First, our validation analyses showed a strong positive correlation between the results obtained in the two different centres confirming the efficiency of the technique used and the occurred technology transfer.

Gut microbiome analyses on Salerno subjects also showed a positive correlation between adherence to MD and some butyrate-producing bacteria. This result seems to support the hypothesis that a greater adherence to the MD promote the growth of beneficial commensal bacteria with a protective action for the cardiovascular system at different stages of CAD prevention.

BLOOD TRANSCRIPTOME AND MEDITERRANEAN DIET

We examined correlations between changes in whole blood transcriptomic genes and bacterial taxa after 3 months of dietary intervention in a randomized subset of our patients. Several nutrigenomic studies have analyzed the effects of MD and its components on the gene modulation in different tissue, to deeply investigate the beneficial role of this diet on human healthy. In a subsample of the PREDIMED study, Castaner et al[186]. showed that 3 months of MD, enriched with nuts or virgin olive oil, can modulate pathways involved in cardiovascular events.

To the best of our knowledges, no study on the relationship between MD, microbiota, and transcriptome in CAD patients has been conducted. In our study, we sought to understand whether the observed effect of MD adherence on gut microbiota in CAD patients, may have an association with MD-induced transcriptomic modulation in blood. We found several genes with modulate expression between T0 and T3. Among these, 39 were strongly associated with modulated bacterial species.

It is interesting to note that some bacteria species showed a correlation with more than one gene. For example, both *Blautia hydrogenotrophica* and *Clostridiales butyrate-producing bacterium*, have a similar correlation with STN1, PJA1, NCL, NUDT9, LINC00937 genes.

NCL gene encoding for nucleolin, a protein involved in the synthesis and maturation of ribosomes. This protein is expressed on the surface of immune cells, tumor cells and vascular endothelial cells. It is a receptor for many proteins, viruses and bacteria and it is involved in inflammation, microbial infection, angiogenesis and other biological processes[187].

Clostridium citroniae is another species correlated with a group of genes, SMARCE1, OGG1, FADS1, PVR.

OGG1 encodes the enzyme involved in the excision of 8-oxoguanine, a mutagenic base resulting from exposure to reactive oxygen. Experiments in transgenic mice have shown that overexpression of this gene in cardiac tissue is associated with a reduction mtDNA content of 8-oxo-dG and in cardiac fibrosis[188].

The FADS1 gene is a key gene in the fatty acid desaturation pathway. Changes in plasma fatty acid profile affect cardiovascular disease risk, and polymorphisms in this gene are known to be associated with various cardiometabolic risk factors[189]. In addition, there

is evidence that a single SNP in the FADS1/FADS2 region may be associated with inflammation[190].

The bacterium *Lachnospiraceae* is positively correlated with the MZB1 gene.

The cardioprotective property of Mzb1 is documented. It is an inflammation-associated factor and protects cardiomyocytes in myocardial infarction in the mouse model by promoting macrophage proliferation and migration, and reducing the release of inflammatory molecules[191].

Considering our preliminary results, it is necessary to validate and deeply investigate the described associations to understand whether they are involved in the positive effects of DM-modulated microbiota on the cardiovascular system.

Furthermore, because at T4 we observed a reduction in hs-CPR, a plasma marker of inflammation, it might be interesting to conduct these experiments also after 1 year to investigate whether the observed modulations are maintained and whether new associations with inflammatory genes emerge.

CONCLUSIONS

The present study has several strengths. Firstly, it involves a large number of patients and the randomized design allows to avoid many confounding factors (sex, age, drugs, etc). Moreover, a good adherence of patients to MD has been obtained.

However, some potential limitations should be considered. The RISMeD study was carried out only in Italia, in a population with a diet that already includes in part the foods of the MD. Moreover, the use of MEDAS score for the assessment of adherence to the Mediterranean diet may also have some limitations. No weighting has been applied to the components of the score, thus we cannot exclude the possibility that two individuals may have the same score but different dietary intakes. In addition, our study lacks a control group that would have allowed comparison studies after 3 months of intervention between CAD subjects and healthy subjects.

In conclusion, our study demonstrated that increased adherence to the MD causes changes in the composition of the gut microbiota in patients with coronary artery disease, already after 3 months of dietary intervention. This remodelling of the microbiota is characterized by an increase in beneficial butyrate-producing bacteria, known to be protective of metabolic health and CVD risk, and a reduction in species considered enemies of the heart. Furthermore, we found the same situation even after 1 year of free diet, during which our population maintained high adherence to MD.

Ii is interesting to note that, although they are two distinct populations, beneficial bacteria positively associated with higher adherence to MD in CAD patients, were found also positively associated with MEDAS score in healthy or in primary prevention Salerno subjects.

It will be necessary to validate and deeply investigated the transcriptomic results to better understand the effects of gut microbiota modulated by MD on gene expression profile.

These results underline the important role of MD not only in primary but also in secondary prevention for CVD and add a step to the understanding of the relationship between this dietary pattern and the gut microbiota.

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