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THE MEDITERRANEAN DIET: INSIGHTS INTO ITS ROLE IN SECONDARY CORONARY ARTERY DISEASE PREVENTION

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Abbreviations

8-iso-PGF$_{2\alpha}$: 8-iso-prostaglandin-F$_{2\alpha}$
8-OHdG: 8-hydroxy-2-deoxyguanosine
ACE: angiotensin-converting enzyme
ACS: acute coronary syndrome
ADMA: asymmetric dimethylarginine
AHA: American heart association
ALA: α-linoleic acid
AU: arbitrary units
BMI: body mass index
CAD: coronary artery disease
CCM: centro cardiologico Monzino
CE: cholesterol ester
Cer: ceramide
CPE: ceramide phosphoethanolamine
CRP: C-reactive protein
CV: coefficient of variation
CVD: cardiovascular disease
DASH: dietary approaches to stop hypertension
DBP: diastolic blood pressure
ECG: electrocardiogram
ED: endothelium dysfunction
eNOS: endothelium nitric oxide synthase
EPIC: European prospective investigation into cancer and nutrition
ESC: European society of cardiology
ESI: electrospray ionization
EVOO: extra-virgin olive oil
FFQ: food frequency questionnaire
FMO-3: flavin-containing monooxygenase-3
GC: gas chromatography
GCer: glucosylceramide
GI: gastrointestinal
GM: ganglioside
GPX: glutathione peroxidase
GSH: reduced glutathione
GSSG: oxidized glutathione
HDL: high-density lipoprotein
HMDB: human metabolome database
HPLC: high performance-LC
hs-CRP: high sensitive C-reactive protein
IL: interleukin
LacCer: lac-ceramide
LC: liquid chromatography
LDL: low-density lipoprotein
LFD: low-fat diet
LLOQ: lower limit of quantification
LOD: limit of detection
LPC: lysophosphatidylcholine
LPS: lipopolysaccharide
m/z: mass-to-charge
MD: Mediterranean diet
ME: matrix effect
MEDAS: Mediterranean diet adherence screener
MI: myocardial infarction
MRM: multiples reaction monitoring
MS/MS: tandem mass
MS: mass spectrometry
MUFA: monounsaturated fatty acid
NADPH: nicotinamide adenine dinucleotide phosphate
NMR: nuclear magnetic resonance
NO: nitric oxide
NSTEMI: non-ST elevation myocardial infarction
OPLS-DA: orthogonal partial least squares discriminant analysis
oxLDL: oxidized low-density lipoprotein
PA: phosphatidic acid
PC: phosphatidylcholine
PCA: principal component analysis
PCSK9: proprotein convertase subtilisin kexin 9
PE: phosphoethanolamine
PEf: process efficiency
PI: phosphatidylinositol
PLS-DA: partial least squares discriminant analysis
PREDIMED: prevencion con dieta mediterranea
PS: phosphatidylserine
PUFA: polyunsaturated fatty acid
QC: quality control
QTOF: quadrupole time of flight
RE: extraction recovery
RF: random forest
RISMeD: Randomized Interventional Study on Mediterranean Diet
ROS: reactive oxygen species
RT: retention time
SA: stable angina
SBP: systolic blood pressure
SD: standard deviation
SFA: saturated fatty acid
SM: sphingomyelin
SMC: smooth muscle cell
SRM: selected reaction-monitoring
STEMI: ST elevation myocardial infarction
Sul: sulfoglycosphingolipid
TG: triglyceride
TC: total cholesterol
TIC: total ion current
TMA: trimethylamine
TMAO: trimethylamine N-oxide
TNF-α: tumor necrosis factor-α
TOF: time of flight
UA: unstable angina
UNESCO: united nations educational, scientific and cultural organization
WHO: world health organization

1. Abstract

Background: Coronary artery disease (CAD) is the leading cause of morbidity and mortality in developed countries. However, incidence rates have clear geographic differences that have been partly ascribed to dietary habits. The Mediterranean Diet (MD) has been proposed to explain this geographical differences. The positive effect of MD on CAD primary prevention is well established. On the contrary, evidence of the MD cardioprotective effect in secondary prevention is much less recognized.

Aim: The primary aim of this study was to explore the effect of a MD in CAD patients in a comprehensive way that includes evaluation of classical CAD risk factors (total cholesterol, LDL and HDL cholesterol, triglycerides, blood pressure, weight, glucose levels) and "*non classical*" factors as inflammation, oxidative stress status, gut microbiota and alternative lipids classes. Secondary aims were to define the correlation between level of MD adherence and the induced modifications. In addition, the application of an untargeted metabolomics approach was used to evidence molecular mechanisms involved in MD cardioprotection.

Materials and methods: This is a parallel-group, randomized, open-label, interventional trial to assess the effects of the MD, on CAD patients, compared to a low-fat diet (LFD). 130 CAD patients have been enrolled and randomized into the two groups (MD *vs* LFD). Diets effects have been evaluated comparing different features before (T0) and three months after the dietary treatments (T3).

Results: The dietary treatments were able to reduce BMI, waist circumference and lipid peroxidation. Specifically, the MD allowed to decrease systolic and diastolic blood pressure, total cholesterol, LDL cholesterol and triglycerides levels. At T3 lower levels of C-reactive protein, reflecting inflammation, have been evidenced in the MD group. Furthermore, the MD intervention induced an increment of betaine, several phosphatidylcholines, lysophosphatidylcholines and phosphatidylethanolamines. These compounds have been previously correlated to lower CVD risk.

Conclusions and relevance: These data suggest a positive effect of MD on CAD patients that can be the results of improving different antiatherogenic features. Their synergy could be the most important determinant of the MD positive effect. This study suggests that, a wider dissemination of Mediterranean diet should be advised as lifestyle change parallel to drug therapy in secondary CAD prevention.

2. Introduction

2.1. Coronary artery disease

2.1.1. Definition and overview

Coronary artery disease (CAD), also known as ischemic heart disease, is defined as a complex chronic inflammatory disease characterized by remodeling and narrowing of the coronary arteries supplying oxygen and nutrients to the heart. It can have various clinical manifestations, including stable angina (SA), acute coronary syndrome (ACS), and sudden cardiac death.

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in developed countries [1, 2], with yearly worldwide deaths expected to increase from 17.7 to 23.6 million by 2030, as reported by the World Health Organization (WHO)[3]. This record is reflected in Europe, where diseases of the heart and circulatory system are, as a whole, responsible for over 3.9 million deaths a year, or 45% of all deaths [4].

As highlighted in Figure 1, among CVDs, CAD is the leading single cause of mortality and morbidity: is responsible for 862,000 deaths a year in Europe (19% of all deaths) [4] and for more than 1,000,000 of new coronary event, or recurrent event, in USA during this year [2].

Take care improvement and the scientific findings in the understanding of the pathophysiology of CAD, have probably led to a decrease in the mortality towards the turn of the 20th century [5], especially in high-income countries [6, 7], and this decline will probably continue (27% reduction by 2030) [2]. Nevertheless, according to American Heart Association (AHA) projections, between 2013 and 2030, medical costs of CAD will increase by about 100 percent and approximately every 40 seconds an American will have a heart attack [2, 8]: the problem is far from resolution.

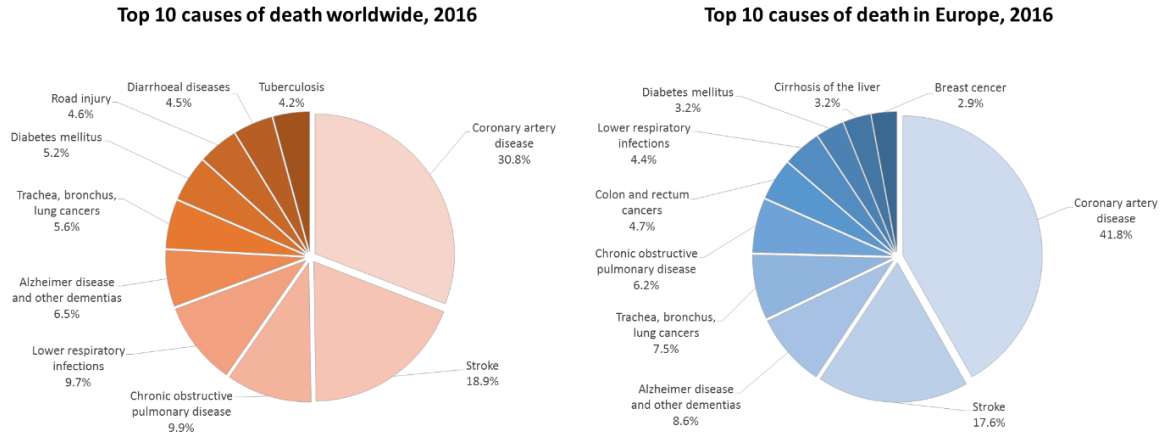


Fig. 1 – Top 10 causes of death in the World and in Europe are reported by the World Health Organization. Data are related to death in 2016, both sexes, all ages.

2.1.2. Coronary artery disease pathogenesis: the atherosclerotic process

CAD has a complex etiopathogenesis and a multifactorial origin resulting from the convergence of environmental factors, such as diet, smoking, and physical activity, and genetic factors that modulate risk of the disease, both individually and through interaction [9].

Atherosclerosis is the main etiopathogenic process that causes CAD. It is a silent progressive chronic process characterized by accumulation of lipids, fibrous elements, and inflammatory molecules in the wall of the large arteries.

The first step leading to atherosclerosis is the **endothelium dysfunction** (ED). The endothelium has a pivotal role in the modulation of vascular function and structure, mainly through the formation of nitric oxide (NO). Pathophysiological states, such as oxidative stress and inflammation, are able to induce ED [10, 11]. ED could be promoted by the switch in signaling from a NO-mediated silencing of cellular processes toward activation by redox signaling which causes increase in reactive oxygen species (ROS) generation (free oxygen radicals, oxygen ions and peroxides) and reduction of NO bioavailability. ROS are able to reduce NO levels directly oxidizing it to peroxynitrite, and indirectly decreasing the enzymatic activity of the endothelium nitric oxide synthase (eNOS) through the oxidative degradation of its essential cofactor, biopterin [12]. Furthermore, ROS can downregulate gene expression of eNOS [13] and increase asymmetric dimethylarginine (ADMA) levels, an inhibitor of NO synthesis, through the reduction of its catabolism [14]. Vascular inflammation is highly associated with ED and plays a major role in the development of atherosclerosis too. Inflammation induces increased expression of redox sensitive molecules, such as vascular adhesion molecule-1, intracellular adhesion molecule-1, E and P-selectin, and augmented secretion of many cytokine [15].

As result of this process, ED induces the increase of endothelium permeability causing the efflux of low-density lipoproteins (LDL) cholesterol to the subendothelial space, which can then be modified and oxidized by various agents. The vascular inflammation and the increased generation of ROS further promote the modification of circulating LDLs in oxidized LDLs (oxLDLs) [16, 17], which exert a crucial role in the pathophysiology of atherosclerosis [18, 19]. Indeed, oxLDLs induce expression of vascular cell and intercellular adhesion molecule at the endothelial surface, and cytokines (i.e. chemoattractant protein-1 and macrophage colony stimulating factor) [20-22]. Furthermore, they promote monocyte adhesion and migration to the subendothelial space, where they differentiate to macrophages, able to turn into foam cells, generate ROS and release

proinflammatory agents [23]. Overall, these steps lead to the formation of the first typical atherosclerotic lesion.

In the subendothelial space, the cross talk between monocytes, macrophages, foam cells, and T-cells results in cellular and humoral immune responses, and ultimately in a chronic inflammatory state with the production of several proinflammatory molecules [24, 25]. This process continues with the migration of smooth muscle cells (SMC) from the medial layer of the artery into the intima, where they will eventually differentiate to form the fibrous coating of the atherosclerotic plaque. A mature atherosclerotic plaque contains a core of dead foam cells and SMCs. The core of the plaque is covered by a fibrous cap, a region of the intimal layer that has become thickened because of medial SMCs depositing collagen fibers. The thickening artery wall of the atherosclerotic plaque gradually encroaches on the arterial lumen and narrows the inner diameter of the artery, resulting in a restriction of blood flow and compromised blood supply to the myocardium (Figure 2).

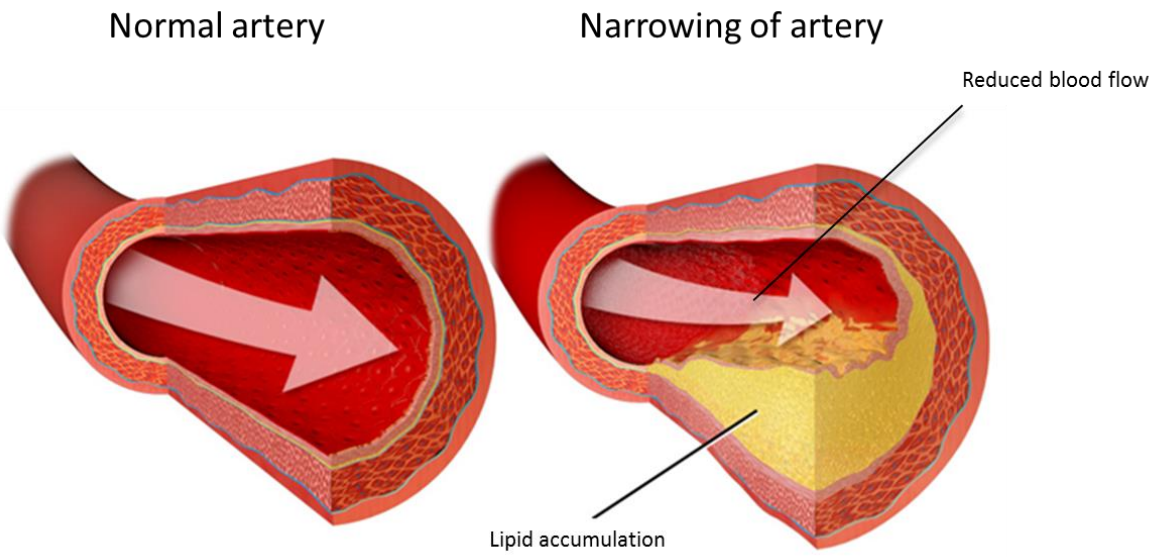


Fig. 2 – Representation of coronary artery disease characterized by lipid accumulation and reduced blood flow.

CAD therefore represents the culmination of cholesterol accumulation, cellular capture, vascular injury, and inflammatory activation. The cellular composition of an atherosclerotic plaque determines whether it will be stable or unstable, and consequently how it will manifest clinically. Stable plaques have an intact, thick fibrous cap composed of abundant SMCs in a matrix rich in type I and III collagen. Usually it expands into the lumen of the artery producing flow-limiting

stenosis, leading to tissue ischemia, reduced myocardial perfusion and usually **stable angina** [26]. Unstable plaques is characterized by thin fibrous cap, composed mostly of type I collagen, and few or no SMCs, but abundant lipid-rich macrophages and proinflammatory and prothrombotic molecules. These plaques are prone to rupture, exposing the core of the plaque to circulating coagulation proteins, causing blood clot formation, artery lumen occlusion and reduced blood flow through the artery [27, 28]. The vascular occlusion can be incomplete/transient or complete, but in any way leads to acute clinical manifestations: unstable angina (UA), ST elevation myocardial infarction (STEMI) and non-ST elevation myocardial infarction (NSTEMI).

2.1.3. Coronary artery disease risk factors

The cause of CAD is not yet completely understood, but many epidemiological studies have identified several risk factors that can contribute to the pathogenesis of the disease.

The concept of “risk factors” in CAD was first coined by the Framingham Heart Study, which in 1957 demonstrated the epidemiologic relations of cigarette smoking, blood pressure, and cholesterol levels to the incidence of CAD [29]. In this study, which took place in the small town of Framingham in Massachusetts USA, researchers followed in a large cohort (5,209 participants between the ages of 30 and 62) the development of CAD over a long-term period. The study has provided substantial insight into the epidemiology of CVD and its risk factors.

As reported in Table 1, different risk factors classification have been proposed: AHA guidelines differentiated CAD risk factors in major independent risk factors, predisposing risk factors and possible risk factors. Instead, European Society of Cardiology (ESC) suggested modifiable (biochemical or physiological characteristics) and non-modifiable (personal characteristics) risk factors, side by side with lifestyle factors [30].

CAD risk factors according to American Heart Association		
Major independent risk factors	Predisposing risk factors	Possible risk factors
• Cigarette smoking • Hypertension • Elevated total and LDL cholesterol • Low HDL cholesterol • Diabetes mellitus • Older age	• Physical inactivity • Obesity • Family history of premature coronary disease • Ethnicity • Psychosocial factors	• Fibrinogen • C-reactive protein • Homocysteine • Lipoprotein-a
CAD risk factors according to European Society of Cardiology		
Non-modifiable	Modifiable	Lifestyle
• Older age • Male gender • Family history of coronary heart disease or other atherosclerotic vascular disease at early age (men < 55, woman <65) • Personal history of coronary heart disease or other atherosclerotic vascular disease	• Elevated blood pressure • Elevated plasma total and LDL cholesterol • Low plasma HDL cholesterol • Elevated plasma triglycerides • Hyperglycemia/diabetes • Obesity • Thrombogenic factors	• Diet high in saturated fat, cholesterol, and calories • Tobacco smoking • Excess alcohol consumption • Physical inactivity

Tab. 1 – Classification of coronary artery disease risk factors according to two of the most relevant cardiac associations: American Heart Association and European Society of Cardiology. Low-density lipoprotein (LDL), high-density lipoproteins (HDL).

To date, no single risk factor has been identified to be responsible for causing CAD; reasonably, multiple interrelated factors seem responsible for its development. Furthermore, the Framingham Heart Study revealed that cardiovascular risk factors tend to cluster [31]. Multiple risk factors have a multiplicative impact [32], even mild to moderate levels of multiple risk factors impart substantial risk, and multiple areas of slight risk can be more important than one area of very high risk. Lifetime risk for CAD increases drastically as a function of risk factor profile. At 55 years of age and with an optimal risk factor profile, risk for CAD is 3.6% for males and <1% for females; with ≥2 major risk factors, it is 37.5% for males and 18.3% for females [33]. In the next paragraphs the main risk factors will be discussed.

2.1.3.1. Age, gender and ethnicity

Age is among the most important unmodifiable risk factors; 4 out of 5 people who die of CVD are at least 65 years old. The risk is progressively increasing with advancing age [34], the incidence is lower in people under 40 years, and increased in elderly people [35]. Both, CAD prevalence and mortality, increase dramatically with age [36].

Another non-modifiable risk factor for cardiovascular disease is **gender**. Women generally have a lower risk for developing CVD compared to men of similar age [37] but CAD frequency increases after menopause, an effect that has been attributed, at least in part, to estrogens [38, 39]. Estrogen therapy lowers LDL cholesterol and raises high-density lipoproteins (HDL) cholesterol, changes that should decrease coronary risk [40]. Furthermore, clinical and experimental data support the consideration of endothelium as a target for estrogens [41].

Ethnicity is another important unmodifiable factor. African-Americans have a tendency towards severe hypertension and a higher rate of CVD than Caucasians. CAD prevalence among black (7.2%) and Hispanic men (6.7%) is lower compared with white men (7.8%). However, in both black (7%) and Hispanic women (5.9%), CAD prevalence is higher than in white women (4.6%) [42].

2.1.3.2. Family and personal history of coronary artery disease

Family CVD history is an acknowledged risk factor. People with a family history of CVD are more likely to develop cardiovascular disease themselves, especially with paternal myocardial infarction (MI) premature history that approximately double the risk of a heart attack in males and increase the risk in females by about 70% [43]. **Genetic alterations** adversely promote the development of atherosclerotic disease in several ways. For example, subjects with a gene mutation in LDL lipoprotein receptor, a pathological condition known as familial hypercholesterolemia, have cholesterol levels twice that of the normal population and they are at high risk to develop cardiovascular disease [44]. However, genetic factors, other than those involved in lipid metabolism, have also been reported to be implicated in atherogenesis. Genetic variations in coagulation factors and fibrinogen are responsible for increased thrombogenicity, alterations of angiotensin-converting enzyme (ACE) [45], angiotensin II receptor type 1 [46] and variants of endothelial NO synthesis have also been associated with hypertension and CAD risk [47, 48].

CAD events are significantly more likely to be fatal in patients with a CAD history than in those without, with a 2.5-fold increase reported in an observational study [49]. Furthermore, the rate of sudden death in patients who have experienced a MI is 4–6 times higher than in the general

population [50]. The AHA 2018 statistics reported that among CAD 17% of males and 21% of females patients who have a first MI, with more than 45 years of age, had a recurrent MI or fatal CAD within 5 years.

2.1.3.3. Blood pressure

Hypertension, defined as a systolic blood pressure (SBP) in excess of 140 mm Hg and/or a diastolic blood pressure (DBP) above 90 mm Hg [51], is a major risk factor for CVD and stroke [52]. It was evidenced for the first time as a relevant CAD risk factor in the Framingham Heart Study demonstrating that the impact of systolic pressure is actually greater than the diastolic component and that even isolated systolic hypertension is dangerous [53, 54]. It is now completely recognized that hypertension increases atherosclerotic CVD incidence at all ages [55]; even high normal blood pressure values, defined prehypertension, are associated with an increased risk of CVD, starting as low as 115/75 mmHg, the risk of heart attack and stroke doubles for every 20-point of SBP or every 10-point of DBP [56]. Therefore, not only hypertension, but also prehypertension should be considered a CVD risk. Recent data regarding hypertension are quite worrying: in USA, there were 78,862 deaths primarily attributable to hypertension in 2015 and the AHA projections indicate that by 2030 more than 40% of USA adults will have hypertension [2, 8].

The mechanism through which hypertension promotes the onset and the progression of CAD is still unclear, but it is known that can produce endothelium damage, with the subsequent increase in its permeability and LDL cholesterol efflux in the vessel wall. In addition, hypertensive patients exhibit elevated levels of angiotensin II, a vasoconstrictor mediator that is involved in the atherosclerotic process [57]. Furthermore, is well recognize that chronic high blood pressure can cause hardening of the artery walls, which can eventually cause decreased blood flow.

2.1.3.4. Plasma cholesterol

The other major risk for CVD is cholesterol [58]. Hypercholesterolemia is a common clinical, metabolic and/or genetic disorder that promotes functional and structural vascular wall injury.

Total cholesterol (TC) association with CVD was evidenced since the ‘50s and was confirmed by epidemiological studies showing a strong relation between serum TC and cardiovascular risk [59-62]. Nevertheless, cholesterol is transported by several classes of lipoproteins, such as very low-density lipoprotein, LDL and HDL. Nowadays, guidelines identify **LDL** cholesterol as the primary

target for high blood cholesterol therapy [63] and **HDL** cholesterol raising as a very relevant factor to decrease CAD incidence rate. The AHA statistics 2018 indicates that about 94.6 million of American adults (39.7 %) have TC of 200 mg/dL, 28.5 million (11.9%) higher than 240 mg/dL and 18.7% have low levels of HDL cholesterol [9].

High levels of plasma TC are related to a high synthesis of LDL cholesterol, that are related to the onset of atherosclerosis [64]. On the contrary, it was estimated that a 1 mg/dL increase in HDL level is associated with a decrease in CAD risk of 2% in men and 3% in women [65]. Moreover, in contrast to the inhibitory effects of LDL cholesterol on eNOS, HDL cholesterol enhances eNOS biosynthesis and activity [66].

2.1.3.5. Glycaemia

Diabetes is accepted as a major cardiovascular risk factor. In the Framingham study, the incidence of CVD was 2-3 times greater in diabetic patients than in the general population [67]. Recent AHA statistics were in accordance with those results: at least 68% of people age 65 or older with diabetes die for CAD; diabetic people are two to four times more likely to die from heart disease than adults without diabetes.

The mechanisms by which hyperglycemia promotes CAD are multiple. Such mechanisms include enhancement of vasoconstrictors activation, such as endothelin -1 [68], oxidative stress increment [69, 70] and non-enzymatic glycosylation processes that could modify lipoprotein structure and induce adverse effects on vascular endothelium [71]. The abnormal lipoprotein profile associated with insulin resistance, known as diabetic dyslipidemia, accounts for part of the elevated cardiovascular risk in patients with type 2 diabetes. Furthermore, the increase in circulating levels of insulin promoted the development of atheromasic plaque by inducing vascular wall changes, promoting SMC proliferation and activating cholesterol synthesis [72].

2.1.3.6. Obesity

The National Heart, Lung, and Blood Institute proposed that values of body mass index (BMI), a surrogate indicator of body fat, above 30 Kg/m^2 should be considered **obesity**. The WHO has referred to the worldwide rise in obesity in developed countries as being a global epidemic. Based on the latest estimates in European region obesity affects 20% of adults, reaching 37% in USA [73]. Probably, the most dangerous data regards children: the worldwide prevalence of childhood

overweight and obesity increased from 4.2% in 1990 to 6.7% in 2010. This trend is expected to reach 9.1%, or 60 million children, in 2020 [74].

Overweight or obese individuals experience greatly elevated morbidity and mortality from nearly all of the common CVD [75]. Therefore, the prevention and control of obesity has become a key element for the prevention of cardiovascular diseases [76, 77], including children. Poirier et al., evidenced how higher BMI during childhood is associated with an increased risk of CAD in adulthood [78].

Excess adipose tissue accumulation produces alterations in metabolic profile and various adaptations in cardiac structure and function, improving not only CAD but also even insulin resistance, metabolic syndrome and type 2 diabetes.

2.1.3.7. Smoking habits

Cigarette smoking is one of the most important risk factors for peripheral vascular diseases and atherosclerosis, and greatly increases the risk of stroke and heart attack [79-81]. Furthermore, risk of morbidity and mortality from CAD is approximately doubled in smokers compared with non-smokers and is related to the duration and amount of smoking [82, 83]. Passive smoking increases the risk of CAD too [84]. Tobacco smoking is the most common form of tobacco use but other forms of tobacco use are becoming increasingly common, such as electronic cigarette cigars, hookahs, and water pipes. Their relationship with CAD will need further investigations.

The key processes in smoking-induced atherogenesis initiation are endothelial dysfunction and damage, increase in oxidation of proatherogenic lipids, as well as decrease of HDL. Moreover, smoke induces inflammation and tissue factor expression that contributes to shift toward a procoagulant status [85]. It was reported that smoking contributed to increased circulating ADMA levels, a NO synthesis inhibitor, and that long-term treatment with nicotine significantly elevated ADMA levels in vascular endothelial cells [86].

2.1.4. Multifactorial aspects involved in coronary artery disease

Nowadays, there is more and more attention not only on "*classical*" CAD risk factors, but also in some multifactorial aspects that are strongly connected to CAD. The multiple risk factors implicated in the development of atheroma appear to act via common biologic pathways that involve inflammation, oxidative stress, lipid profile modification and gut microbiota.

2.1.4.1. Inflammation

Inflammation, from the Latin word *inflammatio*, was described for the first time by the Roman Aulus Cornelius Celsus nearly 2,000 years ago by its four principal effects: *rubor*, *tumor*, *calor et dolor* (redness, swelling, calor and dolor). Only in XIX century, Virchow added the fifth inflammation feature, *functio laesa* (loss of function). Inflammation is defined as a complex beneficial response of the host to harmful stimuli, which involves a well-organized cascade of fluidic and cellular changes. Although inflammation is a defensive mechanism, pathological inflammation is associated with tissue damage and disease [87]. In this context, low-grade systemic inflammation appears to play an important role in the pathophysiology of chronic diseases, including CAD [88].

Inflammatory cells, inflammatory proteins, and inflammatory responses from vascular cells play a pivotal role in all stages of atherosclerosis from lipoprotein retention and immune cell infiltration, to development of vulnerable plaques [89].

There are several markers of systemic inflammation, such as **C-reactive protein** (CRP), tumor necrosis factor-alpha (TNF-α), interleukins (IL) 1, 2, 6, 8 and 10, monocyte chemoattractant protein-1, soluble CD40 ligand or serum amyloid A [90]. Among them, CRP is the most extensively studied inflammatory biomarker in CAD context and is considered to reflect inflammation in the coronary artery. Indeed, activated immune cells produce inflammatory cytokines as interferon-gamma, IL-1 and TNF-α, which subsequently induce substantial IL-6 production. IL-6 stimulates CRP synthesis in the liver (Figure 3). Although cytokines at all steps have relevant biologic effects, their amplification in the cascade makes the measurement of downstream mediators, such as CRP, particularly useful for clinical diagnosis.

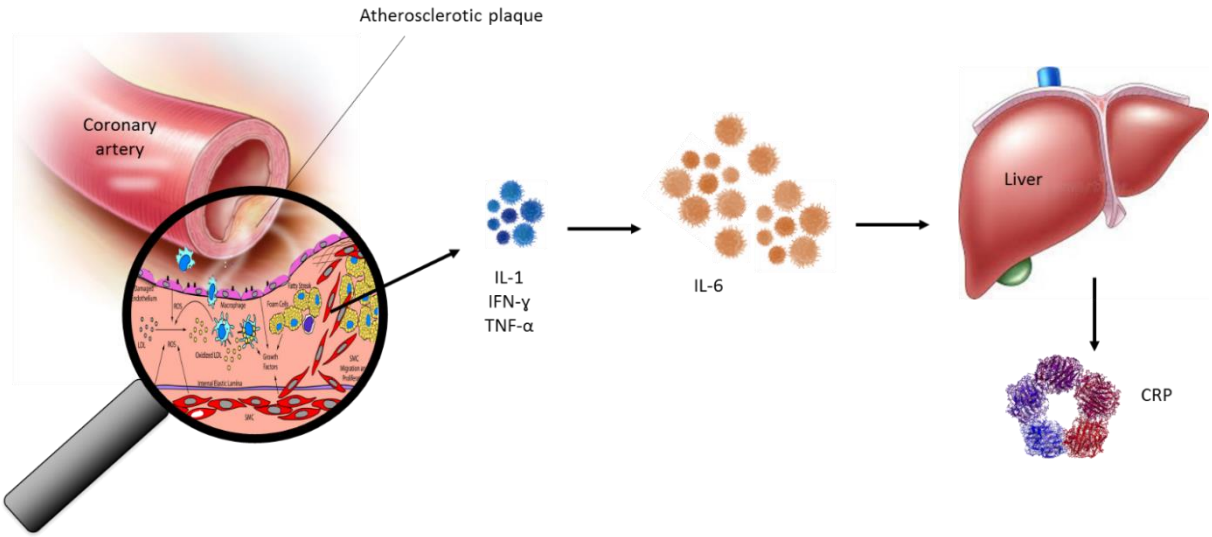


Fig. 3 – The cytokine cascade and CRP synthesis. In the atherosclerotic plaque several cytokines were produces, such as interleukin-1 (IL-1), interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) which induce interleukin-6 (IL-6) production. Then, IL-6 stimulates C-reactive protein (CRP) liver synthesis and release.

Multiple studies have demonstrated the relationship between CRP and different CVD clinical settings. Levels of CRP, and IL-6, have been found elevated in patients with UA and MI, with higher levels predicting worse prognosis [91, 92]. CRP concentration has been associated with the risk of coronary heart disease, ischemic stroke, vascular mortality, and death [93-96]. Moreover, CRP is linked to CVD risk factors. Its concentration is increased in smokers and diabetics, and correlates directly with age, BMI, triglycerides (TGs), blood pressure and inversely with physical activity and HDL [97]. Maraglione et at., showed how healthy individuals with at least one parent with MI had elevated CRP levels compared with those without heredity [98].

Furthermore, increasing evidences suggest that CRP may also directly participate in the inflammatory process of atherogenesis [99, 100]. Ishikawa et al, demonstrates CRP presence in atherosclerotic plaque and suggested its role in the pathogenesis of UA [99].

The US Centers for Disease Control and Prevention and the AHA recommended CRP as inflammatory marker in clinical practice [101]. This choice could be related not only to biological relevance, but also to a very simple, reliable, fully automated, and sensitive assay techniques (high sensitive (hs)-CRP assay) [102].

Considering circulating cells, **leukocytes** have been proposed as biomarker of inflammation in CAD [103]. Total leukocyte count has been evaluated as a risk factor in subjects free of CAD and as a prognostic indicator in patients with CAD. Several prospective studies conducted on CAD-free populations have shown a positive correlation between leukocyte count and CAD risk [104-108].

Moreover, leukocytes count higher than 9000 *per* µL increased risk of MI four times compared to count below 6000 *per* µL in smoking people [104]. A link between leukocyte count and prognosis with stable CAD after MI was found by Schlant et al. [109] and in the Persistent-Aspirin Re-Infarction Study the leucocyte count was associated to coronary event recurrence and total mortality [110]. The differential leukocyte count has been evaluated in several studies but up to now is not well defined which leukocyte subtype (eosinophils, basophils or neutrophils) will be most useful for predicting risk [111]. Nevertheless, elevated levels of **basophils** and eosinophils have been determined in patients with MI, UA and SA, compared to healthy control subjects [112]. As already shown, they play a pathogenic role in CAD due to their ability to cause proteolytic and oxidative damage to coronary arteries. Stimulated neutrophils are able to secrete chemotactic agents [113], proteolytic neutral proteases promoting platelet adherence to subendothelial collagen [114], inflammatory mediators [115] and superoxide anions [116].

2.1.4.2. Oxidative stress

Oxidative stress reflects the imbalance between oxidants, ROS mainly, and the antioxidant system in favor of the former. This state plays a major role in the development of chronic and degenerative diseases such as cancer, arthritis, aging, autoimmune disorders, and neurodegenerative and cardiovascular diseases [117]. In CAD context, one of the most relevant mechanism involving oxidative stress is the reaction between ROS and NO which cause diminished NO bioavailability and, as consequence, ED [118].

ROS include free radicals and non-radical species. The former contain one or more unpaired electrons, such as superoxide anion ($O_2^{\bullet -}$), peroxide ($O_2^{\bullet -2}$) and hydroxyl radical ($^{\bullet}OH$). The latter are produced when 2 free radicals shared their unpaired electrons, for example hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite (ONOO-) and singlet oxygen ($^1O_2$). The balance of many factors determines the amount of free radical production, and ROS can be formed endogenously and exogenously. Physiologically, ROS are produced as a result of normal cell metabolism that includes mitochondrial respiratory chain and cytochrome P-450 metabolism, and different enzymatic reactions involving, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, cyclooxygenases and lipoxygenases. ROS daily production is about four million [119]. The major production source in the cell is the mitochondrial respiratory chain and $O_2^{\bullet -}$ is the primary ROS produced, which is generated from a 1-electron reduction of molecular oxygen.

Among cells, polymorphonuclear leukocytes, monocytes, and macrophages have a consistent ROS production in order to defend the body against invading microorganisms. In these cells, superoxide can be generated either by the NADPH oxidase, a multicomponent enzyme, or in mitochondria by cytochrome c peroxidase or xanthine oxidase. Then, $O_2^{\bullet -}$ is converted to H_2O_2 either spontaneously or mediated by superoxide dismutase. H_2O_2, in relation to different conditions, can be converted to: a) $H_2O + O_2$ in the presence of catalase and glutathione peroxidase (GPX); b) •HO, the most reactive ROS, through Haber–Weiss and Fenton reactions; c) hypochlorite (OCl^-) by myeloperoxidase [120].

In addition to endogenous ROS, there are also exogenous sources of oxidant molecules. Humans are constantly exposed to environmental free radicals, including ROS, in the form of ozone exposure, hyperoxia [121], ionizing radiation [122], ultraviolet light, cigarette smoke [123], heavy metal ions [124] and compounds called as redox-cycling agents, which include some pesticides as well as certain medications used for cancer treatment [125].

ROS may be toxic to cells reacting with most cellular macromolecules, including proteins, lipids, and DNA, and generating different types of secondary radicals like lipid radicals, sugar radicals, amino acid radicals, and thiol radicals.

The **oxidative DNA damage** induced by ROS can generate a number of possible DNA lesions, including DNA bases degradation and modification, single- or double-stranded DNA breaks, loss of purines, deoxyribose sugar damage, mutations, deletions or translocations [126]. These injuries may modulate numerous processes as the activation of stress-induced transcription factors and the production of pro- and anti-inflammatory cytokines. A single double-strand break can induce apoptosis [127] or induce serious chromosomal aberration [128].

Among nucleobases, guanine has the lowest redox potential and thus is the most susceptible to the oxidation [129]. Therefore, its stable urinary end-product, **8-hydroxy-2-deoxyguanosine** (8-OHdG), is one of the most widely recognized biomarker of oxidative DNA damage [130, 131]. This metabolite, mirrored the ROS-damaged guanine mutagenic effect, due to its tendency to preferentially pair with adenine over cytosine during DNA replication, leading to G-to-T point mutation [132]. Studies in patients with atherosclerotic cardiovascular disease highlighted that 8-OHdG is significantly associated with both CAD and other types of atherosclerotic pathologies (stroke, peripheral artery disease, carotid atherosclerosis) [133]. Recently, we found that CAD patients have significant higher urinary 8-OHdG levels compared to healthy controls [134]. Data are in agreement with previously published studies in different matrices such as serum [135] and

DNA extracts from leukocyte or lymphocytes [136, 137]. Moreover increased 8-OHdG concentrations in atherosclerotic plaques, compared to the underlying media or to non-atherosclerotic mammary arteries [138], and elevated DNA strand breaks in cells isolated from the atherosclerotic lesions [139] have also been reported.

Furthermore, ROS can induce **lipid peroxidation**. Polyunsaturated fatty acids (PUFA), major constituents of cellular and subcellular membranes, represent the lipids most vulnerable to oxidation because of the presence of electron-rich double bonds. In particular, ROS, using an electron from PUFA could modifies membrane properties causing alterations in membrane fluidity, deterioration of pores crossing the phospholipid bilayers or variating the physiological functions [140]. Superoxide anion-dependent oxidative modification of PUFA produces isoprostanes, members of prostaglandin family. In particular, isoprostaglandin $F_{2\alpha}$ type III, also known as **8-iso-prostaglandin-$F_{2\alpha}$** (8-iso-$PGF_{2\alpha}$) is produced by a free radical attack on arachidonic acid esterified in membrane phospholipids. Due to its mechanism of formation, specific structural features and stability in urine, 8-iso-$PGF_{2\alpha}$ is considered a reliable index of oxidant stress, representing lipid peroxidation *in vivo* [141]. Moreover, 8-iso-$PGF_{2\alpha}$ is considered a potent vasoconstrictive compound [142, 143] and F_2-isoprostanes are known to be involved in the onset and progression of atherosclerosis through the control of platelet activation and leukocyte-endothelial cell interaction [144, 145]. In accordance with other studies [146], in a study previously published by our group, urinary levels of 8-iso-$PGF_{2\alpha}$ in CAD patients were higher compared to healthy control subjects [147].

Steady-state levels of ROS depend on both their rate of production and the activity of the **antioxidant system**. Indeed, given the huge range of possible oxidative damage, living cells can act different strategies to counteract oxidative damage. Antioxidants, endogenous or exogenous, are those molecules that significantly delays or prevent oxidation of the oxidizable substrate. They can act at three different levels: a) prevention, maintaining ROS production at low levels; b) interception, neutralizing ROS; c) repair, restoring damaged molecules [148].

Glutathione, or γ-L-glutamyl-L-cysteinyl-glycine, chiefly known as **GSH**, is the major scavenging antioxidant in cells being the most abundant intracellular non-protein thiol. It is synthetized *in vivo* from the precursor amino acids cysteine, glutamate and glycine, by the consecutive action of two ATP-dependent enzymes, glutamate–cysteine ligase and glutathione synthase. In contrast to GSH synthesis, which occurs intracellularly, GSH degradation occurs exclusively in the extracellular

space by the γ-glutamyltransferase, which hydrolyzes GSH into glutamic acid and cysteinyl-glycine [149]. Cysteinyl-glycine is further hydrolyzed into amino acids, taken up by cells for regeneration of intracellular GSH [150]. GSH is able to donate reducing equivalent ($H^+ + e^-$), through the thiol cysteine group (-SH), converting into its oxidized form (**GSSG**). The GSH reductase is able to efficiently reduced back GSSG to GSH. Consequently, the balance between its production and consumption determines GSH levels. The measurement of both GSH and GSSG in whole blood is considered an early indicator of oxidative stress [151]. GSH not only reacts directly with oxidants but it also acts as cosubstrate of enzymatic antioxidants like GPX and GSH-s-transferases. The cells within the atherosclerotic plaque try to counteract oxidative stress status using GSH. Yang et al. have demonstrated that macrophages, treated with an inhibitor of GSH synthesis, had increased ROS production [152].

In addition to GSH, other nonenzymatic antioxidants are present in the cells and among them **vitamin E** play a relevant role in preventing oxidative damages. It is found in lipid phase of membranes and acts as a blocker of lipid peroxidation by donating an H-atom. This reaction produces vitamin E radical form that can be reconverted to vitamin E by a reaction involving GSH and ascorbate. Several studies have evaluated the antioxidant effects of vitamin E in CAD primary prevention suggesting a relationship among them. An inverse correlation between vitamin E levels and CAD mortality was evidenced [153]. *In vitro* data suggest that vitamin E is able to protect LDL cholesterol against oxidation and to decrease the deposition of oxidized-LDL in arterial walls [154]. In Figure 4 are summarized the major actors in the balance between ROS and the antioxidant system.

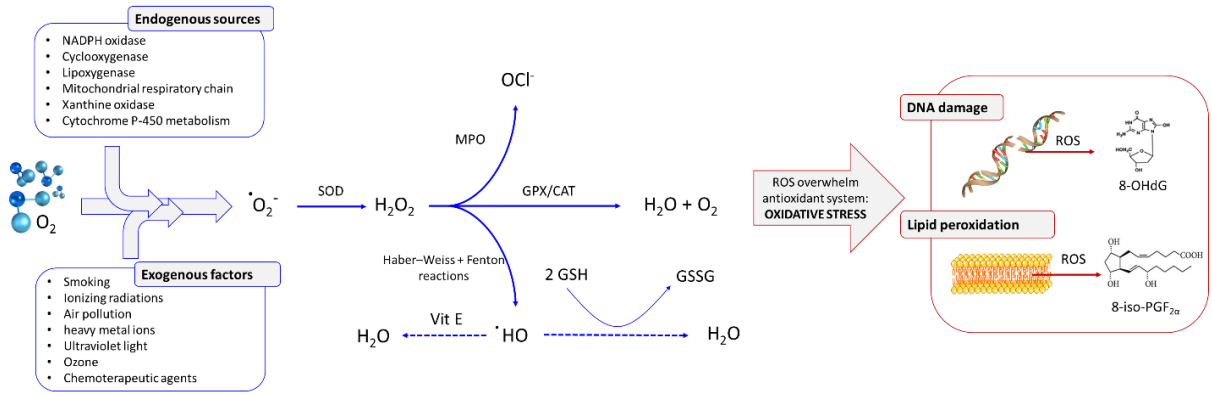


Fig.4 – Schematic representation of reactive oxygen species (ROS) generation, antioxidant system action and oxidative damage if oxidative stress status takes place. ROS production involves: oxygen (O_2), superoxide anion, ($^{\bullet}O_2^-$), superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), myeloperoxidase (MPO), hypochlorite (OCl^-) and hydroxyl radical ($^{\bullet}OH$). The antioxidant system involves: glutathione peroxidase (GPX), catalase (CAT), vitamin E (Vit E), reduced (GSH) and oxidized (GSSG) glutathione. If ROS overwhelm the antioxidant system, oxidative stress status can damage DNA and lipids. 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-iso-prostaglandin-$F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) represents DNA oxidative damage and lipid peroxidation, respectively.

2.1.4.3. Gut microbiota

The gut microbiota is the complex and dynamic population of microorganisms living in the human gastrointestinal (GI) tract. The huge surface of the GI tract (250-400 m^2) hosts more than 10^{14} microorganisms and their gene content (microbioma) exceeds ours by about ten times [155, 156]. A microbiota with high degree of bacterial richness and diversity is considered to be favorable to health through a range of physiological functions such as harvesting energy [157], regulating host immunity [158], protecting against pathogens [159], maintaining mucosal barrier integrity, providing nutrients and strengthening the intestinal epithelium [160]. For example, gut bacteria have the ability to ferment complex carbohydrates generating short-chain fatty acids, such as propionate, butyrate and acetate, which are absorbed and involved in the regulation of chemotaxis, differentiation, proliferation and apoptosis [161]. Quite relevant is the role of microbiota in inflammation process: lipopolysaccharides (LPS), large molecules found in the outer membrane of gram-negative bacteria, induce activation of TLR4, an immune receptor that upregulates the transcription of pro-inflammatory cytokines, and of the adaptive immune system, which results in the production of antibodies [162]. However, these functions can be disrupted as result of microbiota composition alteration, known as dysbiosis, produced by various environmental factors including diet, toxins, drugs and pathogens. Diet seems to be the major determinant of the microbial composition in the gut. There are growing evidences that dysbiosis of the gut microbiota could be associated with the pathogenesis of intestinal, e.g. inflammatory bowel disease or irritable bowel syndrome, or extra-intestinal disorders including allergy, asthma, metabolic syndrome and cardiovascular disease [163].

Trimethylamine N-oxide (**TMAO**), a metabolite originated from gut microbiota, is reported to be strongly associated with increased CVD risk, including CAD risk, and mortality risk [164-168]. TMAO can be introduced directly by seafood or can derive from dietary precursors. Dietary methylamines, such as **choline**, **carnitine**, **betaine** and phosphatidylcholine (PC) are introduced by several foods including red meat, fish, poultry, vegetables, nuts and eggs. The gut microbiota is able to transform them into **trimethylamine** (**TMA**), even if is still unclear which microbes are primarily responsible for this process. This one is absorbed in the small intestine and diffuses into the bloodstream via the hepatic vein to hepatocytes. In the liver, by the hepatic flavin-containing monooxygenase-3 (FMO-3), TMA is oxidized into TMAO (Figure 4). Other FMO forms (FMO-1, FMO-2, FMO-4, FMO-5) are not present in humans nor do they play any important role in TMA metabolism [169].

Up to now, the role of TMAO in atherosclerosis and the mechanism by which it increases CAD risk are not clear. It has been proposed a platelet-mediated mechanism according to which TMAO directly contribute to platelet hyperactivity leading to increased intracellular Ca^{2+} release [170]. Makrecka-Kuka et al. demonstrated TMAO inducing decrease of fatty acids β-oxidation in heart muscle cells [171], while Koeth et al. proposed TMAO as a modulator of cholesterol and sterol metabolism promoting atherosclerosis [172]. Experiments in mice indicated the pro-atherogenic nature of TMAO by augmentation of cholesterol loaded macrophages and foam cell formation [173]. On the contrary, it seems to have beneficial effects by protecting from glutamate neurotoxicity, improving glucose homeostasis by the stimulation of insulin secretion and reducing oxidative stress [174-176].

There are different ways to evaluate microbiota modification such as classical cultural techniques, which is necessarily limited to 'culturable' microorganisms, or the innovative high-throughput DNA based pyrosequencing technology, which classify bacteria according to individual 16S rRNA sequences. Nevertheless, the quantification of pathways directly connected to gut microbiota, as TMAO and its precursors choline, betaine and carnitine, allow to obtain two different information: gut microbiota modification and variation of prognostic CAD marker levels.

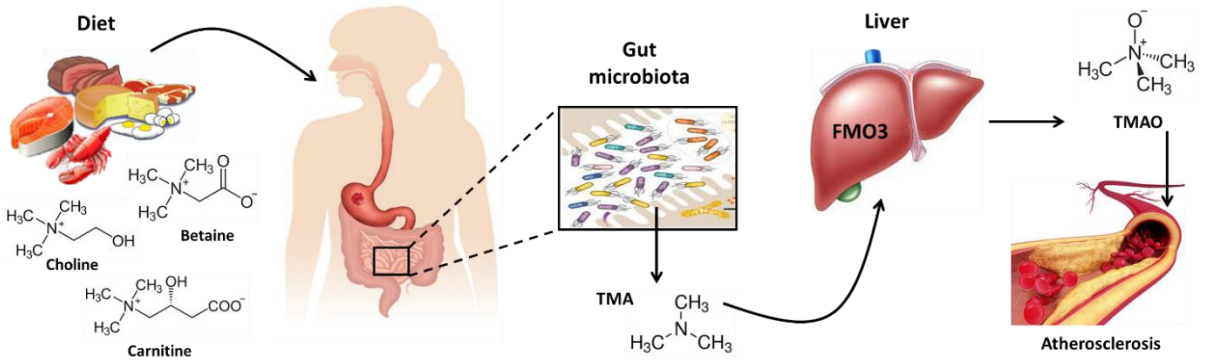


Fig. 5 – Schematic representation of trimethylamine N-oxide (TMAO) metabolism. Its precursors, choline, betaine and carnitine, are introduced by diet and gut microbiota transforms them into trimethylamine (TMA). Hepatic flavin-containing monooxygenase-3 (FMO3) oxidizes TMA into TMAO.

2.1.4.4. Complex lipid profile

Detection and treatment of cholesterol and TGs levels alterations has significantly reduced cardiovascular events. However, several patients with CAD do not have hypercholesterolemia and/or hypertriglyceridemia. The well-known lipid species, such as LDL, HDL, and TC, are able to

explain only a small portion of CVD risk. For this reason, several researchers are focusing their attention on different aspects of lipids and atherosclerosis, such as LDL-HDL lipid composition or other lipid classes involved in CAD [177-179]. In particular, technology evolution and the use of mass spectrometry (MS), allow introducing a new research approach: lipidomics, defined as the comprehensive lipid profile in a sample.

Some studies have found a correlation between CAD events/death and lipid species different from "*classic*" lipids (HDL, LDL, TGs). A combination of lipid species (triacylglycerol 54:2 + cholesterol ester (CE) 16:1 + PC 36:5), lipid classes (such as lysophosphatidylcholine (LPC) 18:1 e 18:2) or single lipids (such as ceramide (Cer) d 18:1 18:0) are found to be predictive of CVD incidence [180-182]. Saturated and monounsaturated PCs species have been reported to have a positive association with total and cardiovascular mortality [183]. Meikle et al. found plasma lipid differences between stable and unstable CAD, in particular, stable CAD patients showed higher levels of phosphatidylethanolamines [184]. Comparing plaque to control arteries, 24 specific lipids have been detected only in atherosclerotic plaque, indicating a specific and exclusive lipid profile of the pathology [185]. Furthermore, decreased PCs and LPCs serum levels were observed in CAD patients compared to healthy subjects [186].

Therefore, dysregulation of "*classic*" lipid metabolism is surely important in CAD, but even other lipid classes have a relevant role in prognosis and pathogenesis of CAD.

2.1.5. Coronary artery disease prevention and treatment

The prevention and treatment of coronary artery disease is not limited to drug therapy. A combination of drugs and lifestyle modifications is the recommended strategy to protect against primary or secondary CAD events.

2.1.5.1. Drug treatement

CAD patients typically receive several medications as part of their treatment to protect against recurrent cardiac events and all-cause mortality. Different therapies have to be defined in relation to CAD risk factors. Patients with CAD commonly have hypertension and hypercholesterolemia, and quite commonly diabetes. Thus, CAD hypertensive patients are usually treated with antihypertensive drug such as diuretics, ACE inhibitors, angiotensin receptor blocker, beta-blockers and calcium-channel blockers and more than one drug is often required. A meta-analysis comparing ACE inhibitors, calcium-channel blockers, diuretics and beta-blockers showed no

differences between drugs in relationship to total cardiovascular mortality [187, 188]. Another meta-analysis investigated the efficacy of beta-blockers in relation to different ages: it was similar to other antihypertensive drugs in younger patients, but lower in older patients [189]. These data and other specific indications (e.g. diuretics are contraindicated for gout, angiotensin receptor blocker in pregnancy) may influence the choice of first-line drug therapy. However, in the absence of any relevant indication, currently available evidences supports the use of any of the following classes as initial therapy: ACE inhibitor, calcium-channel blockers, or diuretic.

Statins are the most hypocholesterolemic drug class used at all. Vrecer et al., through a meta-analysis on 15 trials and 63 410 participants, demonstrated that statin therapy was associated to 22% reduction in TC, 29% reduction in LDL cholesterol, 12% reduction in TGs and 6% increase in HDL cholesterol [190]. In addition to statins, there are others lipid-lowering drugs. Fibrates and nicotinic acid are able to reduce TGs and increase HDL cholesterol, and results of primary and secondary prevention trials support their cardiovascular benefit [191-193]. In 2015, a new class of lipid lowering drugs, defined PCSK9 inhibitors, a monoclonal antibodies class, was approved. They act inhibiting proprotein convertase subtilisin kexin 9 (PCSK9), a hepatic protease that attaches to and internalizes LDL receptors into lysosomes hence promoting their destruction. As consequence, PCSK9 inhibitors are able to reduce LDL cholesterol receptors on the liver cell surface and decrease LDL plasma cholesterol. Clinical trials indicate LDL cholesterol level reduction from 28% to 65% in healthy volunteers [194], but more info are necessary.

WHO prevention guidelines reported that the first approach to controlling glycaemia should be through diet alone; if this is not sufficient, oral medication should be given, followed by insulin if necessary. Metformin is the drug of choice for the initial therapy of type 2 diabetes as it is well defined as safe and effective. The use of the newer insulin secretagogues, the thiazolidinediones, is still being evaluated in clinical trials [195].

Regarding secondary CAD prevention, the use of aspirin and clopidogrel as antithrombotic medications is mandatory. The use of aspirin in primary prevention is still debated: it was associated to 32% reduction in MI and a non-significant increase in the risk of stroke [196, 197]; instead, in the Women's Health study Aspirin had no significant effect on the risk of MI [198]. The novel oral anti-coagulants (dabigatran, edoxaban, rivaroxaban and apixaban) are now approved for clinical use but far from common use.

2.1.5.2. Lifestyle

Despite the cardioprotective therapies, more than 40% of CAD events occur in patients with a previous history of CAD [199]. Lifestyle modifications such as regular physical activity, smoking cessation and diets low in fat are recommended to match polypharmacy regimens in the management of CAD patients. The WHO proposed the "25x25 Global Action Plan", a project aimed at reducing the mortality by 25% by 2025. This project focuses on correction of four mains health-related behaviours: smoking, diet, physical activity, and alcohol [3]. For a similar purpose the AHA promoted the Life's Simple 7, a list of seven recommendation regarding lifestyle and useful to improve quality and lifespan. The seven steps list includes: manage blood pressure, control cholesterol, reduce blood sugar, get active, eat better, lose weight and stop smoking.

Not getting regular **physical activity** is a risk factor for high blood pressure, high cholesterol, and diabetes, all of which are the primary risk factors for CAD. Indeed, regular exercise reduces cardiovascular events through physiological mechanism: reduces plasma TGs, glycemic levels [200], and blood pressure [201], and increases HDL cholesterol but has no effect on LDL cholesterol. Furthermore, physical activity improves insulin sensitivity [202] and endothelial function [203]. The direct relationship between physical inactivity and CAD was published for the first time in 1953 on The Lancet: conductors of double-decker buses (performing physical activity in their working day) have less incidence of MI and CAD death compared to sedentary bus drivers [204]. Observational studies have pointed out how physical activity is associated with reduced cardiovascular risk and cardiovascular mortality in both men and women [205-207] and in middle-aged and older individuals [208, 209]. Regarding secondary prevention, a systematic review of 63 studies determined that exercise cardiac rehabilitation for CAD patients reduced cardiovascular mortality [210].

Tobacco smoking is considered one of the most preventable causes of death in the USA and globally. The WHO reported that, although it is declining worldwide, in 2015 over 1.1 billion people smoked tobacco, far more males than females. There are evidences that CAD risk immediately decrease after stopping smoking, and 20 years after, the risk associated with smoking is completely reversed [211, 212]. More specifically, stop smoking after MI results in 40% reduction of mortality rates and infarct recurrences [213, 214].

A variety of **dietary components and patterns** have been suggested to decrease the risk of CAD. Indeed, a diet rich in saturated fat, sodium, cholesterol and sugar, associated with a sedentary lifestyle, can contribute to increase the incidence of atherosclerosis [215]. Even indirectly, an

incorrect diet can promote the insurgence of various pathological conditions associated with CAD such as diabetes and insulin resistance, obesity and hypertension. The relationship between dietary fat and CAD has been extensively investigates: saturated fats have been shown to increase LDL cholesterol levels [216, 217]. However, saturated fatty acids (SFA) are not all equally hypercholesterolaemic. Lauric acid (12:0), myristic acid (14:0), and palmitic acid (16:0) are the most dangerous [218, 219]. High trans-fatty acids dietary intake can induce LDL cholesterol increase and HDL cholesterol decrease, increasing the risk of CAD [220-222]. It has been reported that replacing saturated and trans-unsaturated fats with monounsaturated and polyunsaturated fats is more effective in preventing CAD events than reducing overall fat intake or cholesterol intake [223, 224]. Diets rich in omega-3 fatty acids, which are abundantly present in fish, fish oil, nuts and plant oils, have a benefic effect on subject with high risk of CAD and on mortality of CAD patients [225-227]. On the contrary, other studies and a recent large meta-analysis did not found any benefit of omega-3 fatty acids [228, 229]. Fruits and vegetables promote cardiovascular health through antioxidants, phytochemicals, flavonoids, fibers and potassium. Joshipura et al. showed that the single each increase of fruit or vegetable *per* day is associated with a 4% lower risk of CAD [230]. As well known, high salt intake is associated with an increased risk of high blood pressure [231], CVD morbidity and mortality [232]. The WHO prevention of cardiovascular disease guidelines, reported that reducing intake of total fat (to less than 30% of calories), saturated fat (to less than 10% of calories) and salt (to less than 5 g *per* day), and increasing fruits and vegetables (to 400-500 g daily) are likely to have a cardioprotective effect [195].

2.2. Mediterranean diet: an alternative prevention approach

There is increasing research on diet effects on CAD, assessing individual nutrients or food items and dietary patterns. This last one is considered superior, as people do not consume isolated nutrients, but various food, which are a complex combination of nutrients that could exert synergistic or antagonistic effects [233]. Furthermore, dietary patterns also may overcome potential confounding by specific nutrients or foods, and avoid the problem of concomitance between foods.

It has been reported that 80-90 % of CVD primary risk could be prevent with a healthy diet [234]. Different dietary patterns have been evaluated, such as the dietary approaches to stop hypertension (DASH) diet [235], the vegetarian diet [236], the low-fat diet (LFD), high-carbohydrate diet and the Mediterranean Diet (MD). The Mediterranean diet pattern is the one with the highest evidence for protection against CVD risk factors and outcomes.

As previously shown, CAD is the main cause of death, but incidence rates have clear geographic differences. Epidemiological studies have shown that CVD mortality is higher in Central and Eastern European countries than in Northern, Southern and Western countries. These data has been partly ascribed to dietary habits [237-241].

2.2.1. Definition and overview

In 2013, the United Nations Educational, Scientific and Cultural Organization (UNESCO) recognized the Mediterranean Diet as an "Intangible Cultural Heritage of Italy, Portugal, Spain, Morocco, Greece, Cyprus and Croatia". Despite different habits in the area, there are several common features in the dietary patterns of Mediterranean countries. The **Mediterranean diet pattern** is characterized by: high intake of fruits, vegetables, legumes, unrefined cereals, and nuts; moderate consumption of seafood and red wine (especially during meals); a low-to-moderate consumption of dairy products (mostly as cheese and yogurt); low consumption of poultry, red meat and meat products and the use of olive oil as the main source of fat. Olive oil is important not only for its own health benefits, but also because it is usually consumed with great quantities of vegetables in the form of salads and large quantities of legumes in the form of cooked foods. In addition to olive oil and olives, other essential components of the MD are wheat, grapes, and their derivatives. The MD shows some differences among countries. For example, the Italian MD variant is characterized by high pasta consumption and a total fat content ≥ 30% of total energy intake, while

in Spain fish consumption is higher and in Greece there is high consumption of fruits and the total fat content is ≥ 30% [242, 243]. Historically, MD was defined as the dietary pattern in the olive-growing areas of the Mediterranean regions in the late 1950s and early 1960s, when the consequences of World War II were over and the fast-food culture had not yet started [242]. Nowadays, due to the social and cultural changes in alimentary habits, the MD is not the same one described by Trichopoulou. Thus, it could be appropriate to consider MD dietary patterns as variants of a single entity, and to define MD as a modern dietary approach inspired by the traditional diets of the countries bordering the Mediterranean Sea. Recently, the typical MD features have been summarize in the so call "*MD pyramid*", which was constructed on scientific evidences and epidemiological studies in order to be applied to present days and adapted to different geographical contexts (Figure 6) [244].

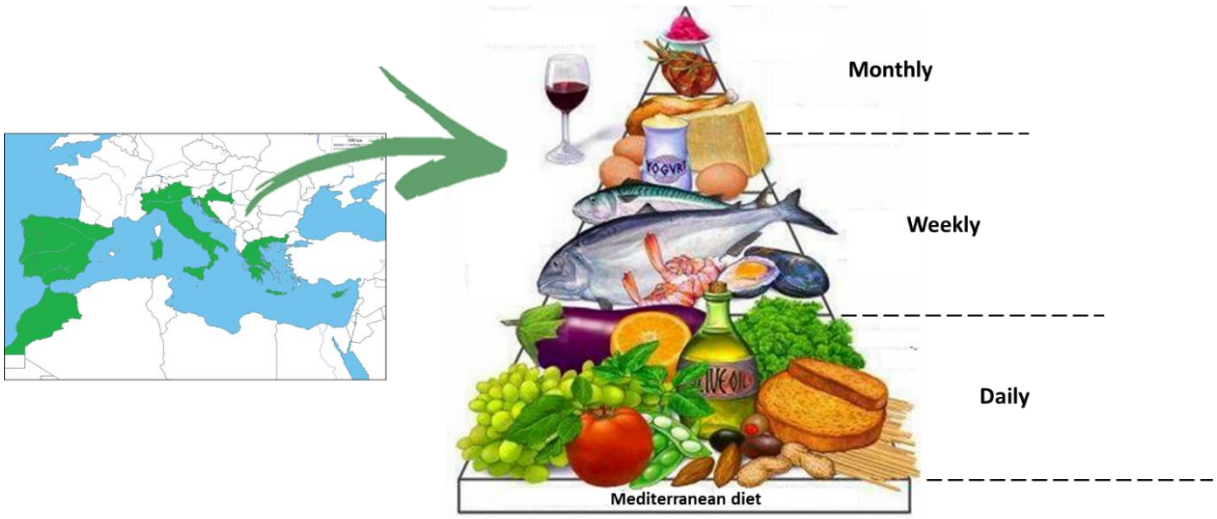


Fig. 6 – Schematic representation of the Mediterranean diet composition and consumption frequency.

The positive effect of MD on **primary prevention** is well established through a lot of observational studies and several interventional trials. On the contrary, evidence of the MD cardioprotective effect in **secondary prevention** is much less recognized.

The very first observation about the cardioprotective effect of MD was the result of the **Seven Countries Study**. Ancel Keys, an American biologist, during his long-stay in Italy, developed the concept that the rate of CAD was lower in Mediterranean regions and tested it in the Seven Country Study. It was a longitudinal multinational observational study, involving healthy population from Finland, Greece, Italy, Japan, The Netherlands, U.S. and Yugoslavia, with at least

5-year follow-up. His hypothesis was confirmed, the populations on the Mediterranean Sea (Greece, Italy and Yugoslavia) had lower incidence of CAD and CVD in general. Furthermore, the results pointed out a three-way relationship: favourable rates of CAD in Mediterranean countries were associated with lower dietary intakes of SFAs and lower levels of serum TC [239, 245]. In cross-population analyses, ten years CAD mortality rate was strongly associated to average dietary SFA intake. Similar findings were found for serum cholesterol: the highest the sample average cholesterol level, the highest the coronary mortality rate. In addition, dietary SFAs were closely related to serum cholesterol. Since then substantial body of evidence, observational and interventional studies, indicates that the MD promotes optimal cardiovascular health.

In particular, the European Prospective Investigation into Cancer and Nutrition (**EPIC**) **Study** is the largest single resource worldwide prospective study. It is an ongoing multicenter prospective study designed to investigate the relationship between nutrition and cancer, but with the potential for studying other diseases, including CAD [246]. The study currently includes 519, 978 participants (366,521 women and 153,457 men, mostly aged 35–70 years) in 23 structures located in 10 European countries, to be followed for cancer incidence and cause-specific mortality for several decades. On 22,043 Greek adults from the EPIC cohort, Trichopoulou et al. demonstrated that a high adherence to the MD was associated with a lower total mortality and found an inverse association with high adherence to MD diet for both CAD or cancer death. Interestingly, no association emerged between single MD food component and mortality, indicating greater strength of the whole dietary pattern compared to single food components [247]. The contribution of individual food components on survival was evidenced after 8-years follow-up indicating moderate ethanol consumption, low meat consumption and high consumption of fruit and vegetables as the major food components influencing MD effect [248].

These results have been confirmed by several cohorts worldwide. A systematic meta-analysis on 35 prospective cohort studies, with a total of over 4 million subjects, evaluated the MD relationship with various health outcomes in a primary prevention setting. A two point increase in the adherence to MD scores (ranging from 0 to 18) was associated with a 8% reduction of overall mortality and 10% reduction of the risk of cardiovascular disease [249]. Among the most relevant observational studies taken into account the CVD primary prevention context there are the Spanish EPIC study about overall and CAD mortality [250, 251], studies focused on stroke mortality in Italy and USA [252, 253], the Spanish SUN Study on CVD mortality [254] and the Northern

Manhattan Study on CVD risk [255]. Furthermore, several gender prospective studies relating MD and CVD have also been performed in Netherland, Greece and Sweden [256-260].

Instead, few interventional studies are available, but among them, the PREvencion con DIeta MEDiterranea (**PREDIMED**) is one of the most interestingly. It is a multicenter interventional randomized clinical trial on CVD primary prevention carried out in Spain between 2003 and 2011 which tested the long-term effects of the MD on incident CVD in subjects at high CVD risk [261]. The effect of MD was evaluated randomizing individuals (7,500) into three group of diets: two different MDs, MD supplemented with extra-virgin olive oil (EVOO) or MD supplemented with nuts, and a control LFD. Now the follow up is over a 5-year period and it was observed that, MD reduces incidence of major CV events (MI, stroke or CV death) by almost 30% compared to a LFD [262]. Nevertheless, this study allow to obtain not only prospective results, but even to go inside different mechanisms probably involved in the MD positive effects. For example, the reduced expression of CD40 on monocytes surface, of soluble intracellular cell adhesion molecule and of P-selectin, in the MDs groups, was pointed out [263]. The PREDIMED study is now moving to a second part, the PREDIMED-PLUS clinical trial. It is an ongoing, randomized, multicentre, primary prevention study with 6919 participants. Subjects are randomized in two groups: Mediterranean diet supplemented with EVOO and nuts or hypocaloric Mediterranean diet supplemented with EVOO and nuts together with intensive physical activity. The combination of MD and physical activity was previously demonstrated to be high effective in the Spanish SUN study. Alvarez-Alvarez et al., observed a 75% CVD incidence reduction in the highest followers of MD and physical activity [264]. A recent meta-analysis investigated the MD effect on different type of CVD of cohort case-control studies. It provided evidence that people with highest adherence to MD had incidence and mortality decrease of 30 % for cardiovascular heart disease and MI, and 27% for stroke [265].

Regarding CVD **secondary prevention**, at the end of the 1990s, the **Lyon Diet Heart Study** demonstrated, for the first time, the protective role of MD in secondary prevention of CAD [266]. This study was a randomized multicentre clinical trial examining whether a MD enriched with margarine rich in α-linoleic acid (ALA), compared to a prudent Western-type diet, may improve the prognosis and reduce recurrence after a first MI. First results, 2 year of follow-up, indicated that there were 16 CV deaths in the control group and 3 in the MD one. After correction for other factors, a surprisingly high (76%) reduction in the risk of cardiac death was found during the observation period [267]. Due to this unexpected result the trials was stopped as considered non-

ethic. Data were confirmed up to 4 years after the first MI, the MD pattern is associated with a 72% reduction in cardiac death and non-fatal MI incidence [268]. Such reduction was associated with enhanced plasma levels of eicosapentaenoic acid and its parent fatty acid, ALA. However, in the Lyon study, the first source of fat was canola oil, and not olive oil as usually indicated in the MD.

Since then, no other relevant randomized interventional clinical trials, regarding secondary CAD prevention ad MD, were carried out. The Italian GISSI-Prevenzione study involved more than 10,000 subjects with recent MI, but only diet "Mediterranean" advices have been promoted and no control group have been used. Nevertheless, it showed a 15% decrease in mortality each unit increase in MD score adherence [269]. Two recent CAD secondary prevention have been registered: the Coronary Diet Intervention with Olive oil and cardiovascular PREVention study [270] and the AUStralian MEDiterranean Diet Heart Trial [271]. The first one is still ongoing and results are not available yet. It is a prospective, randomized clinical trial including 1002 CAD patients with a long-term period of follow-up (7 years). The second one is a multicenter, parallel design, randomized controlled trial in a multi-ethnic population. Both studies are comparing a MD to a LFD. Prospective studies have confirmed the findings of the Lyon Heart Study, providing more evidence concerning the protective role of the Mediterranean diet against recurrent cardiac events [272-274]. The analysis of the INTERCATH cohort, an observational study on patients undergoing coronary angiography, found an independent association of CAD complexity and MD adherence [275]. However, further information on MD benefits in CAD secondary prevention are necessary [276].

2.2.2. The protective role of the Mediterranean diet on coronary artery disease

Biochemical, clinical, and epidemiologic research during the last 15 years has provided a rationale for the health benefits of the MD against CAD. [277, 278]. The cardioprotective MD action is not solely due to the beneficial effects of this diet on classical risk factors such as hypertension, plasma cholesterol, obesity and insulin resistance. Now the benefits of MD have been recognized to be mediated even by emerging factors such as other lipid fractions, oxidative stress, inflammatory response and endothelial dysfunction [279-281]. Probably, additional pathways will be elucidated in the future.

The mechanisms responsible of such protection have not been completely established yet. Although the MD beneficial effects are attributed to its high content in anti-oxidant and anti-

inflammatory nutrients, fiber, poly and mono-unsaturated fats and to its moderate content in ethanol, along with the low content in trans/saturated fat ratio and dietary cholesterol. Perhaps, the synergy among these components attenuates the intermediate CVD pathways of atherosclerosis and thrombosis, as well as multiple CVD risk factors [282].

2.2.2.1. Coronary artery disease risk factors

Regarding CAD risk factors, MD have been shown to have a positive effect on several modifiable risk factors [261].

Blood pressure. The DASH clinical trial have demonstrated that a diet rich in fruits, vegetables, and low-fat dairy products with reduced saturated and total fat was able to reduce SBP and DBP of 3.5 and 2.1 mm Hg, respectively, in healthy subjects. Furthermore, the diet was able to reduce SBP and DBP of 11.4 and 5.5 mm Hg, respectively, in hypertensive subjects [283]. The DASH diet, as described above, is quite similar to a MD. A cross-sectional analysis of the SUN cohort showed that fruit and vegetable consumption, main component of MD, is inversely associated with blood pressure [284]. Furthermore, olive oil consumption was demonstrated to reduce blood pressure in hypertensive elderly subjects [285]. Finally, the MD effect on blood pressure, as a dietary pattern, was evidenced in the PREDIMED study where a reduction of both SBP and DBP was observed in MD groups, but not in the control LFD group [261].

Plasma cholesterol. The MD effects on cholesterol is not limited to lowering its plasma level. Lowering total and LDL cholesterol concentration is the primary target in CVD prevention, however LDL physico-chemical properties (e.g., size and oxidation) should be considered too. Indeed, subjects with a predominance of small and dense LDL particles showed increased CAD risk compared to those with larger and buoyant LDL particles [286]. Compared with large LDL, small LDL cholesterol has a lower affinity for the LDL receptor [287], penetrate the arterial sub-endothelium more easily [288] and are more susceptible to oxidation [289]. MD can reduce LDL cholesterol atherogenicity enhancing LDL characteristics such as resistance against oxidative stress, size, composition and cytotoxicity, particularly with MD EVOO enriched [177]. Another study showed that MD, in addition to LDL cholesterol concentration reduction, induces a redistribution from smaller to larger LDL in men, but not in women [290]. At the same time, MD

improves several HDL cholesterol functions, such as cholesterol efflux capacity, cholesterol metabolism, antioxidant/anti-inflammatory properties, and vasodilatory capacity [178].
Moderate alcoholic beverages consumption was reported to reduce the risk of CAD probably by increasing levels of serum HDL cholesterol [291, 292]. MD is characteristic by moderate red wine drinking. Moreover, monounsaturated fat, including olive oil, increases HDL cholesterol more than polyunsaturated [293] and carbohydrates [293-295], making it an optimal energy-generating nutrient [277, 278].
Furthermore, in the PREDIMED study was observed that MD diet, supplemented with walnuts, reduced TGs by 0.15 mmol/L compared with a low-fat control diet [261].

Glycaemia. There are controversial opinion concerning the effect of MD on hyperglycemia. Some studies in diabetic patients reported a positive effect of the MD on glycemic control [296, 297], others did not [298, 299]. In the PREDIMED study a decrease in glucose levels was observed in groups adhering to a MD but not in the LFD group [261]. Olive oil and complex carbohydrates derived from legumes, whole-wheat bread, and cereals, are able to modulate postprandial hyperglycemia, which could explain the glucose reduction.

Obesity. The results of observational studies in Mediterranean countries have shown that increasing MD adherence is associated with decreasing obesity rates [300, 301]. Accordingly, a recent cohort study demonstrated, after 6 years of follow-up, a lower incidence of obesity in those who consumed olive oil than in those who consumed sunflower oil [302]. Probably, a satiating effect of olive oil could explain its lack of a fattening effect. In a very huge study, including 500,000 individuals from 10 Mediterranean, Central, and Northern European countries, MD adherence was associated with lower waist circumference in men and women after controlling for BMI, total energy intake, and other potential confounders [303]. A prospective study on the EPIC-PANACEA cohort (373,809 subjects from 10 European countries), demonstrated after 5 years of follow-up that a high MD adherence reduces 10% risk to develop obesity [304]. The PREDIMED study, instead, showed no weight changes after 3 months of MD treatment [261].

Smoking habits. A literature review investigated if MD might have a role in modifying deleterious effects on human health of active and passive smoking [305]. Both epidemiological and experimental studies, demonstrated that MD has a protective effect against biochemical and

molecular smoking-related processes that lead to CVD. Based on the high daily intake of vitamins and antioxidants, MD provides a variety of compounds that positively affect outcomes related to smoking, but further research is needed.

2.2.2.2. Multifactorial coronary artery disease aspects

As reported before, the health effects of the MD goes beyond the traditional risk factors, others interrelated and overlapping factors play a relevant role.

Inflammation. Healthy dietary patterns are considered anti-inflammatory [306-308], but, particularly MD has the strongest evidences for reduction in markers of inflammation in randomized controlled trials. It has been associated with significantly decrease of CRP levels [309-311], proinflammatory ILs (IL-1, IL-6, IL-7, IL-8, and IL-18), TNF-α and its receptors, chemoattractant molecules (MCP-1), and soluble endothelial adhesion molecules (sVCAM-1, sE-, and sP-selectins) [263, 309, 312-314]. Furthermore, an increase adherence to MD was associated to lower levels of leukocyte and platelets on healthy subjects [315].

A meta-analysis performed by Schwingshackl et al. on 17 studies pointed out the decrease in inflammatory markers (CRP, adiponectin and IL-6) induce by MD in different pathological cohorts [310]. Contrasting results were reported by Mayr et al. in a recent meta-analysis focused on MD anti-inflammatory effect on CAD patients. Unexpectedly, it revealed that even if most studies reported a reduction in CRP, it is not significant. They suggested that, probably, this result is related to small sample sizes and very different period MD treatment in the studies evaluated [316].

The main MD nutrients, such as fiber, monounsaturated fatty acids (MUFA), n-3 PUFA, vitamin C, vitamin E, and carotenoids, have been associated with lower inflammation [312]. Omega-3 fatty acids intake was negatively correlated with circulating inflammatory markers and this relationship seems to be mediated by the binding to the G-protein-coupled receptor 120 and inhibition of Nod-like receptor pyrin domain-containing protein (NLRP) 3 inflammasome activity [317, 318]. Polyphenols hydroxyl groups can interact with proinflammatory pathways, such as the nuclear factor kappa B(NFkB), the NLRP3 inflammasome, and the mitogen-activated protein kinase, reducing inflammation. In mouse, the downregulation of NFkB inhibits inflammation in macrophages [319]. *In vitro* studies suggested that polyphenols, inhibiting these pathways, reduces the synthesis and release of proinflammatory cytokines and TNF-α [320-322]. Indeed, recently the diet polyphenol contents have been negatively associated with an inflammation score

in a healthy cohort [323]. Wheat bran has several antioxidant and anti-inflammatory compounds, such as ferulic acid, apigenin, lignans and phytic acid [324].

Oxidative stress. Increased dietary antioxidants has been associated to reduction in the incidence of heart failure [325], stroke [326] and coronary artery disease [327]. Mediterranean diet is particularly rich in antioxidants (i.e., vitamins and polyohenols) and is able to protect against oxidative stress. Vitamins and polyphenols are mainly contained in fruits, vegetables, whole grain, nuts, EVOO and red wine. Among vitamins, betacarotene (vitamin A precursor), vitamins E, vitamin C, and mineral selenium are abundantly present. In the polyphenol group, there are a large number of secondary plant metabolites, for example flavonoid as quercetin, kaempferol, myricetin, apigenin, and luteolin, hydroxytyrosol and oleuropein. The hydroxyl groups, characterizing polyphenols, are responsible for their antioxidant and anti-inflammatory properties. Flavonoids are able to prevent ROS generation, and consequently DNA damage, chelating metal ions [328]. Hydroxytyrosol, an antioxidant phenol of EVOO, and resveratrol, a major antioxidant of red wine, activate the Nrf2 pathway, which, in turn, promotes the expression of antioxidants, e.g. NAD(P)H deidrogenase [329, 330]. Spermidine, contained in whole grains germs and some vegetables, is known to enhance the resistance to oxidative stress and reduce inflammation inhibiting histone acetyltransferases [331].

Two randomized trials assess the antioxidant effect of a dietary supplemented with EVOO, the Italian VOLOS [332] and the EUROLIVE [333]. The first one demonstrated that in dyslipidemic patients the plasma total antioxidant capacity increased after 7-week treatment. Interestingly, the EUROLIVE study, found a positive correlation between EVOO phenolic content (low, medium or high) and oxidative stress markers reduction. In metabolic syndrome individuals, it was demonstrated that a MD, compared to a LFD, is able to protect against DNA oxidative damage and lipid peroxidation [334]. Wang et al., in a meta-analysis on 14 observational cohorts, demonstrated that the increase intake of flavonoids decreased risk of CVD [335]. Similarly, in the PREDIMED study, the total dietary antioxidant capacity was evaluated and a non-significant reduction in CV mortality was found in subjects in highest quintile of total antioxidants consumption [336]. A prolonged MD adherence, with or without caloric restriction, in overweight men, was associated with reduced oxidative stress. The result is amplified when MD is complemented with calorie restriction and physical activity [337].

Gut microbiota. The studies on the possible modification induced by a MD to the microbiota are still incomplete, and only very few of them evaluated the impact on CAD context. A major MD characteristic is the high content of fiber. Thornburn et al., showed how a high dietary fiber intake promotes gut microbiota composition modifications with decreased *Firmicutes*, increased *Bacteroidetes* and higher levels of short-chain fatty acids [338]. In accordance, a study evaluating the relationship between microbiota composition and MD adherence score highlighted an increase of *Firmicutes*, *Baceroidetes* and short-chain fatty acids correlating to MD adherence [339]. In a very recent study, Holcher et al. evaluated the gut microbiota modification induced by diet enriched by walnut. Significant changes have been found in *Faecalibacterium* and *Bifidobacteria* families and were associated to LDL cholesterol reduction [340]. In obese population, a 2-years MD treatment produced a huge reshaping of gut microbiota compared to a LFD. Was observed an increase in *Bacteroides*, *Prevotella*, *Faecalibacterium*, *Roseburia* and *Ruminococcus*. Some of them are involved in the metabolism of carbohydrates to short-chain fatty acids, but not all the bacteria functions have been elucidated [341].

A potential mechanism underlying the association of MD, inflammation and CAD can be identified in the gut microbiota production of LPS. Fiber and fat, two important MD components, have differential effects on the gut microbiota. It was demonstrated that high-saturated fat diet may be involved in inflammation increasing gram-negative bacteria in the gut (LPS producers) [342] and LPS transport [343]. On the contrary, high-fiber diets decrease the proportion of gram-negative bacteria [344] and improve gut barrier function blocking both trans cellular and paracellular LPS translocation [345]. A recent prospective study evaluated the relation between consumption of a MD and LPS reporting that plasma LPS concentrations were negatively associated with the MD adherence in adults with atrial fibrillation [346].

Of specific concern is the microbial-derived compound TMAO, which has been linked to the development of atherosclerosis but few data on MD diet impact on TMAO have been reported. The intake of choline and carnitine, TMAO precursors present in red meat, eggs and cheese, is 50 % lower in a MD than in a Western diet. However, seafood, usually recommended in CVD prevention and present in MD diet, is rich in TMAO. In a recent study, TMAO levels modification was evaluated in healthy subjects adhering to a healthy diet (reduced in fat intake, saturated fat intake and increased in fiber intake) for 9 months: no changes have been found [347]. Likewise, no changes have been observed even after 2-week high-fat diet in fasting TMAO levels, but only in postprandial ones [348].

Complex lipid profile. If the impact of MD on cholesterol and TGs have been deeply evaluated, few data are available MD effects on non-classical lipids. Very recently, a targeted lipidomic analysis was performed on a PREDIMED study subgroup (983 participants). Plasma samples have been analyzed at baseline and 1-year after MD or LFD treatment. Several lipid families (PC, CE, triglyceride (TG), diacylglyceride, monoacylglyceride, phosphoethanolamine (PE)) and created lipid groups (lipids inversely or directly associated with CVD) have been assessed: unexpectedly, no changes induced by diets treatment have been observed [349]. However, a previously lipidomic study, performed on the same subgroup cohort, at the same time points, and evaluating 202 single lipids, reported that MD supplemented with nuts decreased levels of CE 20:3, compared to LFD group. They also revealed MD supplemented with EVOO induced greater increase of lipids with a longer mean acyl chain length than those with shorter acyl chain length, compared to controls [350]. Furthermore, MD can act ameliorating the HDL cholesterol functionality and this result have been associated to an increased expression of phospholipids in HDL surface, which lead to greater HDL fluidity [178].

The observations supported the interest to better understand the MD effects on lipids of CAD patients.

2.2.3. Mediterranean diet assessment

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 [351, 352]. Traditionally, they are used to describe habitual dietary intake, particularly in epidemiologic studies. However, in the last 10 years, they were also used to stratify patients according to their healthy dietary habit and sometimes to better characterize the risk to develop different pathology (e.g. cancer and CVD). FFQ are usually composed by 100-200 questions covering food and frequency consumption, portion size and beverage intake [353-356].

Diet indexes, instead, are a simple summary of the complexity of a diet. They attempt to evaluate the overall quality of a diet by the combination of nutrients and/or foods. Regarding Mediterranean diet adherence, several diet indexes, or scores, have been proposed. The differences among these scores are mainly determined by the selection of variables and the different cut-off points or algorithm developed. A recent meta-analysis found 28 different scores meant to reflect the adherence to MD [357]. Most of the scores (n=18) are based on positive and negative components of MD. Five are based on the "*MD pyramid*" structure [244] [358]. Usually,

higher score indicates good adherence, however, two scores used higher levels to indicate poor adherence [359, 360].

One of the most used, and the first one proposed at all, is the **Trichopoulou MD score**, created in 1995 and modified in 2003 [247, 361]. It includes nine food groups, beneficial components (vegetables, legumes, fruits and nuts, cereal, and fish), detrimental components (meat and dairy products), alcohol intake and lipid intake expressed as the ratio of MUFA to SFAs (SFA). Thus, the total Mediterranean diet score ranged from zero (minimal adherence to the traditional MD) to nine (maximal adherence). From this score, several other similar definitions have been proposed [250, 255, 306, 362-366].

Another very common MD adherence index is the Mediterranean Diet Adherence Screener (**MEDAS**) score, developed and validated in the PREDIMED study [367]. It is obtained by a quick and easy questionnaire bases on 14 questions (questions are reported in the Material and Methods section). The MEDAS score ranged from 0 (minimal adherence to the traditional MD) to 14 (maximal adherence). It specifically addressed normative or absolute cutoff points for the consumption of food items typical of the Mediterranean diet, such as nuts, legumes, and olive oil, and also inquires about the consumption of foods that do not fit the traditional Mediterranean diet, such as sugary soft drinks and pastries. Similarly, Abellan Aleman et al. proposed a scored based on 15 questions but with a score range of 0-10 [368].

Cavalieri developed a very recent and completely different MD score with 0-32 points range [369]. It has three indicators, the food consumption index, the fat and salt consumption index, and the drink consumption index, each one comprising several items (16, 2 and 4 questions, respectively). Instead, Sofi et al. created an algorithm based on literature in order to obtain a more comparable score [249]. Even scores quantifying the Mediterranean lifestyle (e.g. sociability, sleep, conviviality and water consumption) have been proposed [370].

2.3. New investigation approach: metabolomics

The biological mechanisms involved in the beneficial effects of the Mediterranean diet on CAD prevention are not yet fully understood. In the last two decades a new era of research, established through technological developments, including MS, have emerged. These advances in technology, as the metabolomics approach, could help in understanding how the MD acts on atherosclerotic disease.

2.3.1. Definition and overview

Metabolomics is the youngest in the "omics family" (genomics, transcriptomics and proteomics). Indeed, the term metabolome was coined only in 1998 by S.G. Oliver and his colleagues [371] indicating the whole set of small-molecules (usually < 1200 Da) found within a biological sample. The biological sample could be a cellular organelle, a cell, a tissue, an organ, an entire organism or different biological fluids (plasma, urine, serum, etc.). The small-molecules set includes endogenous metabolites physiologically produced (such as fatty acids, amines, amino acids, organic co-factors, acids, nucleic acids, sugars, vitamins, etc.) as well as exogenous chemicals introduced and not usually produced (such as environmental contaminants, food additives, drugs, toxins and other xenobiotics). The metabolome includes a large number of metabolites: in human serum approximately 4000 endogenous metabolites have been found [372] but this number can reach 40,000 metabolites when exogenous metabolites from drugs, food and the microbiota are included [373].

The study of the metabolome is defined **metabolomics**. Among the omics family it is considered the closest representation to the phenotype, indeed, metabolites can be regarded as the ultimate response of biological system to genetic, environmental or dietary changes. Nowadays, the interest in the metabolomics approach is growing exponentially and it can be appreciated by the number of publications in the area. In 1999 three publications with the keyword metabolomic could be found, in 2004 this number increase to 203 whilst in 2017 there were 3649 publications about metabolomics. Metabolomics approach is applicable to a wide range of areas such as human diseases [374], environmental system [375], microbial [376] and plant [377] sciences, nutrition field [378], drugs [379] and more.

In relation to the underlying biological question, two different metabolomics approaches exist: targeted or untargeted metabolomics. **Targeted metabolomics** is the measurement of defined

groups of annotated metabolites, usually related to one or more pathways (usually not more than 100-200 metabolites at time). The most relevant advantages of targeted metabolomics is absolute quantification, higher sensitivity and huge selectivity, because metabolite extraction, compound chromatographic separation, and instrumentation parameters can be optimized for the metabolites of interest. Quantification is usually obtained using labelled standard and through calibration curves with external standard. This approach is defined as hypothesis driven because a pre-defined set of metabolites is measured in a sample in order to answer a specific question. On the contrary, the **untargeted metabolomics** approach is defined as hypothesis generating because only at the end of the experiments a hypothesis can be proposed and deeper investigated with targeted approaches or others.

2.3.2. Untargeted metabolomics workflow

The untargeted metabolomics is a comprehensive analytic approach that aims to evaluate the whole metabolome of a sample or, due to technologies limitations, as many metabolites as possible, including chemical unknowns. In this technique, metabolites are compared using a relative quantification, typically involving peak area comparison of the analytes. In Figure 7 are reported the general steps involved in any untargeted metabolomics study, briefly explained in the next paragraphs.

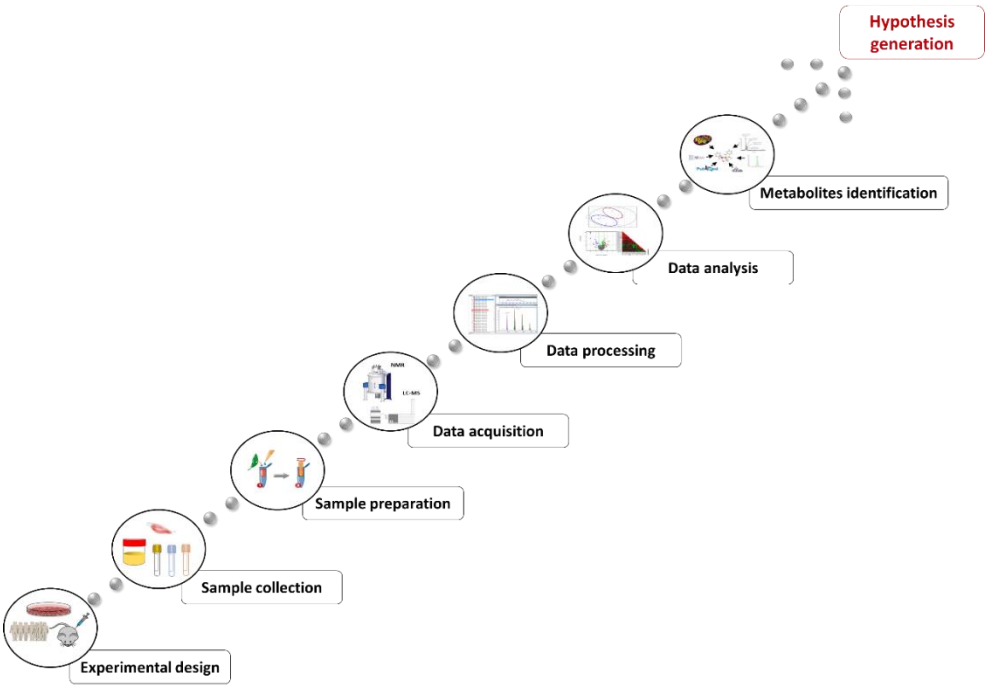


Fig. 7 – Schematic representation of metabolomics untargeted workflow, from experimental design to hypothesis generation.

2.3.2.1. Sample collection and preparation

The wide range of different metabolite classes with different chemical/physical properties makes sample collection and preparation a relevant step in metabolomics analysis. Many aspects may affect metabolites measurement. For example, metabolites with labile functional groups can quickly degrade, other metabolites can be sensible to pH, oxygen or light. Nevertheless, in an untargeted metabolomics approach is only possible to limit as much as possible these aspects using uniform preparation techniques and minimal preparation protocol to avoid procedural artefacts. Up to now, an extraction technique able to recover the whole metabolome was not described. As a result, the choice of extraction solvents and solution conditions will influence the spectrum of metabolites.

2.3.2.2. Data acquisition

The most common platforms for metabolomic profiling are nuclear magnetic resonance (NMR) and MS instruments with different benefits and challenges. Nevertheless, the number of mass spectrometers installed worldwide is higher than NMR instruments, probably due to their lower cost [380].

NMR manipulates the magnetic properties of atomic nuclei exciting nuclear spin through rapid changes in an external magnetic field and then recording electromagnetic radiation released as a result of nuclei relaxation, which is compound specific. The main advantages of NMR include highly reproducible quantitation, even across different instruments, simple sample preparation, detailed structural information and being non-destructive. However, sensitivity is poor making it non-suitable for low-abundance metabolites (µmol/L by NMR *vs* pmol/L by MS) and the metabolite coverage is lower than MS (hundreds of metabolites in NMR *vs* thousands in MS).

MS is based on the ionization of analytes followed by measurement of the intensity of the ions produced, which is recorded according to mass-to-charge ratios (*m/z*). MS is more sensitive than NMR and can measure several hundreds of metabolites with different properties and concentrations at the same time in minimal sample size, but is a destructive technique. This technique can be used alone, with direct injection of the sample into the mass spectrometer, or can be coupled to a separation technique, usually gas (GC) or liquid chromatography (LC). The use of a chromatographic separation increases selectivity and decreases compound coelution avoiding ineffective ionization. Among them, **LC** is preferred in metabolomics approach because it does not require derivatization and allow a huger metabolite coverage. Mass spectrometers are essentially

composed of ionizer and mass analyser. Different MS ionization technique are available (electrospray ionization, atmospheric pressure chemical ionization, atmospheric pressure photoionization, electron impact ionization or matrix assisted laser desorption ionization) and electrospray ionization (ESI) is one of the most applied in metabolomics as it offers the greatest extent of global metabolome coverage and improved limit of detection (LOD) of several compounds. Mass analyser can be the Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), the Orbitrap or the time of flight (TOF), all characterized by high mass accuracy, a very important feature to reduce the list of candidate molecule identities of potential metabolites. Even if FT-ICR-MS and Orbitrap are currently able to achieve high resolution and mass accuracy in the 1 ppm range they are less frequently used for untargeted metabolomics analysis than TOF, due to their higher cost and lower scanning speed.

2.3.2.3. Data processing and data analysis

Beyond sample preparation and data generation, appropriate processing of metabolomics data is essential to produce high-quality data sets that will ultimately be used for statistical analyses. Raw data are pre-processed using software from instrument companies (e.g. Mass Profiler by Agilent, SIEVE by Thermo Fisher or Marker view by Sciex) or from open sources (e.g. XCMS, MathDAMP, Metalign or MZmine). The first step is data extraction: the identification and integration of features (expressed as chromatographic peaks) with a specific mass and retention time (RT) using extraction algorithms. Often, this step is not perfectly performed by software and thus requires a manual inspection of the data. The number of features detected is not a direct measure of metabolites present in a sample. Depending on the data processing, peak list can include adducts, isomers or fragments leading to several features detected *per* metabolite. Adduct-finding scripts and de-isotope procedure are usually performed to avoid multiple features for one metabolite. Then, all features common between different samples are aligned by the software as shifts in RT may arise from degradation of the column, change in mobile phase pH and sample carry over. As result, each feature has the same *m/z* and RT in each sample. Then, scaling and normalization procedure can be applied. Scaling is performed in each sample in order to put all variables (features) on a comparable scale. There are many different scaling methods, but auto-scaling and Pareto-scaling are commonly used in metabolomics. Normalization is performed on single feature signal intensity to minimize unwanted variations from environmental or instrument factors, especially in large number of samples analysis. Different strategies have been proposed and,

among them, quality control strategy is one of the most used [381]. Quality control (QC) is a sample, prepared mixing small and equal amount of all samples, that should be analized repeatedly throughout an individual batch and across different batches. The signal variation of each feature in QCs during the run allow correcting the signal of the same feature in each sample in relationship to the order of injection. Third additional step could be a quality assurance (QA) protocol to remove features with poor repeatability across QCs analysis. For example, features detected in less than 80% of QCs and those with a relative standard deviation (SD) more than 30% can be removed. Finally, missing values imputation could be performed by different approaches as introducing zero, the mean or the median of other samples, k-nearest neighbours and random forest (RF) [382].

After data processing, multivariate data analysis is usually applied to highlight metabolites that change most significantly in order to define a list of compounds to identify. A traditional statistical analysis is not suitable to big data set like those produced through the untargeted metabolomics approach as the chance of false association increases with so many metabolites. For this reason, the statistical analysis is adjusted for multiple comparisons across the number of metabolites studied with Bonferroni or with false discovery rate thresholds. Other commonly used analytical approaches are principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), orthogonal partial least squares discriminant analysis (OPLS-DA) and hierarchical cluster analysis.

2.3.2.4. Metabolites identification

Probably, the major challenge of untargeted metabolomics is the identification of compounds. In this sense, NMR have a significant advantage compared to MS as it can provide structural and functional group information. Identification using MS is based on the accurate mass, RT (indicating hydro-lipophilic properties), tandem mass (MS/MS) spectra and isotope distribution. Spectra and RT libraries of known metabolites are the resources to identify metabolites. Libraries could be in-house or free databases such as Human Metabolome Database (HMDB), composed by more than 100,000 metabolites, Lipidmaps, specialized in lipids, the MetaboLights database, developed by the European Bioinformatics Institute, METLIN, Mass bank and many others. In addition, are emerging new tools for *in silico* mass spectra generation using quantum chemistry coupled to machine learning methods [383]. The Metabolomics Standard Initiative defined four levels of identification [384]: identified metabolites (level 1), putatively annotated compounds (level 2),

putatively characterised compound classes (level 3), and unknown compounds (level 4). Level 1 requires the comparison of experimental metabolite to an authentic chemical standard. By contrast, level 2 and 3 annotation does not require matching to data for authentic chemical standards but is enough the comparison to spectra databases.

2.3.3. Metabolomics and coronary artery disease

Several cardiovascular diseases involve disturbances in cardiac metabolism. Moreover, disorders in metabolism that occur in other diseases, such as diabetes mellitus, can directly affect cardiac metabolism. On the other hand, heart disease *per se* can disturb systemic metabolism [385], creating a vicious cycle. Targeted metabolomics was usually applied to investigate specific pathways involved in CAD but it could be a reductionist approach compared to untargeted metabolomics that allow a more unbiased and comprehensive view of the molecular changes. Therefore, the untargeted metabolomics have been used to investigate deeply inside coronary artery disease. Different metabolites have been correlated to CAD using the untargeted approach, for example an NMR serum profiling allow to distinguish, with a specificity of >90%, the presence and the severity of CAD from subjects with angiographically normal coronary arteries using OPLS-DA [386]. A LC-MS application on the TwinGene cohort, pointed out 4 unsaturated lipids (LPC 18:1, LPC 18:2, monoglyceride 18:2 and sphingomyelin (SM) 28:1) that are associated to CVD independently from traditional CVD risk factors [181]. A very recent LC-MS untargeted plasma metabolomics analysis performed on subjects without evidence of ACS, who underwent elective diagnostic coronary angiography, identifies trimethyllysine, a nutrient TMAO-producing precursor, as predictor of incident cardiovascular disease risk [387]. Furthermore, metabolomics profiling data have been associated not only to global CVD outcomes, but even to specific CVD subtypes, such as myocardial ischemia and infarction [388], congestive heart failure [389] and stroke [390]. In addition, it was successfully used to discriminate among different CAD groups such as nonobstructive coronary atherosclerosis, SA, UA and acute MI through the profiling of circulating metabolites [391].

Untargeted metabolomics applications allowed to evidence alterations of tricarboxylic acid cycle and glucose utilization in CAD patients. Plasma profile of individuals subjected to exercise stress demonstrated that patients exhibiting myocardial ischemia had reductions in 6 metabolites of tricarboxylic acid cycle, consistent with the reductions in oxidative metabolism induced by myocardial ischemia [392]. A serum profiling study on patients undergoing transitory acute

myocardial ischemia revealed that the event induces increase of circulating glucose, lactate, glutamine, glycine, glycerol, phenylalanine, tyrosine, and PE and decrease of choline-containing compounds and triacylglycerols [393].

Regarding studies on atherosclerosis, untargeted metabolomics was used by Vorkas et al. to investigate the metabolic phenotyping of atherosclerotic tissue compared to intimal thickening tissue, considered immediate preplaque stage. They observed alteration in β-oxidation process in atherosclerotic plaque tissue and proposed phosphatidylethanolamine-ceramide as novel biomarker of atherogenesis, [394]. All these studies confirmed the potential use of untargeted metabolomics in monitoring CAD.

2.3.4. Metabolomics and Mediterranean diet

Diet can act in two different ways: modifying the endogenous metabolome and introducing exogenous metabolites. Thus, an untargeted metabolomic approach can provide a comprehensive picture of the overall dietary intake effects. Nevertheless, there are few studies of untargeted metabolomics in the diet field and, specifically, in the Mediterranean diet field. Metabolomics approach was used to identify new biomarkers of nutritional exposure or to investigate biological mechanisms of diet. It can be applied to evaluate nutrients or single food intake and dietary patterns, usually in plasma, serum o urine samples. The most extensively studied food groups include meat, fish, fruits, vegetables, coffee, tea, wine, bread, whole-grain cereals, cocoa, chocolate and nuts [395]. Regarding dietary interventional trials, Vazquez-Fresno et al. evaluated, using a NMR untargeted metabolomics approach on urine samples, in a nondiabetic subcohort of the PREDIMED study, the modification induced by 1- and 3-year MD treatment period compared to a LFD [396]. They observed higher impact of MD after 3 years of follow-up, specifically, changes in carbohydrate (3-hydroxybutyrate, citrate and cisaconitate) and lipid metabolism (oleic and suberic acids), amino acids (proline, glycine and leucine) and microbial cometabolites (*p*-cresol and phenylacetylglutamine). The LFD was instead associated to hippurate, TMAO, and metabolites related to histidine and xanthosine metabolism. Recently, in another PREDIMED subpopulation, applying NMR untargeted metabolomics approach on urine samples, microbial metabolites discriminating high or low MD adherence have been determined [397].

Another study, using LC-MS, evaluated the impact of MD treatment in patients with metabolic syndrome compared to a control diet based on AHA guidelines [398]. Plasma metabolomics evidenced changes in phospholipids and lysophospholipids after 2 month of MD diet. These

differences disappeared after 6 month of MD. Recently, an untargeted NMR metabolomic approach was used to evidence the impact of a weight loss lifestyle (including Mediterranean diet) on healthy obese women [399]. After 3 months of intervention, plasma profiling evidenced modification in energy, amino acid, lipoprotein and microbial metabolism: higher levels of 3-hydroxybutyrate, formate, methylguanidine, myoinositol and phosphocreatine, and lower levels of proline and TMA. Up to now, no studies evaluating the effect of MD on CAD patients using an untargeted metabolomics approach have been published.

3. Objective

Since the first results from the Seven Countries Study, several studies, carried out in different populations, have established the beneficial role of Mediterranean diet in terms of morbidity and mortality. Particularly attention has been focus on MD cardioprotective effects due to very promising epidemiological studies. However, epidemiological studies are by nature observational rather than experimental and observed associations do not imply a cause-effect relationship.

At first, the relationship between MD and its cardioprotective effect was almost entirely connected to its lowering plasma TC capabilities. More recently, was recognized that the effect of MD can be also mediated by the action on other lipids fractions, such as HDL, LDL and TGs, and on other CVD risk factors, such as blood pressure, insulin resistance, weight and even smoke. Moreover, further factors, processes and pathways have been proposed to be involved in MD outcome, such as thrombotic tendency, oxidative stress, homocysteine pathway, inflammation, endothelial dysfunction, and last in chronological order, gut microbiota. These factors can be, probably, interrelated or overlapping or synergistic, so the MD effect can not be defined on the basis of one factor alone, but a more comprehensive view is necessary.

Concerning coronary artery disease, some interventional trials have been performed to evaluate MD effects in primary prevention setting, but the role of MD for secondary coronary artery disease prevention remains to be deeply explored.

The primary aim of this study was to investigate the effect of a Mediterranean diet in coronary artery disease patients in a comprehensive way. Thus, the aim includes to assess the modification induced not only on classical CAD risk factors (such as TC, LDL and HDL cholesterol, TGs, blood pressure, weight, glucose levels) but even on inflammation, oxidative stress status, microbiota and alternative lipids classes. In order to better understand the MD benefits, two different data analysis strategies have been proposed: "*intention to treat*", defined as the analysis of the whole population, and "*per protocol*", defined as the analysis of a subgroup population selected afterwards on most MD adhering patients. Secondary aims were to define the correlation between level of MD adherence and the induced modifications. In addition, this study aims to evidence the molecular mechanisms involved in MD cardioprotection going beyond already defined factors, by using an untargeted metabolomics approach.

This study could be of particular relevance because, by increasing knowledge on mechanisms of MD effects, it can offer enormous chances to improve secondary prevention by MD in addition to drug therapy.

4. Materials and Methods

4.1. Study design

4.1.1. Patients and recruitment

The Randomized Interventional Study on Mediterranean Diet (RISMeD) is a parallel-group, randomized, open-label, interventional trial that aims to assess the effects of the MD, on CAD patients, compared to a classic LFD. From July 2015 to May 2018, 130 CAD patients have been enrolled at Centro Cardiologico Monzino IRCCS, Milano (CCM). Eligible patients were males and females, age 30-75, with a recent history of coronary revascularization (ACS, angina pectoris, coronary artery bypass grafting or percutaneous coronary intervention). Exclusion criteria were patients with diabetes mellitus, food intolerance, BMI < 19 or > 33, or those who assume drugs or food supplements with omega-3 fatty acids or natural or synthetic antioxidants.

After a clinical stabilization period, at least 60 days after any coronary procedure or event, patients were randomized, by a computerized randomization list, into two groups: MD group or LFD group. According to the assigned group, patients have adhered to MD or LFD diet for three months. Patients underwent a medical visit, venous blood drawing, urine collection and they filled out a validated FFQ at randomization (T0) and at the end of the diet treatment (T3).

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.

4.1.2. Diet interventions

In both study groups, registered nutritionists managed the dietary interventions by giving personalized dietary advices. Patients who were allocated to the LFD were instructed to follow the standard diet recommendations provided to cardiac patients. They were advised to reduce intake of saturated fats and sugar rich foods. While the participants who were allocated to the LFD did not receive further instructions, those assigned to the MD group had access to a more controlled intervention. MD was personalized in term of total calories, total lipids, saturated/unsaturated fatty acids balance and was characterized by: fish at least 3 times a week; legumes 2-3 times a week; raw or cooked vegetables (preferably antioxidant-rich), twice a day; fresh fruits twice a day; 30 to 45 g olive oil a day; not more than 150 g red meat a week. For participants choosing to consume alcohol, red wine was suggested to be consumed in moderation (men 1–2 and woman 1 glass a day). Cold cuts, sweets, cakes, butter, fatty cheese were discouraged. All patients were

monitored monthly with a reinforcement of the dietary recommendations, to ensure the maintenance of adequate adherence to the assigned diet. The composition of these diets is shown in Table 2.

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	0.600
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

Tab. 2 – 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.

4.1.3. Dietary assessment

Dietary assessment was performed at enrolment by administering EPIC FFQ [247], validated for Italian population. The EPIC FFQ consists of 248 questions concerning foods and beverages commonly consumed 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. Furthermore, to assess the degree of adherence to the traditional Mediterranean diet, at T0 and T3, a 14-item questionnaire was administered. This tool, developed and validated by the Spanish PREDIMED study [367], allows to create the MEDAS score, a score ranged from 0 (no MD adherence) to 14 (complete MD adherence). The PREDIMED questionnaire is reported in Table 3.

	Food and frequency of consumption	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 tablespoons
3	How many vegetable servings do you consume *per* day? (1 serving 200 g [consider side dishes as half a serving])	≥2 (≥1 portion raw or as salad)
4	How many fruit units (including natural fruit juices) do you consume *per* day?	≥3
5	How many servings of red meat, hamburger, or meat products (ham, sausage, etc.) do you consume *per* day? (1 serving 100–150 g)	<1
6	How many servings of butter, margarine, or cream do you consume *per* day? (1 serving 12 g)	<1
7	How many sweet or carbonated beverages do you drink *per* day?	<1
8	How much wine do you drink *per* week?	≥3 glasses
9	How many servings of legumes do you consume *per* week? (1 serving 150 g)	≥3
10	How many servings 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 servings of nuts (including peanuts) do you consume *per* week? (1 serving 30 g)	≥1
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

Tab. 3 – 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.

4.1.4. Population subgroups

This diet trial could have some limitations. Even if participants were randomized in the two groups, MD and LFD, it is not possible to complete discriminate a Mediterranean diet *vs* a general Italian low fat diet as different nutritional aspects are shared. Indeed, a significant increase in the score of adherence to Mediterranean diet in the LFD group was observed after three months of diet (data showed in the Results section). Perhaps, an untreated control group would have been better to highlight the effects of a Mediterranean diet, but a large body of clinical evidences and professional organization guidelines pointed to the importance of heart-healthy diets for high-risk patients [268, 313, 400-402]. Consequently, in order to better point out the specific effects of a Mediterranean diet on CAD patients, a subgroup analysis was designed. At the end of the study, 19 CAD patients from each group, MD and LFD group, were selected, so that:

a. Demographic and clinical characteristics at baseline were comparable among the two subgroups;
b. Mediterranean diet adherence at baseline, evaluated through the MEDAS score, was comparable and not more than 9 (9 points is considered acceptable MD adherence [403]);
c. Mediterranean diet adherence in the control group (LFD subgroup), after three months of dietary intervention, evaluated through the MEDAS score, was unchanged;

Mediterranean diet adherence in the MD group (MD subgroup), after three months of dietary intervention, evaluated through the MEDAS score, was significantly increased.

4.2. Clinical measurements

Medical conditions related to eligibility were collected in the screening process using hospital records and by questionnaire at a pre-baseline appointment. Sociodemographic and clinical characteristics, type, dose and frequency of medication and supplement use were collected at baseline and checked at T3.

Weight and height were measured, after an 8 hours fast, using international standards for anthropometric assessment. Body weight was measured to the nearest 0.1 kg using calibrated digital scales and height was measured to the nearest 0.1 cm, while barefoot using a wall-mounted stadiometer. BMI was calculated as weight (kg)/height (m^2) and obesity was classified as BMI >30 kg/m^2. Waist circumference was measured midway between the lowest rib and the iliac crest by using an anthropometric tape and blood pressure with a validated semiautomatic oscillometer. Patients were considered hypertensive if they were treated with antihypertensive drugs or if blood pressure measurements were higher than 90/140 mmHg.

4.3. Laboratory measurements

To evaluate classic lipid metabolism, TC, LDL, HDL, TAG were determined in plasma samples. TC, HDL and TAG were measured in CCM clinical laboratory by a colorimetric enzymatic method. LDL concentrations were determined by Friedewald formula [HDL= TC - (HDL + (TAG/5))]. Patients were considered dyslipidemic if they were treated with lipid lowering drugs or if TC > 200 mg/dL and/or LDL > 115 mg/dL and/or TAG > 180 mg/dL.

Fasting glycaemia was measured on plasma samples by enzymatic glucose– oxidase method and urinary creatinine was measured by the Jaffe's reaction. Patients were considered hyperglycemic if glycaemia was more than 100 mg/dL.

4.4. Targeted analysis

4.4.1. Methods development and validation

For the evaluation of DNA oxidative damage and the gut microbiota metabolism, two different LC-MS methods have been developed and validated: measurement of 8-OHdG and TMAO pathway, respectively.

The method was validated according to U.S. Food and Drug Administration guidelines for validation of bioanalytical methods [404]. Briefly:

- Imprecision

  Due to the lack of a "blank" matrix samples, ten human samples (urine for 8-OHdG and plasma for TMAO pathway) were pooled together (pooled sample). For assessment of assay imprecision, QC samples were prepared at three different nominal concentrations using pooled sample: low QC concentration was made by diluting pooled sample 1:4 v/v (with water for 8-OHdG and with physiological saline solution for TMAO pathway), medium QC concentration consisted of the pooled sample while high QC concentration was prepared by fortifying the endogenous pooled sample metabolites with known amount of standard solution (spiked concentrations 25 ng/mL). The intra-assay imprecision was determined by assaying 9 separate aliquots of each QC sample in a single batch. Inter-assay imprecision was determined by testing a single aliquot of each QC in 5 consecutive days.

- Linearity

  The linearity and range of the calibration curve were evaluated with ten standard calibrators over the concentration range 0.1-100 ng/mL for 8-OHdG and 0.15-125 ng/mL for TMAO pathway. Each calibrator was spiked with internal standard. Linearity of the assay was assessed by repeated analysis (n=5) of calibrators and linear regression analysis was used to determine the slope, intercept, and correlation coefficient (r^2).

- Lower limit of quantification

  The lower limit of quantification (LLOQ) was calculated as the lowest concentration providing a coefficient of variation (CV) <20% and an accuracy between 80% and 120%.

- Limit of detection

  The LOD was defined as the lowest concentration that gave a signal-to-noise of at least 3.

- Stability

  The stability of the analytes at different temperatures was tested by analysing pooled sample aliquots kept at -80°C, +4°C and room temperature for 24h, 72h and 6 months. For the freeze/thaw stability study, two aliquots of pooled samples were used. The first aliquot was immediately analysed for the quantification while the second one was frozen at -80°C and assayed after being freeze/thawed three times in three consecutive days.

The absolute matrix effect (ME) was investigated by comparing the slope of the standard calibration curve with the slope of matrix-matched standard curve [405]. For this purpose, the calibration curves were prepared in methanol and in pooled sample.

Relative ME, extraction recovery (RE), and process efficiency (PEf) were evaluated for 8-OHdG measurement method according to Matuszewski et al [406]. All these parameters were assessed at three different concentrations of 8-OHdG (0.5, 5 and 50 ng/mL added) and the analysis was repeated 5 different times. Three sets of each concentration levels were prepared as follows: neat 8-OHdG standard solution (set A); pooled sample spiked with 8-OHdG standard after centrifugal filtration (set B); pooled sample spiked with 8-OHdG standard before centrifugal filtration (set C). The values of set A, B and C are expressed, in arbitrary units (AU), as mean of area ratio. For set B and C the spiked area ratio of 8-OHdG was calculated by subtracting the basal endogenous 8-OHdG value (unspiked) from the measured area ratio. The rates of ME, RE and the overall PEf were determined at each concentration tested as follows: ME (%) = peak area ratio from set B/peak area ratio from set A × 100; RE (%) = peak area ratio from set C/peak area ratio from set B × 100; PEf (%) = peak area ratio from set C/peak area ratio from set A × 100.

4.4.2. Inflammatory markers measurement

High sensitive-C reactive protein (hs-CRP) was determined on serum samples in CCM clinical laboratory by immunoturbidimetry.

Leukocytes and basophils counts was performed on EDTA-anticoagulated blood samples using an automated hematology system Sysmex XE 2100 (Sysmex, Kobe, Japan).

4.4.3. Antioxidants and oxidative stress evaluation

4.4.3.1. α -tocopherol and ɣ-tocopherol measurement

α-tocopherol and ɣ-tocopherol were measured on plasma samples by a fluorimetric method developed and validated in our group [407]. Briefly, 100 µL of plasma were precipitated with 50% ethanol and analytes were extracted with 1 mL of n-hexane. The organic extract was evaporated to dryness under nitrogen flow and the residue was redissolved in methanol (2.5 mL). An aliquot (25 µL) was chromatographically separated by a XBridge C18 column (4.6x150 mm, 5 µm, Waters) and methanol (100%) at a flow rate of 1 mL/min for a total run time of 10 minutes. The analysis was performed on an high performance-LC (HPLC) Accela (Thermo Fisher Scientific) system coupled to the Dionex Ultimate 3000 fluorimeter (Thermo Fisher Scientific) (λecc 292 nm, λem 335 nm). Data acquisition and analysis were performed with Xcalibur®software, version 2.0 (Thermo Fisher Scientific). The analytes values were expressed as concentration (µg/mL) or as the ratio between analyte concentration and TC concentration.

4.4.3.2. Glutathione measurement

The determination of GSH and GSSG was performed using a LC-MS/MS method previously developed and validated in our group [408]. Briefly, EDTA-anticoagulated blood samples were diluted with 10% trichloroacetic acid containing 1 mM EDTA solution in a 1:1 (v/v) ratio and stored at -80°C until analysis . After centrifugation at 12000*xg* for 10 minutes at RT, the supernatant was diluted 1:200 with 0.1% formic acid and analyzed. Liquid chromatography was performed using an Accela HPLC pump system (Thermo Fisher Scientific). The separation of analytes was conducted on a Luna analytical PFP column (100x2.0 mm, 3 µm, Phenomenex) maintained at 35°C. The mobile phase was composed by two solvents: solvent A (ammonium formate 0.75 mM adjusted to pH 3.5 with formic acid) and solvent B (methanol). Separation was performed under isocratic conditions with 99% mobile phase A at flow rate of 0.2 mL/min for a total run time of 10 minutes per sample. Mass spectrometric analysis was performed using a TSQ Quantum Access (Thermo Fisher Scientific) triple quadrupole mass spectrometer coupled with ESI operating in positive mode. The selected reaction-monitoring (SRM) was performed by monitoring the transitions *m/z* 308.1 → *m/z* 76.2 + 84.2 + 161.9 (GSH) and *m/z* 613.2 → *m/z* 230.5 + 234.6 + 354.8 (GSSG). The operating MS conditions analysis were as follows: spray voltage, 2500 V; capillary temperature and voltage, 280 °C and 35 V, respectively; sheat gas and auxiliary gas flow, 30 and 5 AU, respectively; tube lens

offset, 84 V for GSH and 115 V for GSSG. The mass spectrometer was employed in MS/MS mode using argon as collision gas. Data acquisition and analysis were performed with Xcalibur®software, version 2.0 (Thermo Fisher Scientific).

4.4.3.3. 8-iso-prostaglandin $F_{2\alpha}$ measurement

8-iso-$PGF_{2\alpha}$ determination was performed modifying a previously developed and validated LC-MS/MS method [147]. Briefly, 2 mL of urine were spiked with 50 µL of the deuterated internal standards solution (8-iso-$PGF_{2\alpha}$-d_4) and purified using a solid phase extraction cartridge. The eluate was evaporated to dryness and reconstituted with 100 µL of acetonitrile:water (90:10 v/v). The chromatographic separation was performed using an Accela HPLC pump system (Thermo Fisher Scientific) with a XBridge® C18 column (100x2.1 mm, 3.5 µm, Waters) as stationary phase. The mobile phase was composed by two solvents: solvent A (water with 0.1% ammonium hydroxide) and solvent B (methanol:acetonitrile 50:50 v/v with 0.1% ammonium hydroxide). The following gradient was used: 0 min – 15%B, 14 min – 50%B, 16 min – 90%B, 22 min– 15%B and 40 min – 15%B. Mass spectrometric analysis was performed using a TSQ Quantum Access (Thermo Fisher Scientific) triple quadrupole mass spectrometer coupled with ESI operating in negative mode. The SRM was performed monitoring the transitions *m/z* 353.1 → *m/z* 192.8 (8-iso-$PGF_{2\alpha}$) and *m/z* 357.05 → *m/z* 197.1 (8-iso-$PGF_{2\alpha}$-d_4). The operating MS conditions analysis were the following: spray voltage, 2500 V; capillary temperature, and voltage, 270 °C and 30 V, respectively; sheath gas and auxiliary gas, 25 and 10 AU, respectively; tube lens offset, 103 V for 8-iso-$PGF_{2\alpha}$ and 100 V for 8-iso-$PGF_{2\alpha}$-d_4. The mass spectrometer was employed in MS/MS mode using argon as collision gas. Data acquisition and analysis were performed with Xcalibur®software, version 2.0 (Thermo Fisher Scientific). The estimated analytes values were corrected for the urinary creatinine levels, to control for variation in urinary output, and expressed as pg/mg of creatinine.

4.4.3.4. 8-hydroxy-2'-deoxyguanosine measurement

The determination of 8-OHdG has been performed by the LC-MS/MS method developed and validated in the first year of the current project and described in the Results section [134]. Briefly, 200 µL of urine were diluted with 200 µL of internal standard solution ($^{15}N_5$-8-OHdG) and filtered through a 30000 NMWL (Nominal Molecular Weight Limit) centrifugal filter at 10000*xg* for 30 minutes and analyzed. The chromatographic separation was performed using an Accela HPLC

pump system (Thermo Fisher Scientific) with a PFP Kinetex F5 analytical column (100x2.1 mm, 2.6 µm, Phenomenex), maintained at 30°C. The mobile phase was set at a flow rate of 0.25 mL/min using as eluents: solvent A (ammonium acetate 10 mmol/L) and solvent B (ammonium acetate 10 mmol/L in acetonitrile/water 50:50 v/v). Samples were eluted with the following gradient of mobile phase: 0 min – 5% B, 4 min – 50%B, 4.5 min – 100%B, 5.5 min 100%B, 6 min – 5%B and 14 min – 5%B. Mass spectrometric analysis was performed using a TSQ Quantum Access (Thermo Fisher Scientific) triple quadrupole mass spectrometer coupled with ESI operating in positive mode. The SRM was performed monitoring the transitions *m/z* 284.0 → *m/z* 168.1 (8-OHdG) and *m/z* 289.0 → *m/z* 173.0 ($^{15}N_5$-8-OHdG). The operating MS conditions analysis were the following: spray voltage, 2200 V; capillary temperature, and voltage, 280 °C and 35 V, respectively; sheath gas and auxiliary gas, 25 and 10 AU, respectively; tube lens offset, 76 V for 8-OHdG and 84 V for $^{15}N_5$-8-OHdG. The mass spectrometer was employed in MS/MS mode using argon as collision gas. Data acquisition and analysis were performed with Xcalibur®software, version 2.0 (Thermo Fisher Scientific).

8-OHdG values were corrected for the urinary creatinine levels, to control for variation in urinary output, and expressed as ng/mg of creatinine.

4.4.4. Gut microbiota: TMAO pathway measurement

The determination of gut microbiota metabolism variation was performed measuring carnitine, choline, betaine, TMA and TMAO plasma levels. An LC-MS/MS method developed and validated in the second year of the current project and described in the Results section. Briefly, 50 µL of plasma were diluted with 450 µL of methanol internal standard solution (carnitine-d_9, choline-d_9, betaine-d_{11}, TMA-d_9 and TMAO-d_9) and centrifuged at 12,000*xg* for 10 minutes. The supernatant is further diluted 1:10 with methanol. The chromatographic separation was performed using an ExionLC™ AC system (Sciex) with an Acquity BEH HILIC column (100x2.1 mm, 1.7 µm, Waters) maintained at 40°C. The mobile phase was set at a flow rate of 0.4 mL/min using as eluents: solvent A (ammonium formate 10 mmol/L, pH 3.5 with formic acid) and solvent B (acetonitrile). Samples were eluted with the following gradient of mobile phase: 0 min – 90% B, 4 min – 45%B, 4.5 min – 30%B, 5.5 min 30%B, 6 min – 90%B and 14 min – 90%B. Mass spectrometric analysis was performed using a 5500 QTrap linear ion trap quadrupole mass spectrometer (Sciex) outfitted with ESI source operating in positive mode. The SRM was performed monitoring the transitions *m/z* 162.0 → *m/z* 103.0 (carnitine), *m/z* 171.1 → *m/z* 69.1 (carnitine-d_9), *m/z* 104.0 → *m/z* 60.0

(choline), *m/z* 113.0 → *m/z* 69.0 (choline-d_9), *m/z* 118.0 → *m/z* 58.0 (betaine), *m/z* 129.1 → *m/z* 66.0 (betaine-d_{11}), *m/z* 60.3 → *m/z* 44.0 (TMA), *m/z* 69.0 → *m/z* 49.1 (TMA-d_9), *m/z* 76.1 → *m/z* 58.1 (TMAO) and *m/z* 85.1 → *m/z* 66.0 (TMAO-d_9). Operating MS conditions were the following: gas 1, nitrogen 50 psi; gas 2, nitrogen 60 psi; ion spray voltage, 2500 V; ion source temperature, 500°C; curtain gas, nitrogen 35 psi; collision gas, medium. The mass spectrometer was employed in MS/MS mode using nitrogen as collision gas. Data acquisition and analysis were performed with MultiQuant™ software (Sciex).

4.4.5. Complex lipid profile measurement

Plasma complex lipid profile was performed on MD subgroup (n=19) and LFD subgroup (n=19) by LC-MS analysis of plasma samples. Briefly, lipids in 100 µL of plasma were extracted with 1 mL of acetonitrile:methanol (50:50 v/v) and centrifuged at 12,000*xg* for 10 minutes at 4°C. The eluate was evaporated to dryness and reconstituted with 200 µL of water:methanol (50:50 v/v). Samples were analyzed two times: positive and negative ionization mode.

The chromatographic separation was performed using Agilent 1200 binary Pump (Agilent Technologies) with a Synergie Hydro RP column (Phenomenex) for positive mode or Luna CN column (Phenomenex) for negative mode. The mobile phase was 0.1% formic acid in methanol for positive analysis and 5 mM ammonium acetate pH 7 in methanol for negative. Total run time was 5 minutes for both analysis. Mass spectrometric analysis was performed using an API 4000 (Sciex) triple quadrupole mass spectrometer coupled with ESI with a 268 multiples reaction monitoring (MRM) transition in positive mode and 88 MRM transition in negative mode.

The operating condition for MS analysis were as follow: ion spray voltage , 4500 V; temperature , 500 °C; ion source gas 1 and ion sourcesGas 2 , 50 and 55 AU, respectively; curtain gas, 40 L/min; collision gas, 3 L/min gas. Data acquisition and analysis were performed with MultiQuant™ software (Sciex).

4.5. Untargeted analysis

Plasma untargeted analysis was performed on MD subgroup (n=19) and LFD subgroup (n=19).

4.5.1. Sample preparation

Plasma samples (100 µL) were diluted with 400 µL cold acetonitrile:methanol 50:50 v/v reference standards solution containing 11 labeled standards at a final concentration 2 µg/mL each (trimethylamine-N-oxide-d_9 (TMAO-d_9), L-4-tyrosine-$^{13}C_9$ (L-4-tyr-$^{13}C_9$), Methionine-^{13}C, 8-hydroxy-2-deoxyguanosine-$^{15}N_5$ (8-OHdG-$^{15}N_5$), 3-nitro-tyrosine-$^{13}C_9$ (3-nitro-tyr-$^{13}C_9$), Rivastigmine, acetylsalicylic acid-d_4 (ASA- d_4), Reserpine, 8-iso-prostaglandin $F_{2\alpha}$-d_4 (8-iso-$PGF_{2\alpha}$-d_4), 11-dehydro-thromboxane B_2-d_4 (11-DH-TXB_2-d_4) and 12-hydroxyeicosatetraenoic acid-d_8 (12-HETE-$_{d8}$)). Then, samples were centrifuged at 12,000*xg* for 20 minutes at 4° C. Supernatant was aliquoted in two vials for the LC-quadrupole time of flight (QTOF)-MS metabolic fingerprinting and the LC-QTOF-MS/MS analysis.

4.5.2. LC-QTOF-MS sample analysis

LC-QTOF-MS analysis was performed by an ultra-HPLC system (1290 Infinity series, Agilent Technologies) coupled to a QTOF MS detector (Agilent 6550 iFunnel Q-TOF) outfitted with ESI source. Samples were analysed both in positive and in negative detection mode. Two µL was injected onto the Zorbax Eclipse Plus C18 reverse phase column (2.1 x 150 mm, 1.8 µm, Agilent Technologies), maintained at 60°C, using a constant flow rate of 0.5 mL/min. For both positive and negative ion mode, the mobile phases were: water:acetonitrile (95:5 v/v) with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Samples were eluted with the following gradient of mobile phase: 0 min 10%B, 1 min – 10%B, 8 min – 80%B, 11.5 min 100%B, 12.5 min – 100%B, 13.5 min 10%B and 23.5 min – 10%B. The detector operated in full scan mode, acquiring mass spectra over the *m/z* range 40-1100 Da with a scan rate of 2 scan per second. Source parameters for both ion modes were: drying gas temperature 250°C, drying gas flow 12 l/min, nebulizer pressure 45 psig, sheath gas temperature 370 °C, sheath gas flow 11 l/min, nozzle voltage 1000 V and fragmentor 150 V. Capillary voltage was set at 3000 V in positive ionization mode and 4000 V in negative one. The references masses, 121.0509 *m/z*, 922.0098 *m/z* (ESI+) and 112.9856 *m/z*, 1033.9881 *m/z* (ESI-), were continuously infused to correct instrument variability. Data were acquired by MassHunter Workstation Data Acquisition software (Agilent Technologies).

4.5.3. Performance evaluation

Performance evaluation was assessed using two different approaches:

- QC samples [409];
- reference standards solution spiked in plasma and QC samples [410].

QC samples were obtained by pooling together equal volumes of all plasma samples (MD T0 + MD T3 + LFD T0 + LFD T3) and spiked with the reference standards solution. Subsequently, QCs were injected at the beginning of the analytical sequence (n=9), every six injections and at the end of the analysis.

4.5.4. Data processing

Raw acquired data were analyzed by MassHunter Profinder software (Agilent Technologies) using the "Batch Recursive Feature Extraction" algorithm that provides a list of features, which represent possible metabolites, combining different information from co-eluting ions such as charge-state, isotopic distribution, presence of adducts and/or dimers. The most relevant parameters selected for the features extraction were: 1000 counts for peak filter (to clean background noise), charge state limited at 2, allowed ion species +H, +Na, +K in positive ion mode and –H, +Cl, +CH_3COO, +HCOO in negative ion mode, and neutral loss of water for both ion modes. Through the same software was performed the peak alignment using a RT window of 0% + 0.15 min and a mass window of 15 ppm + 2 mDa. To improve data quality, a manual feature evaluation procedure was performed: features present in the blank (water) or with a RT lower than 0.6 min or subjected to carry over or reference standards solution's peaks, were excluded. Features with more than 20% missing values within the same group (MD T0, MD T3, LFD T0 or LFD T3) were removed. Missing values imputation was performed using RF algorithm [382] and the dataset were normalized using the support vector regression algorithm [411] which considers the intensity of the QCs throughout the entire analysis. Furthermore a filtering quality assurance procedure was applied to deem as reliable features those present in at least 90% of the QCs with a CV < 20% [381]. The robust lists finally obtained, for positive and negative mode, were defined as compound lists.

4.5.5. Compound identification

The metabolite identification, performed only for statistically significant compounds, was based on their measured accurate *m/z* values (10 ppm mass error window) and on the comparison of their acquired LC-QTOF-MS/MS spectra with those available on different databases such as Metlin (http://metlin.scripps.edu), Kyoto Encyclopedia of Genes and Genomes (http://www.kegg.jp/kegg), HMDB (http://www.hmdb.ca) and Personal Compound Database and Library (Agilent Technologies). The fragment elucidation, performed by MassHunter Molecular Structure Correlator (Agilent Technologies), the agreement between RT and compounds' polarity, and the biological significance also contributed to define putative matches. Annotation or identification was determined following official classification defined by Metabolomics Standard Initiative [384].

4.5.5.1. LC-QTOF-MS/MS sample analysis

The LC-QTOF-MS/MS experiments were performed only for statistically significant compounds, using the same chromatographic separation and ionization conditions previous described. Compounds were targeted using *m/z* value (isolation width 4 Da) and RT (ΔRT 0.9 min), and data were collected applying two fixed collision energy, 10 and 40 eV. Moreover, samples with the highest intensity of each compound were analyzed to optimize MS/MS spectra quality. Different runs were performed in order to avoid fragmentation of significant compounds co-eluting in the same analysis. Subsequently, spectra were processed through MassHunter Qualitative software (Agilent Technologies).

4.6. Statistical analysis

Participants who did not complete the intervention (n=10) were excluded from all analyses. For the present study, a final sample size of 60 subjects *per* group (accounting for 20% drop-out) allowed a statistical power of 80% to deem as significant (alpha= 0.05) a between-group difference in any analyte approximately equal to one SD.

The distribution of continuous variables was assessed by visual inspection of frequency histograms and with the use of the Shapiro–Wilk test. If necessary data were log transformed.

Numerical variables were summarized as mean and SD, unless otherwise stated, and categorical variables were summarized as frequencies and percentages. Continuous variables were compared with paired or unpaired t-test, as appropriate, whereas categorical variables were compared using the Chi square test or Fisher's exact test, as appropriate. No adjustment was deemed necessary as no confounding parameters were found between groups.

Two different data analysis strategies have been applied: "***intention to treat***", defined as the analysis of the whole population (n=120), and "***per protocol***", defined as the analysis of the subgroup population (n=38).

For complex lipid profile analysis paired or unpaired t-test, as appropriate, was used and Storey correction for multiple comparison was applied.

For untargeted metabolomics analysis, the compound lists obtained from positive and negative modes were treated independently for statistical analysis. Compounds were compared using paired or unpaired t-test, as appropriate, and Storey correction for multiple comparison was applied. Unsupervised PCA, PLS-DA and heatmap were obtained using Mass Profiler Professional software (Agilent Technologies) and MetaboAnalyst (http://www.metaboanalyst.ca).

Correlations between variables were performed using the Pearson test. A p value <0.05 was considered to indicate statistical significance.

All calculations were computed by SAS software package (Version 9.2; SAS Institute Inc., Cary, NC).

5. Results

Part I

Methodological results

5.1. DNA oxidative damage evaluation: 8-hydroxy-2'-deoxyguanosine

5.1.1. Sample preparation and analytical method conditions

An LC-MS/MS method was set up and validated [134] to measure urinary 8-OHdG.

Several experiments were performed, for example different purification methods and different sample dilutions, to define the best urine sample preparation protocol for the quantification of 8-OHdG (data not shown). The final developed protocol, here reported, is simple and fast. Frozen urine sample is thawed at room temperature, heated at 37°C for 10 minutes to re-dissolve possible analyte precipitates [412] and centrifuged at 1,700*xg* for 10 minutes. Subsequently, 200 µL aliquot is diluted with 200 µL of internal standard $^{15}N_5$-8-OHdG solution (final concentration 5 ng/mL) and filtered through a 30,000 NMWL centrifugal filters at 10,000*xg* for 30 minutes. The filtrate is injected into the LC-MS/MS system.

An Accela HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a triple-quadrupole mass spectrometer TSQ Quantum Access (Thermo Fisher Scientific) outfitted with ESI source operating in positive mode was used. Different stationary and mobile phases have been evaluated in order to optimize the chromatographic separation of the analytes (data not shown). The optimized chromatographic separation conditions were:

- stationary phase was pentafluorophenyl Kinetex F5 100Å analytical column (100x2.1 mm, 2.6 µm, Phenomenex, Torrance, CA, USA) maintained at 30°C;
- gradient mobile phase was composed by ammonium acetate 10 mmol/L (solvent A) and ammonium acetate 10 mmol/L in acetonitrile/water 50:50 v/v (solvent B), at a flow rate of 0.25 mL/min for a total run time of 14 minutes (Table 4).

Time (min)	% Solvent A (NH_4COOCH_3 10 mM)	% Solvent B (CH_3CN 50% + NH_4COOCH_3 10 mM)	Analysis step
0.0	95	5	Separation
0.5	95	5	
4.0	50	50	
4.5	0	100	Washing
5.5	0	100	
6.0	95	5	Reconditioning
14.0	95	5	

Tab. 4 – Developed mobile phase gradient for the chromatographic separation of urinary 8-hydroxy-2'-deoxyguanosine.

The SRM is performed by monitoring the transitions *m/z* 284.0 → *m/z* 168.1 (8-OHdG) and *m/z* 289.0 → *m/z* 173.0 ($^{15}N_5$-8-OHdG). The operating optimized conditions for MS analysis were the following: spray voltage, 2200 V; capillary temperature, 280°C; sheath gas, 25 UA; auxiliary gas, 10 UA.

A representative chromatogram of 8-OHdG and of its internal standard $^{15}N_5$-8-OHdG in urine pool sample, resulting from the chromatographic conditions and the selected transitions, is shown in Figure 8. The peaks eluted at 3.33 min, in a region of the chromatogram without any interfering background peaks.

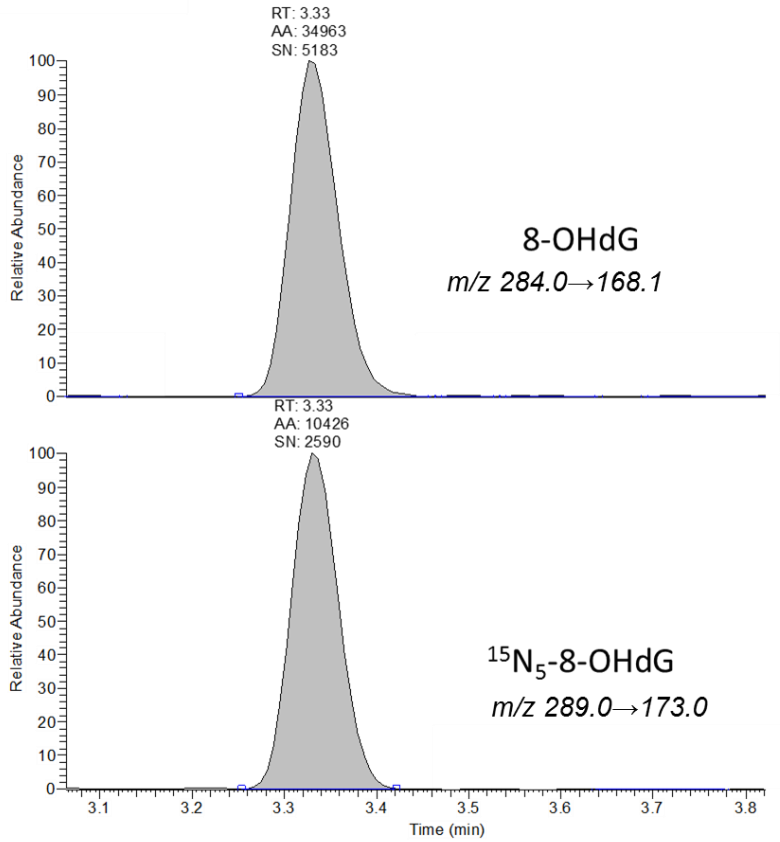


Fig. 8 – Representative chromatogram of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and of its internal standard $^{15}N_5$-8-OHdG obtained with the developed method. Relative abundance, expressed as percentage, is reported on the Y-axis and time, expressed as minutes, on X-axis.

5.1.2. Method validation

The 10-point calibrator concentrations, plotted against the ratio of analyte/internal standard areas for five consecutive assays, showed linear and reproducible curves with the following non-zero forced linear regression equations: y= (0.1609±0.006) x - (0.0143±0.035) (r^2=0.999). Over the entire concentration range of the curve, the mean observed percentage deviation of back-calculated concentrations was between -0.7% and +3.8% with an imprecision CV <15%. Intra-assay and inter-assay imprecisions were <10% for all QCs tested; the LLOQ was 0.25 ng/mL while LOD value was 0.1 ng/mL (Table 5).

QC level	Intra-assay imprecision (CV%)	Inter-assay imprecision (CV%)	LLOQ ng/mL	LOD ng/mL
QC Low	6.7	8.7		
QC Medium	6.4	9.1	0.25	0.10
QC High	7.6	8.0		

Tab. 5 – Imprecision, lower limit of quantification (LLOQ) and lower limit of detection (LOD) of the method. Imprecision, evaluated at three different concentration quality control (QC) levels, is expressed as coefficient of variation (CV).

Table 6 shows the relative ME, the RE and the overall PEf of the method. No relative ME was observed at the three concentrations evaluated. Recovery and PEf values complied with the acceptability requirements, indicating a good reliability of the developed method.

Concentration spiked (ng/mL)	Mean peak area ratio			ME (%)	RE (%)	PEf (%)
	Set A	Set B	Set C			
0.5	0.10	0.09	0.10	86.6	101.0	87.5
5.0	0.78	0.82	0.84	105.0	107.8	113.2
50.0	7.57	7.94	8.02	104.9	105.9	111.2

Tab. 6 – Matrix effect (ME), recovery (RE) and process efficiency (PEf) values for the validated method. The values of set A, B and C are expressed, in arbitrary units, as mean of area ratio (area ratio 8-OHdH/area ratio $^{15}N_5$-8-OHdG). ME, RE and PEf are expressed as percentage and defined in the material and method section.

The analyte was highly stable in urine at different temperatures of storage for at least 6 months and even throughout three freeze-thaw cycles (Table 7).

Temperature	8-OHdG (%)		
	24 h	3 days	6 months
-80°C	101.4	103.5	105.1
+4°C	101.9	98.2	-
RT	100.9	96.4	-
Freeze-thaw	97.7		

Tab. 7 – Stability of 8-hydroxy-2'-deoxyguanosine (8-OHdG) at different times (24 hours, 3 days and6 months) and at different temperatures (-80°C, +4°C and room temperature). The displayed values are percent related to sample analyzed immediately after the collection.

5.2. Gut microbiota evaluation: TMAO pathway

5.2.1. Sample preparation and analytical method conditions

An LC-MS/MS method was set up and validated to measure plasma levels of carnitine, choline, betaine, TMA and TMAO. Sample diluition was optimized in order to detect all the analites of interest as they have quite different concentration ranges in plasma samples (data not shown). The final developed protocol is here reported. Frozen plasma is thawed at room temperature and 50 µL aliquot is diluted 1:10 with a methanol labeled standard solution containing carnitine-d_9 (80 ng/mL final concentration), choline-d_9 (40 ng/mL final concentration), betaine-d_{11} (80 ng/mL final concentration), TMA-d_9 (20 ng/mL final concentration) and TMAO-d_9 (40 ng/mL final concentration) and and then centrifuged at 12,000*xg* for 10 minutes. The supernatant is further diluted 1:10 with methanol before being injected into the LC-MS/MS system.

An ExionLC™ AC system (Sciex, Darmstadt, Germany) coupled to a 5500 QTrap linear ion trap quadrupole mass spectrometer (Sciex) outfitted with ESI source operating in positive mode was used. Different stationary and mobile phases have been evaluated in order to optimize the chromatographic separation of the analytes (data not shown). The optimized chromatographic separation conditions were:

- stationary phase was an Acquity UPLC BEH HILIC analytical column (100x2.1 mm, 1.7 µm, Waters, Milford, MA, USA) with an Acquity HILIC VanGuard pre-column (5x2.1 mm, 1.7 µm, Waters) maintained at 40°C;
- gradient mobile phase was composed by ammonium formate 10 mmol/L, pH 3.5 with formic acid (solvent A) and a acetonitrile (solvent B), at flow rate of 0.40 mL/min for a total run time of 12 minutes (Table 8).

Time (min)	% Solvent A (NH_4COOH 10 mM pH 3.5)	% Solvent B (CH_3CN)	Analysis step
0.0	10	90	Separation
0.5	10	90	
4.0	55	45	
4.5	70	30	Washing
5.5	70	30	
6.0	10	90	Reconditioning
14.0	10	90	

Tab. 8 – Developed mobile phase gradient for the chromatographic separation of the analytes.

The operating conditions for MS analysis were the following: gas 1, nitrogen 50 psi; gas 2, nitrogen 60psi; ion spray voltage, 2500 V, ion source temperature, 500°C; curtain gas, nitrogen 35 psi; collision gas, medium. The SRM and the specific MS settings for each analyte are reported in Table 9.

	Q1 (*m/z*)	Q3 (*m/z*)	DP (V)	EP (V)	CE (eV)	CCEP (V)
Carnitine	162.0	103.0	80	10	24	8
Carnitine-d_9	171.1	69.1	80	10	24	8
Choline	104.0	60.0	60	5	30	7
Choline-d_9	113.0	69.0	60	5	30	7
Betaine	118.0	58.0	100	6	40	7
Betaine-d_{11}	129.1	66.0	100	9	40	11
TMA	60.3	44.0	80	8	26	8
TMA-d_9	69.0	49.1	80	8	29	8
TMAO	76.1	58.1	60	5	24	7
TMAO-d_9	85.1	66.0	60	5	24	7

Tab. 9 – Mass spectrometer settings (Q1 parent ion, Q3 ion fragment, DP declustering potential, EP entrance potential, CE collision energy, CXP collision cell exit potential) of the measured analytes.

A representative chromatogram of plasma sample, obtained through the developed LC-MS method, is reported in Figure 9. All compounds eluted in less than 4 minutes with good separation.

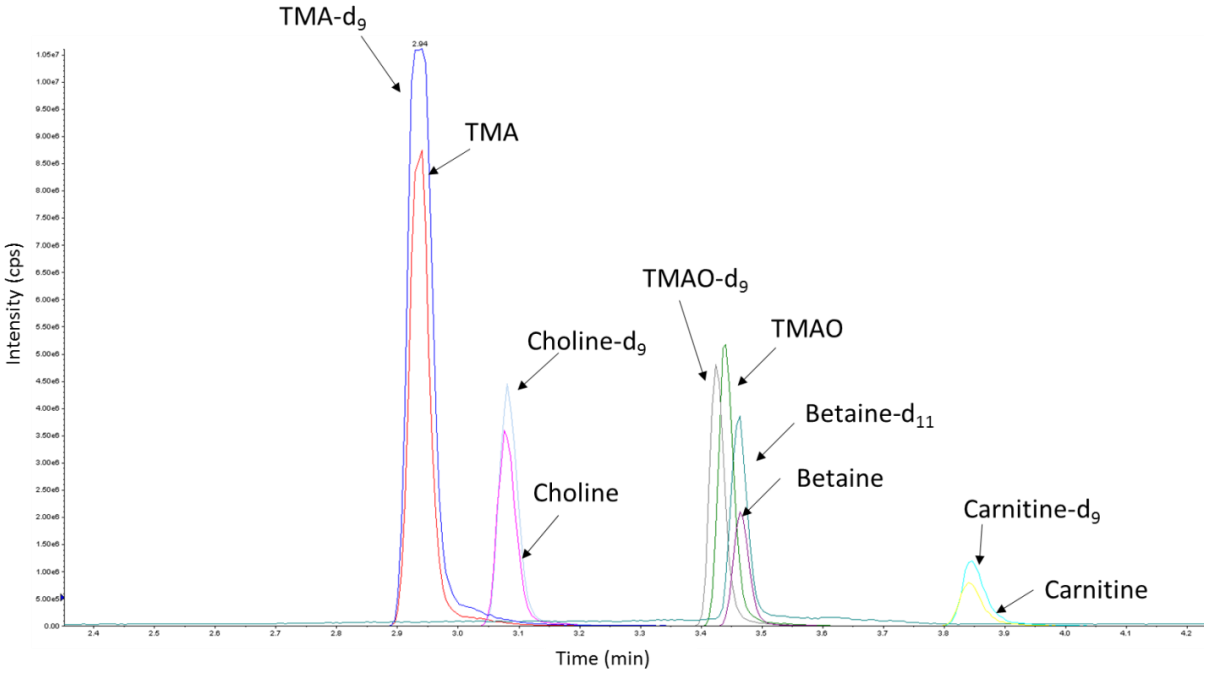


Fig. 9 – Representative chromatogram of trimethylamine (TMA) and its internal standard (TMA-d9), choline and its internal standard (choline-d9), trimethylamine-N-oxide (TMAO) and its internal standard (TMAO-d9), betaine and its internal standard (betaine-d11) and carnitine and its internal standard (carnitine-d9). Intensity, expressed as count *per* second, is reported on the Y-axis and time, expressed as minutes, on X-axis.

5.2.2. Method validation

The five analytes has quite different plasma values ranges and human inter-variability, so different ranges of linearity and calibration curve have been evaluated. The calibrant ranges, the equation curves obtained, LLOQ and LOD, for each analyte, are reported in Table 10.

Analyte	Calibration curve range (ng/mL)	Linear regression equation	R^2	LLOQ ng/mL	LOD ng/mL
Carnitine	6.5-100	y= (0.121±0.016 x + (0.406±0.121)	0.999±0.001	1.5	0.15
Choline	0.8-25	y= (0.169±0.005) x + (0.032±0.011)	0.999±0.001	0.8	0.15
Betaine	12.5-100	y= (0.060±0.006) x + (0.799±0.141)	0.994±0.004	12.5	0.8
TMA	0.4-6.25	y= (0.204±0.027) x + (1.006±0.234)	0.992±0.018	0.4	0.15
TMAO	0.4-80	y= (0.138±0.013) x + (0.034±0.014)	0.999±0.001	0.4	0.15

Tab. 10 – For each analyte, calibration curve range, linear regression equation, correlation coefficient (R^2), lower limit of quantification (LLOQ) and limit of detection (LOD) are reported. The equation was assessed by repeated analysis (n=5) of calibrators.

All analytes showed linear and reproducible curves, very low LOD and a satisfactory LLOQ. Intra-assay and inter-assay imprecisions for different QC levels are reported in Table 11. Imprecision was acceptable for all the analytes.

Analyte	QC low		QC medium		QC high	
	Intra-assay imprecision (CV%)	Inter-assay imprecision (CV%)	Intra-assay imprecision (CV%)	Inter-assay imprecision (CV%)	Intra-assay imprecision (CV%)	Inter-assay imprecision (CV%)
Carnitine	2.1	2.3	2.7	2.5	1.3	2.1
Choline	2.3	4.1	2.5	2.5	2.1	2.3
Betaine	5.2	5.2	2.8	3.4	2.3	3.3
TMA	3.9	6.1	6.7	9.9	4.9	7.4
TMAO	3.1	4.0	2.2	3.1	2.8	2.4

Tab. 11 – Imprecision, evaluated at three different quality control (QC) concentration levels, is expressed as coefficient of variation (CV) for each analyte: carnitine, choline, betaine, trimethylamine (TMA) and trimethylamine-N-oxide (TMAO).

The ME, assessed through the overlapping of solvent calibration curve and plasma matrix calibration curve, was remarkable for all analytes except betaine (Figure 10). As consequence, the quantification of those metabolites must be performed using plasma matrix calibration curves.

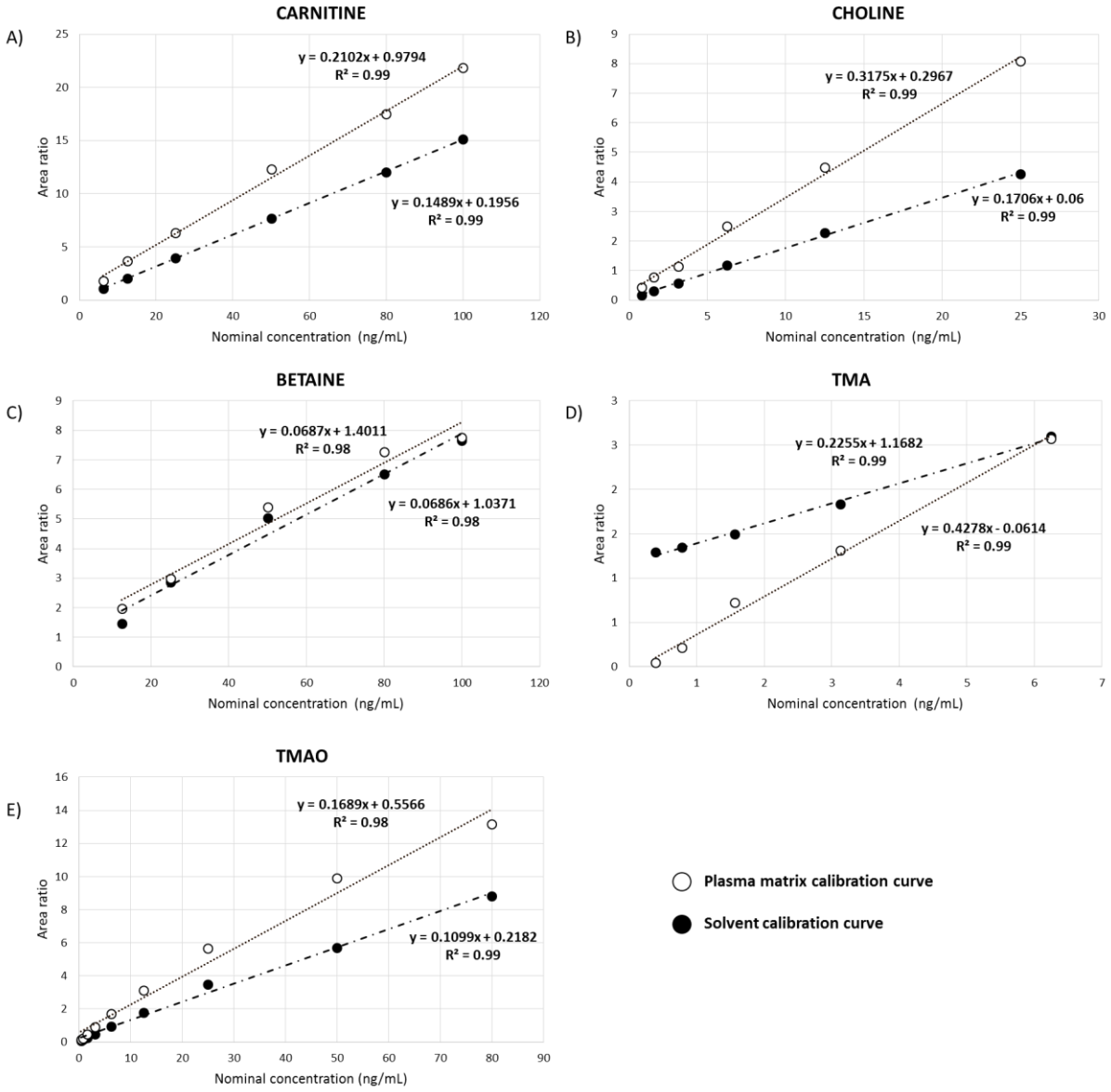


Fig 10 – Evaluation of matrix effect for each analyte in plasma: carnitine (panel A), choline (panel B), betaine (panel C), trimethylamine (TMA) (panel D) and trimethylamine-N-oxide (TMAO) (panel E). Area ratio, expressed as ratio between external standard area and internal standard area, is reported on the Y-axis and nominal concentration, expressed as ng/mL, on X-axis.

The analytes were highly stable in plasma at different temperatures of storage for at least 6 months and even throughout three freeze-thaw cycles (Table 12).

T	TMAO			TMA			Carnitine			Coline			Betaine		
	24 h	**3 d**	**6 m**	**24 h**	**3 d**	**6 m**	**24 h**	**3 d**	**6 m**	**24 h**	**3 d**	**6 m**	**24 h**	**3 d**	**6 m**
-80°C	98.3	100.6	98.2	100.1	102.8	101.5	99.2	98.5	96.5	99.8	102.5	100.7	101.2	98.8	99.7
+4°C	99.3	99.2	-	102.1	100.3	-	97.5	98.2	-	106.2	98.2	-	100.9	100.2	-
RT	95.8	96.2	-	98.8	100.2	-	95.4	95.8	-	94.5	95.5	-	98.1	99.4	-
Freeze-thaw	99.6			100.2			96.5			97.5			98.5		

Tab. 12 – Stability of carnitine, choline, betaine, trimethylamine (TMA) and trimethylamine-N-oxide (TMAO) at different times (24 hours, 3 days and 6 months) and at different temperatures (T; -80°C, +4°C and room temperature). The displayed values are percent related to sample analyzed immediately after the collection.

Part II

Experimental results

5.3. Study population

5.3.1. Patients enrollment

Figure 11 demonstrates the randomization to diet study groups and completion of study appointments. Among the participants randomized in the two groups, 64 started the mediterranean diet intervention and 66 the LFD one. Among them 58 and 62 completed the interventional treatment period, respectively.

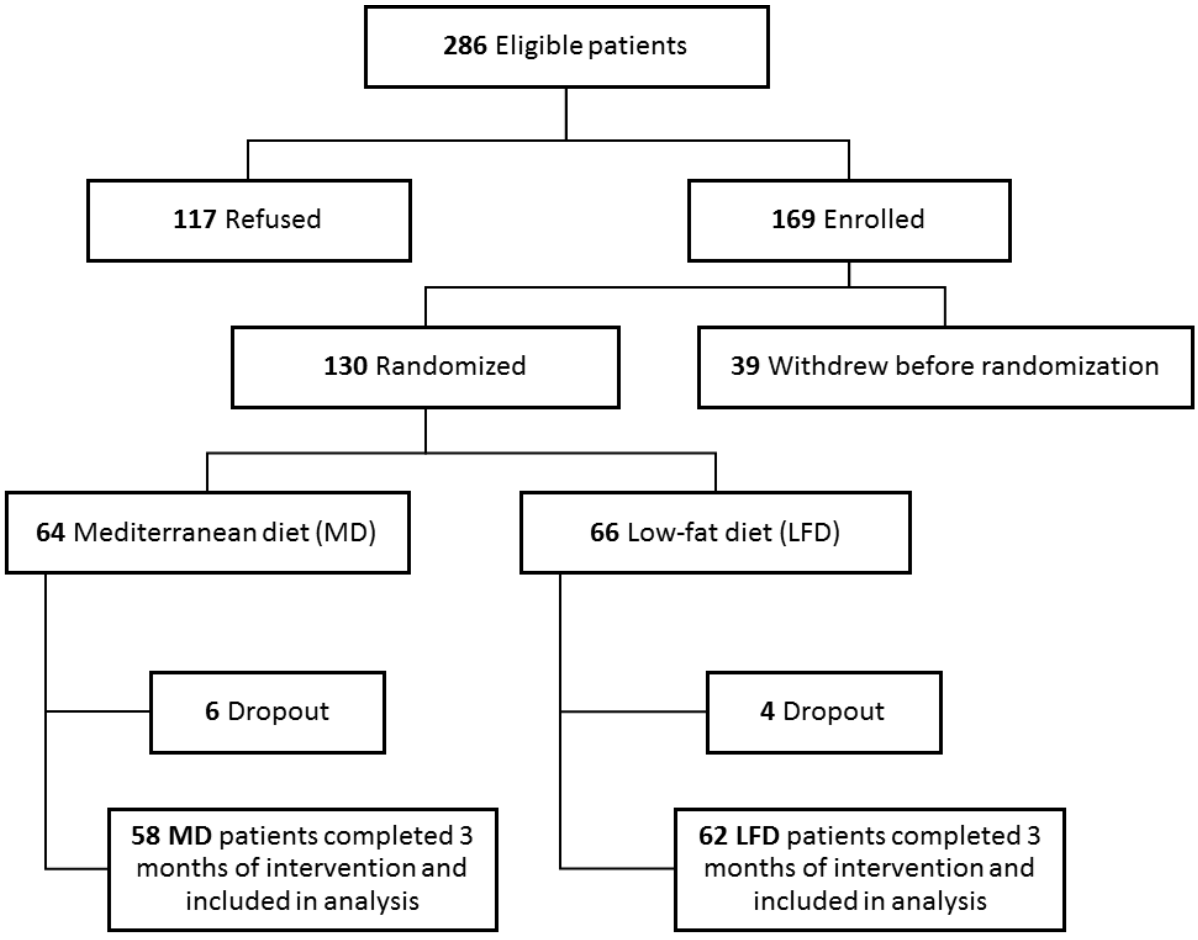


Fig. 11 – Study flow diagram of RISMeD patients from enrollement to diet study groups to end of diet intervention: Mediterranean diet (MD) or low-fat diet (LFD).

5.3.2. Patients baseline characteristics

Demographic characteristics, laboratory parameters, clinical and pharmacological features of the whole population and of the diet study groups, at baseline, are reported in Table 13.

Variable	All population (n=120)	MD group (n=58)	LFD group (n=62)	*p value* (MD *vs* LFD)
Demographic characteristics				
Age - years	62.6±8.5	62.7±7.1	62.5±9.6	0.90
Male gender - no. (%)	104 (86.7)	49 (84.5)	55 (88.7)	0.89
Laboratory parameters				
Hemoglobin - (g/dL)	14.9±1.1	14.9±1.2	15.0±1.1	0.81
Urine creatinine - (mg/dL)	107.7±51.9	103.0±48.5	112.2±55.0	0.34
hs-CRP - (mg/L)	1.6±2.1	1.4±1.6	1.7±2.5	0.46
Total cholesterol - (mg/dL)	167.6±29.2	172.7±29.1	162.6±28.5	0.06
HDL cholesterol - (mg/dL)	51.2±13.2	50.6±12.4	51.8±13.9	0.61
LDL cholesterol - (mg/dL)	93.8±25.7	99.2±26.6	88.7±23.9	*0.02*
Triglycerides - (mg/dL)	111.0±50.8	112.7±53.5	109.4±48.6	0.72
Fasting glycaemia - (mg/dL)	102.3±10.6	100.4±9.2	104.0±11.5	0.07
Cardiovascular risk factors				
Obesity - no. (%)	23 (19.2)	11 (19.0)	12 (19.4)	1.00
Hypertension - no. (%)	86 (71.7)	43 (74.1)	53 (69.4)	0.89
Dyslipidemia - no. (%)	95 (79.2)	44 (75.9)	51 (82.3)	0.79
Hyperglycemia - no. (%)	62 (51.7)	28 (48.3)	34 (54.8)	0.76
Smoke				
smoker - no. (%)	14 (11.7)	4 (6.9)	10 (16.1)	0.26
ex-smoker - no. (%)	73 (60.8)	36 (62.1)	37 (59.7)	1.00
non-smoker - no. (%)	33 (27.5)	18 (31.0)	15 (24.2)	0.56
Coronary artery disease				
Acute myocardial infarction - no. (%)	30 (25.0)	14 (24.1)	16 (25.8)	1.00
Stable angina - no. (%)	81 (67.5)	41 (70.7)	40 (64.4)	0.78
Unstable angina - no. (%)	9 (7.5)	3 (5.2)	6 (9.7)	0.50
Pharmacological treatments				
Hypoglycemic drugs - no. (%)	0 (0)	0 (0)	0 (0)	1.00
Lipid lowering drugs - no. (%)	110 (91.7)	50 (86.2)	60 (96.8)	0.70
Antiplatelet drugs - no. (%)	120 (100)	58 (100)	62 (100)	1.00
Antihypertensive drugs - no. (%)	109 (90.8)	54 (93.1)	55 (88.7)	0.90
MEDAS - no.	7.2±1.6	7.3±1.5	7.1±1.8	0.55

Tab. 13 – Baseline characteristics of the whole population (n=120). 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* CTR). For continuos variables the t-test and for binary variables the Fisher's exact test were used. High sensitive-C reactive protein (hs-CRP), high-density lipoprotein (HDL), low-density lipoprotein (LDL), mediterranean diet adherence score (MEDAS).

Overall, the participants represented a middle to older-aged adult (mean age 62.6 ± 8.5 years), mostly male (86.7%). Laboratory parameters were included within the normal ranges except for

fasting glycemia which exceed the limit usually recognize for normoglycemia (100 mg/dL). Most of the population was hypertensive (71.7%) and dyslipidemic (79.2%), more than half was hyperglycemic (51.7%) and only 20% was obese. Few participants (11.7%) were current smokers but most of the population was ex-smoker (60.8%). Regarding coronary artery disease, among enrolled patients stable angine was the most frequent disease (67.5%), than acute MI (25%) and UA (7.5%). All patients were treated with multiple medications during the trial, of which the most common were anti-platelets (100%), lipid lowering drugs (91.7%) and a range of anti-hypertensives (90.8%). Nobody were treated with hypoglicemic drugs as no diabetic patient was enrolled. The pharmacological treatment did not change during the diet period.

There were no significant differences between the study groups for any of these characteristics at baseline except for LDL cholesterol level which was higher in the MD than LFD group (99.2±26.6 *vs* 88.7±23.9, *p*=0.02).

The mean calculated adherence to MD at baseline was exactly half of the entire range (0-14 points). The distribution of MEDAS score at baseline is reported in Figure 12.

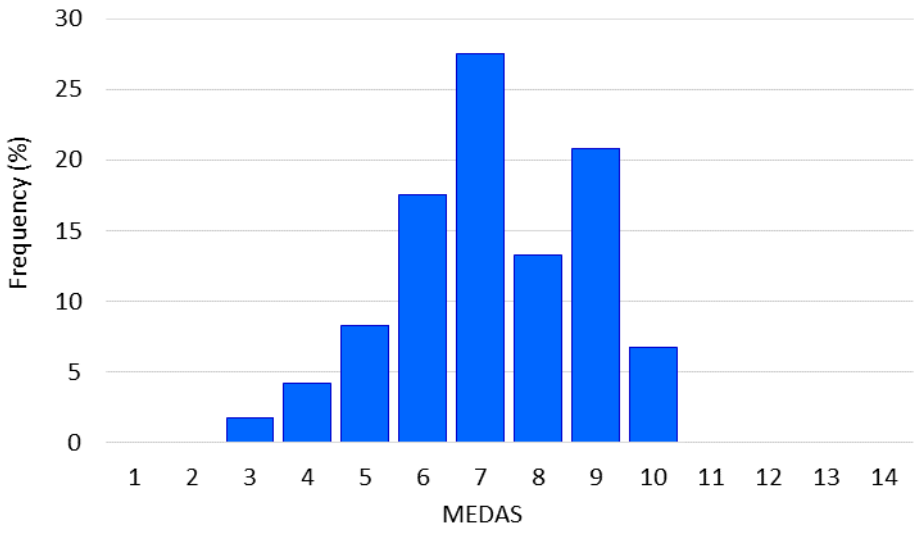


Fig. 12 – Distribution of the mediterranean diet adherence score (MEDAS) in the whole population (n=120). Frequency percentage is reported on the Y-axis and MEDAS value on X-axis.

5.3.3. Population subgroups

In Table 14 are described the diet subgroups, MD-subgroup and LFD-subgroup, in terms of demographic characteristics, laboratory parameters, clinical and pharmacological features. None of the considered variables differed between the two subgroups, MD subgroup and LFD subgroup, at baseline.

Variable	**MD subgroup (n=19)**	**LFD subgroup (n=19)**	***p value* (MD *vs* LFD)**
Demographic characteristics			
Age - years	61.3±6.3	58.9±7.6	0.30
Male gender - no. (%)	17 (89.5)	16 (84.2)	1.00
Laboratory parameters			
Hemoglobin - (g/dL)	15.3±1.1	14.8±1.0	0.22
Urine creatinine - (mg/dL)	120.7±50.3	115.1±61.3	0.76
hs-CRP - (mg/L)	1.4±1.3	1.1±1.0	0.48
Total cholesterol - (mg/dL)	170.1±30.6	153.1±23.7	0.06
HDL cholesterol - (mg/dL)	50.4±14.0	49.0±15.7	0.78
LDL cholesterol - (mg/dL)	97.0±27.6	82.3±18.8	0.06
Triglycerides - (mg/dL)	113.9±63.0	108.9±47.0	0.79
Fasting glycaemia - (mg/dL)	102.2±10.2	104.0±11.9	0.61
Cardiovascular risk factors			
Obesity - no. (%)	4 (21.1)	2 (10.5)	0.67
Hypertension - no. (%)	13 (68.4)	12 (63.2)	1.00
Dyslipidemia - no. (%)	13 (68.4)	15 (79.0)	0.81
Hyperglycemia - no. (%)	10 (52.6)	12 (63.2)	0.79
Smoke			
smoker - no. (%)	1 (5.3)	4 (21.1)	0.35
ex-smoker - no. (%)	10 (52.6)	12 (63.2)	0.79
non-smoker - no. (%)	8 (42.1)	3 (15.7)	0.30
Coronary artery disease			
Acute myocardial infarction - no. (%)	6 (31.6)	4 (21.1)	0.73
Stable angina - no. (%)	12 (63.2)	13 (68.4)	1.00
Unstable angina - no. (%)	1 (5.3)	2 (10.5)	1.00
Pharmacological treatments			
Hypoglycemic drugs - no. (%)	0 (0)	0 (0)	1.00
Lipid lowering drugs - no. (%)	18 (94.7)	19 (100)	1.00
Antiplatelet drugs - no. (%)	19 (100)	19 (100)	1.00
Antihypertensive drugs - no. (%)	18 (94.7)	18 (94.7)	1.00
MEDAS - no.	6.5±1.0	6.7±1.1	0.53

Tab. 14 – Baseline characteristics of the subgroups (n=38). 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 subgroup *vs* CTR subgroup). For continuos variables the t-test and for binary variables the Fisher's exact test were used. High sensitive-C reactive protein (hs-CRP), high-density lipoprotein (HDL), low-density lipoprotein (LDL), mediterranean diet adherence score (MEDAS).

5.4. Coronary artery disease risk factors and diet adherence

5.4.1. MD and LFD groups

In Table 15 MEDAS, clinical and hematological parameters of the whole population, at baseline and three months after diet intervention, are reported. To complete the information, in Figure 13 is shown the comparison among percentage of variation of the same features between groups.

Variable	**MD group T0 (n=58)**	**MD group T3 (n=58)**	***p value***	**LFD group T0 (n=62)**	**LFD group T3 (n=62)**	***p value***
MEDAS - no.	7.3±1.5	9.9±1.7	*<0.001*	7.1±1.8	8.2±1.7	*<0.001*
BMI - (Kg/m2)	27.2±3.4	26.2±3.3	*<0.001*	27.8±3.6	26.6±3.3	*<0.001*
Waist circumference - (cm)	97.7±9.7	94.3±9.3	*<0.001*	99.0±10.0	95.8±9.2	*<0.001*
SBP - (mmHg)	133.2±16.0	124.9±17.5	*0.002*	132.2±19.6	126.6±15.3	*0.008*
DBP - (mmHg)	78.8±10.2	74.8±11.2	*0.003*	80.0±9.6	76.6±9.0	*0.001*
Total cholesterol - (mg/dL)	172.7±29.1	162.2±36.4	*0.005*	162.6±28.5	154.8±32.0	*0.013*
HDL cholesterol - (mg/dL)	50.6±12.4	50.7±11.9	0.904	51.8±13.9	51.3±14.5	0.575
LDL cholesterol - (mg/dL)	99.2±26.6	91.7±30.7	*0.013*	88.7±23.9	83.5±25.3	*0.036*
Triglycerides - (mg/dL)	112.7±53.5	99.4±48.3	*0.013*	109.4±48.6	99.9±45.4	*0.033*
Fasting glycaemia - (mg/dL)	100.4±9.2	100.2±8.3	0.835	104.0±11.5	103.8±12.0	0.841

Tab. 15 – Variation in the whole population clinical parameters between T0 (baseline) and T3 (three months after diet) (n=120). Variables are expressed as mean±SD. *p*-values are shown for comparison of T3 *vs* T0. Paired t-test was used. Mediterranean diet adherence score (MEDAS), body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), high-density lipoprotein (HDL) and low-density lipoprotein (LDL).

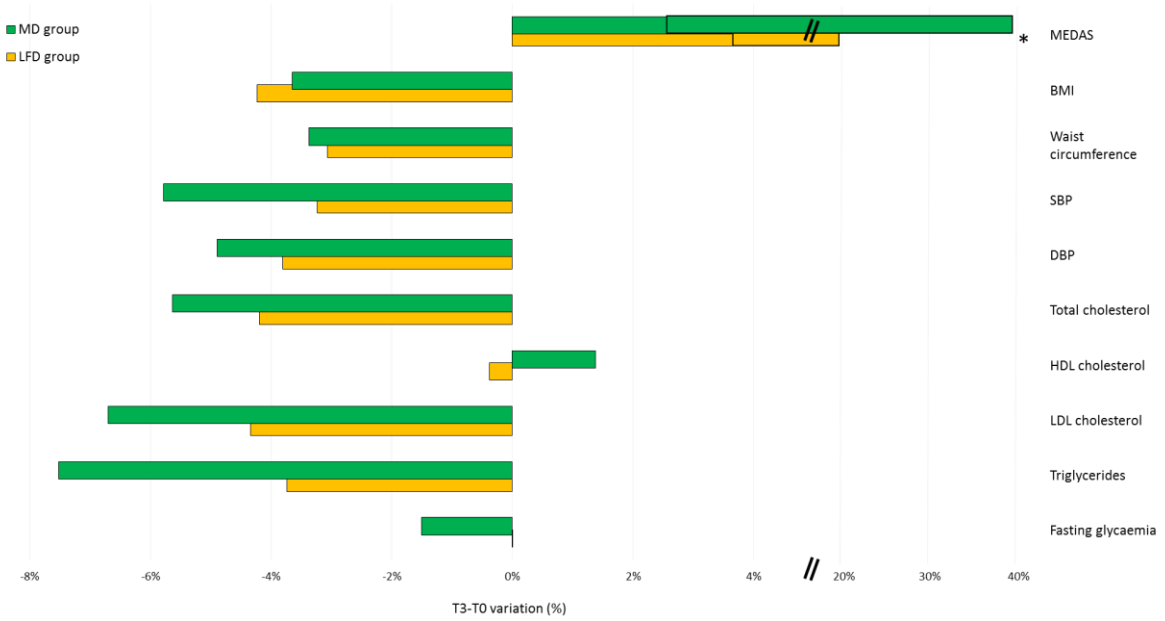


Fig. 13 – Comparison of T3-T0 clinical parameters variation between the two diet groups considering the whole population (n=120). Variables are expressed as average percentage variation value. Unpaired t-test was used. * p<0.0001 MD *vs* LFD. Mediterranean diet adherence score (MEDAS), body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), high-density lipoprotein (HDL) and low-density lipoprotein (LDL).

The adherence to MD, according to MEDAS score, increased in both MD and LFD groups from T0 to T3 (7.3 > 9.9, p<0.001 and 7.1 > 8.2, p<0.001, respectively). Nevertheless, the increase in MD group was significantly higher than in CTR group, as shown in Figure 3 (38.9% and 19.9%, respectively, p<0.0001).

Both diet interventions were able to considerably reduce BMI, waist circumferene, SBP and DBP, TC, LDL cholesterol and TGs. Even if no significant difference between groups was reached, MD induced a greater percentage decrease of these features, except for BMI which was more reduced by LFD. None of the diet intervention was able to increase HDL cholesterol and reduce fasting glycaemia.

As shown in Figure 14, the correlation analysis evidenced the strong negative relationship among MEDAS and BMI (r=-0.234, p<0.001), waist circumference (r=-0.245, p<0.001) and TGs (r=-0.193, p=0.003) in the whole population, considering both T0 and T3 (n=240). Consistently, we observed a negative correlation between MEDAS variation (delta T3-T0) and BMI variation (r=-0.351, p<0.001), waist circumference variation (r=-0.253, p=0.005), TC variation (r=-0.405, p<0.001), LDL cholesterol variation (r=-0.373, p<0.001) and TGs (r=-0.255, p=0.005) in the whole population (n=120) (Figure 15).

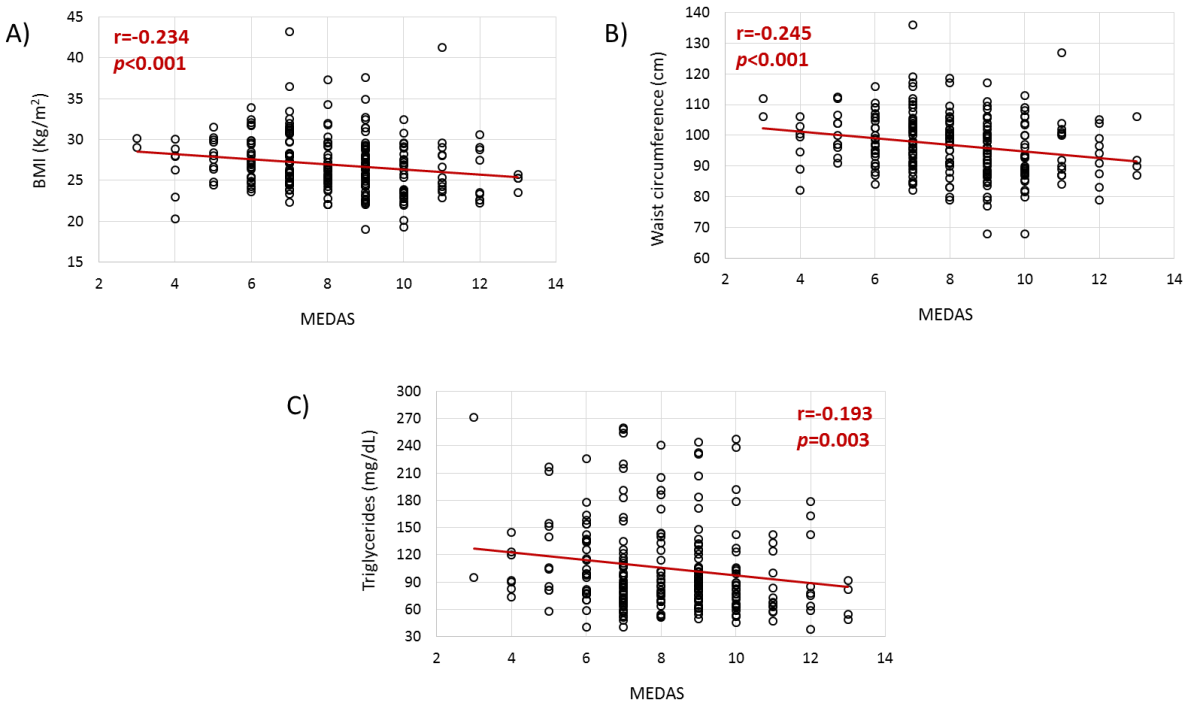


Fig. 14 – Correlations between MEDAS values and BMI (panel A), waist circumference (panel B) and triglycerides (panel C) in the whole population both at baseline (T0) and three months after diet intervention (T3) (n=240). Spearman correlation was used. Mediterranean diet adherence score (MEDAS) and body mass index (BMI).

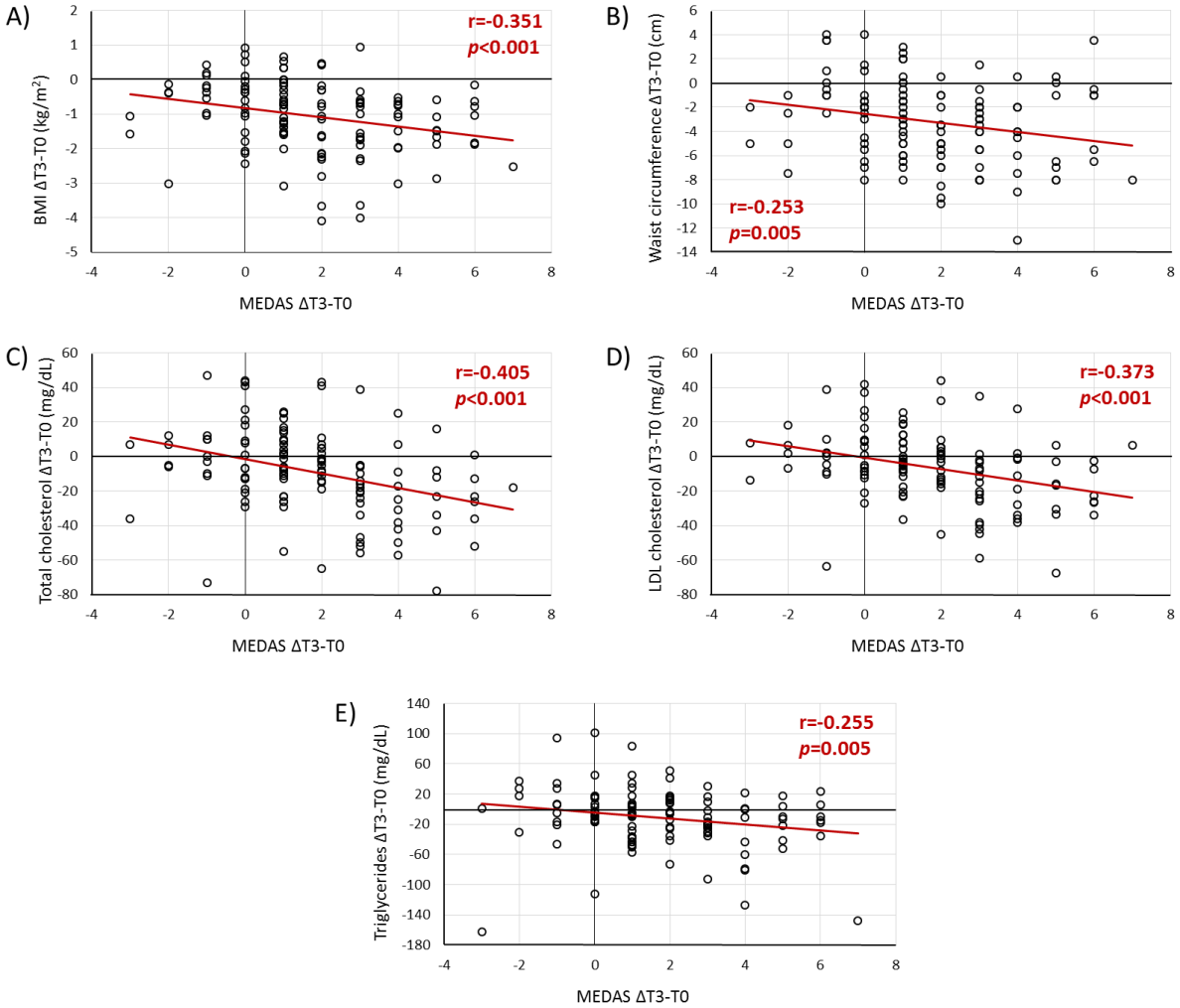


Fig. 15 – Correlations between delta T3-T0 MEDAS values and delta T3-T0 BMI (panel A), delta T3-T0 waist circumference (panel B), delta T3-T0 total cholesterol (panel C), delta T3-T0 LDL cholesterol (panel D) and delta T3-T0 triglycerides (panel E) in the whole population (n=120). Spearman correlation was used. Mediterranean diet adherence score (MEDAS), body mass index (BMI) and low-density lipoprotein (LDL).

5.4.2. MD and LFD subgroups

In Table 16 are reported the values of clinical and hematological variables related to diet interventions, before and after the diet period, in the subgroups. In Figure 16 are shown the values of percentage modification betweem T0 and T3 of the same variables.

Variable	**MD subgroup T0 (n=19)**	**MD subgroup T3 (n=19)**	***p value***	**LFD subgroup T0 (n=19)**	**LFD subgroup T3 (n=19)**	***p value***
MEDAS - no.	6.5±1.0	11.0±1.3	*<0.001*	6.7±1.1	6.7±1.4	1.000
BMI - (Kg/m2)	27.6±2.9	26.1±2.5	*<0.001*	27.5±2.7	26.5±2.7	*<0.001*
Waist circumference - (cm)	98.3±8.2	93.5±7.6	*<0.001*	100.0±8.1	96.8±7.8	*<0.001*
SBP - (mmHg)	130.1±16.6	121.8±16.8	*0.015*	124.7±14.3	122.8±15.3	0.565
DBP - (mmHg)	76.5±9.4	72.7±10.6	*0.041*	77.6±7.9	75.9±9.0	0.398
Total cholesterol - (mg/dL)	170.1±30.6	145.0±25.1	*<0.001*	153.1±23.7	148.6±31.6	0.398
HDL cholesterol - (mg/dL)	50.4±14.0	49.6±11.6	0.624	49.0±15.7	48.2±17.2	0.574
LDL cholesterol - (mg/dL)	97.0±27.6	78.0±21.3	*0.003*	82.3±18.8	80.0±24.8	0.597
Triglycerides - (mg/dL)	113.9±63.0	86.6±38.9	*0.013*	100.9±32.1	102.4±36.8	0.874
Fasting glycaemia - (mg/dL)	102.2±10.2	98.9±8.5	0.095	104.0±11.9	106.6±15.0	0.248

Tab. 16 – Variation in the subgroups (n=38) clinical parameters between T0 (baseline) and T3 (three months after diet). Variables are expressed as mean±SD. *p*-values are shown for comparison of T3 *vs* T0. Paired t-test was used. Mediterranean diet adherence score (MEDAS), body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), high-density lipoprotein (HDL) and low-density lipoprotein (LDL).

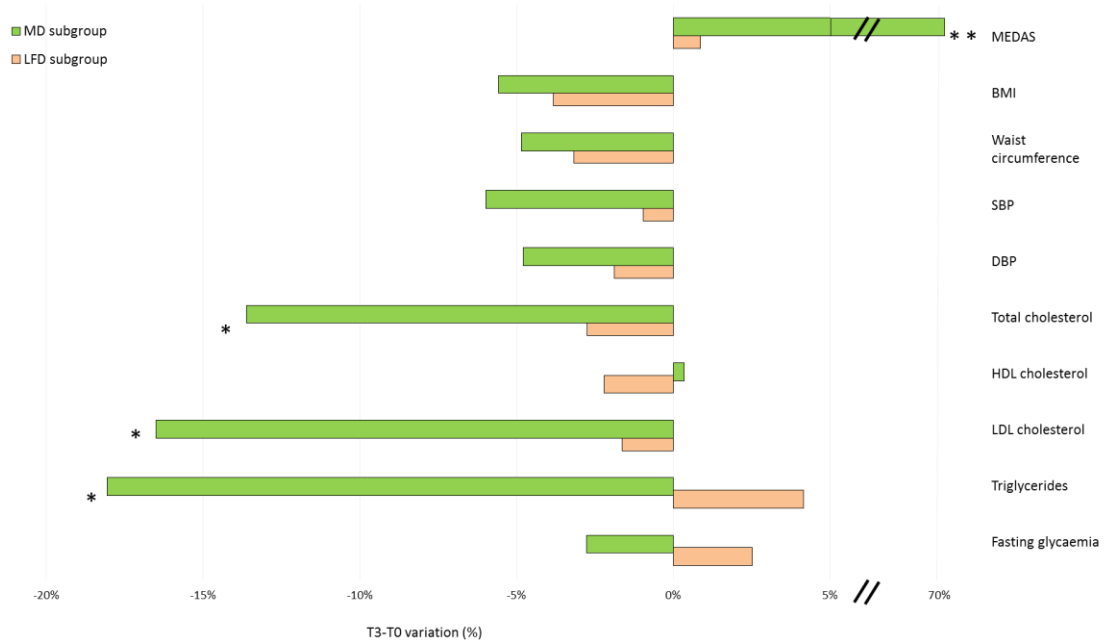


Fig. 16 – Comparison of T3-T0 clinical parameters variation between the subgroups, MD subgroup and LFD subgroup (n=38). Variables are expressed as average percentage variation value. Unpaired t-test was used. ** p<0.0001, *p<0.05 MD *vs* LFD. Mediterranean diet adherence score (MEDAS), body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), high-density lipoprotein (HDL) and low-density lipoprotein (LDL).

As subgroup definition, the adherence to MD from T0 to T3, according to MEDAS score, increased only in MD subgroup and was unchanged in LFD subgroup (6.5 > 11.0, p<0.001 and 6.7 > 6.7, p=1, respectively). The increase in MD adherence in the MD subgroup was more than 70% (Figure 16). Either, MD and LFD subgroups, showed a comparable significant decrease of BMI and waist circunference values. On the contrary, only MD subgroup significantly decreased in terms of SBP, DBP, TC, LDL cholesterol and TG.

As in the MD and LFD group, even in the subgroups HDL cholesterol and fasting glycaemia were not significantly modified.

5.5. Targeted analysis

5.5.1. Inflammatory markers

5.5.1.1. MD and LFD groups

Baseline levels of inflammatory markers did not differed between MD and LFD groups considering the whole population (leukocytes *p*=0.329; basophils *p*=0.310; hsCR, *p*=0.722).

Variation in inflammatory markers, induced by diet interventions, is reported in Table 17. No modifications have been observed after MD or LFD diet. However, a significant negative correlation between MEDAS variation (delta T3-T0) and hs-CRP variation (delta T3-T0) on the whole population was found (r= -0.223, *p*=0.015, n=120) (Figure 17).

Variable	**MD group T0 (n=58)**	**MD group T3 (n=58)**	***p value***	**LFD group T0 (n=62)**	**LFD group T3 (n=62)**	***p value***
Leukocytes - (10^3 cells/µL)	6.5±1.7	6.4±2.0	0.172	6.7±1.6	6.8±1.9	0.471
Basophils - (cells/µL)	29.3±15.0	29.8±27.4	0.496	31.8±18.4	29.7±16.0	0.128
hs-CRP - (mg/L)	1.4±1.6	1.2±1.2	0.237	1.4±1.3	1.4±1.8	0.335

Tab. 17 – Variation of inflammatory markers between T0 (baseline) and T3 (three months after diet) in the whole population (n=120). Variables are expressed as mean±SD. *p*-values are shown for comparison of T3 *vs* T0. Paired t-test was used on log transformed data. High sensitivity c-reactive protein (hs-CRP).

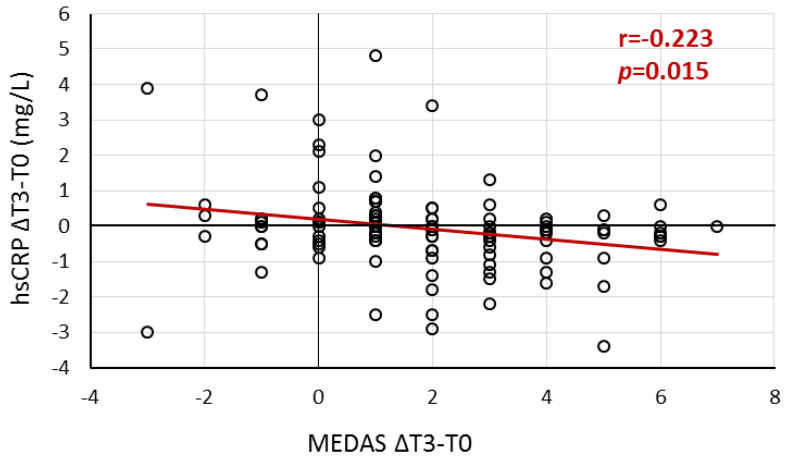


Fig. 17 – Correlations between delta T3-T0 mediterranean diet adherence score (MEDAS) values and delta T3-T0 high sensitivity c-reactive protein (hs-CRP) in the whole population (n=120). Spearman correlation was used.

5.5.1.2. MD and LFD subgroups

Baseline levels of inflammatory markers did not differed between MD and LFD subgroups (leukocytes *p*=0.847; basophils *p*=0.932; hs-CRP *p*=0.534).

The subgroup anlysis confirmed the inefficacy of LFD to decrease inflammatory markers and pointed out a very significant reduction in hs-CRP and basophils levels in MD group (Table 18). MD induced more than 40% average reduction of baseline hs-CRP levels and 15% basophils count. As in the whole population analysis, considering the subgroups population there is significant negative correlation between MEDAS variation (delta T3-T0) and hs-CRP variation (delta T3-T0) (r= -0.339, *p*=0.037, n=38).

Variable	MD subgroup T0 (n=19)	MD subgroup T3 (n=19)	*p value*	LFD subgroup T0 (n=19)	LFD subgroup T3 (n=19)	*p value*
Leukocytes - (10^3 cells/µL)	6.7±1.7	6.5±1.6	0.367	6.5±1.4	6.7±2.3	0.460
Basophils - (cells/µL)	31.1±18.5	27.4±23.3	*0.037*	31.7±20.1	28.8±15.3	0.182
hs-CRP - (mg/L)	1.4±1.3	0.8±0.8	*0.001*	1.1±1.0	1.6±1.9	0.327

Tab. 18 – Variation of inflammatory markers between T0 (baseline) and T3 (three months after diet) in MD and LFD subgroups (n=38). Variables are expressed as mean±SD. *p*-values are shown for comparison of T3 *vs* T0. Paired t-test was used on log transformed data. High sensitivity c-reactive protein (hs-CRP).

5.5.2. Antioxidants and oxidative stress

5.5.2.1. MD and LFD groups

Considering the whole population, baseline levels of antioxidants and oxidative stress markers did not differed between MD and LFD groups (ɣ-tocopherol *p*=0.672; α-tocopherol *p*=0.057; ɣ-tocopherol/TC *p*=0.246; α-tocopherol/TC *p*=0.354; GSH/GSSG *p*=0.711; 8-OHdG *p*=0.935; 8-iso-$PGF_{2\alpha}$ *p*=0.157). Variation between T3 and T0 in antioxidants, ɣ-tocopherol and α-tocopherol themselves or TC corrected, and oxidative stress biomarkers, GSH/GSSG ratio, 8-OHdG and 8-iso-$PGF_{2\alpha}$, are described in Table 16. ɣ-tocopherol, GSH/GSSG ratio and 8-OHdG levels have been not modified by diet intervention. ɣ-tocopherol and α-tocopherol corrected for TC levels did not change at T3. A decreased of α-tocopherol concentration was observed in both groups, but statistically significant only for MD group. Both diet were able to significantly decrease lipid peroxidation, in terms of 8-iso-$PGF_{2\alpha}$ urinary concentrations (Table 19). No difference in the

induced amount reduction between MD and LFD was observed (-49.4±144.9 *vs* -36.9±153.3 pg/mg creatinine, respectively, *p*=0.666).

Variable	MD group T0 (n=58)	MD group T3 (n=58)	*p value*	LFD group T0 (n=62)	LFD group T3 (n=62)	*p value*
ɣ-tocopherol - (µg/mL)	0.32±0.13	0.30±0.14	0.123	0.38±0.47	0.30±0.16	0.056
α-tocopherol - (µg/mL)	11.5±2.6	10.7±2.5	*<0.001*	10.6±2.6	10.2±2.8	0.091
ɣ-tocopherol/TC - (10^{-3})	0.18±0.07	0.18±0.08	0.875	0.17±0.06	0.18±0.10	0.293
α-tocopherol/TC - (10^{-3})	6.7±1.2	6.6±1.1	0.577	6.3±1.2	6.6±1.3	0.488
GSH/GSSG	12.5±8.0	13.9±10.4	0.636	12.2±8.8	13.5±9.0	0.245
8-OHdG - (ng/mg creatinine)	1.7±1.0	1.7±1.3	0.586	1.7±1.1	1.6±1.1	0.210
8-iso-$PGF_{2\alpha}$ -(pg/mg creatinine)	227.6±155.6	177.5±98.3	*0.016*	195.4±148.0	160.1±94.1	*0.025*

Tab. 19 – Variation of antioxidants and oxidative stress markers between T0 (baseline) and T3 (three months after diet) in the whole population (n=120). Variables are expressed as mean±SD. *p*-values are shown for comparison of T3 *vs* T0. Paired t-test was used on log transformed data. Total cholesterol (TC), ratio reduced/oxidized glutathione forms (GSH/GSSG), 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$).

A significant negative correlation between MEDAS variation (delta T3-T0) and α-tocopherol variation (delta T3-T0) on the whole population was found (r= -0.361, *p*<0.001, n=120) (Figure 18). Furthermore, α-tocopherol showed a positive correlation with ɣ-tocopherol both as single values (T0 and T3 determinations, n=240) and as variations between T3-T0 (delta T3-T0, n=120) on the whole population (r= 0.532, *p*<0.001; r= 0.267, *p*=0.003, respectively) (Figure 19).

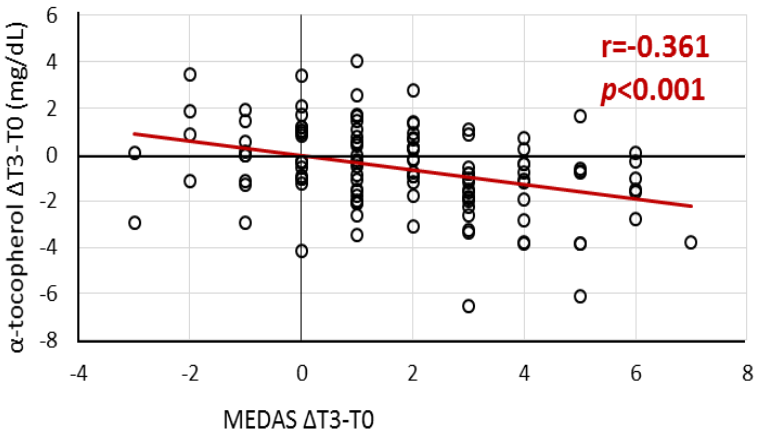


Fig. 18 – Correlations between delta T3-T0 mediterranean diet adherence score (MEDAS) values and delta T3-T0 α-tocopherol in the whole population (n=120). Spearman correlation was used.

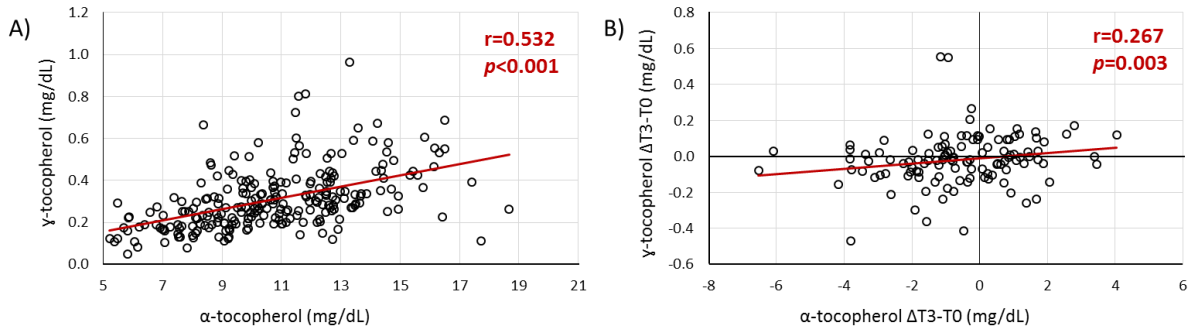


Fig. 19 – Correlations between gamma and alpha tocopherol: in the whole population both at baseline (T0) and three months after diet intervention (T3) (n=240) (panel A); as delta T3-T0 in the whole population (120). Spearman correlation was used.

5.5.2.2. MD and LFD subgroups

Baseline levels of antioxidants and oxidative stress markers did not differed between MD and LFD subgroups (γ-tocopherol p=0.538; α-tocopherol p=0.116; γ-tocopherol/TC p=0.940; α-tocopherol/TC p=0.565; GSH/GSSG p=0.247; 8-OHdG p=0.649; 8-iso-$PGF_{2\alpha}$ p=0.472).

The antioxidants and oxidative stress biomarkers analysis in the MD and LFD subgroups, reflected and confirmed the results obtained in the whole population analysis (Table 20). The data showed the significant decrease of α-tocopherol and 8-iso-$PGF_{2\alpha}$ at T3 in MD group. The latter diminished in LFD group too. No difference in the delta T3-T0 between MD and LFD was observed (-28.6% *vs* -18.8%, respectively, p=0.117). As regard to the correlations, MEDAS variation (delta T3-T0) negatively correlated with α-tocopherol variation (delta T3-T0) on the whole subgroups population (r= -0.431, p=0.007, n=38). Furthermore, α-tocopherol showed a positive correlation with γ-tocopherol both as single values (T0 and T3 determinations, n=76) and as variations between T3-T0 (delta T3-T0, n=38) on the whole subgroup population (r= 0.481, p<0.001; r= 0.366, p=0.024, respectively).

Variable	MD subgroup T0 (n=19)	MD subgroup T3 (n=19)	*p value*	LFD subgroup T0 (n=19)	LFD subgroup T3 (n=19)	*p value*
ɣ-tocopherol – (µg/mL)	0.29±0.14	0.26±0.10	0.430	0.26±0.10	0.27±0.15	0.800
α-tocopherol - (µg/mL)	11.2±3.0	9.5±2.2	*0.001*	9.7±2.6	9.8±2.9	0.888
ɣ-tocopherol/TC - (10^{-3})	0.17±0.09	0.18±0.07	0.391	0.23±0.29	0.20±0.09	0.877
α-tocopherol/TC - (10^{-3})	6.6±1.4	6.5±1.3	0.920	6.5±1.1	6.6±1.4	0.161
GSH/GSSG	12.8±8.1	13.2±9.4	0.922	15.3±10.0	13.9±9.6	0.352
8-OHdG - (ng/mg creatinine)	1.7±0.9	1.8±1.0	0.805	1.7±1.2	1.3±0.9	0.292
8-iso-$PGF_{2\alpha}$ -(pg/mg creatinine)	194.4±91.5	156.4±80.0	*0.039*	230.8±139.8	149.3±94.0	*0.004*

Tab. 20 – Variation of antioxidants and oxidative stress markers between T0 (baseline) and T3 (three months after diet) in MD and LFD subgroups (n=38). Variables are expressed as mean±SD. *p*-values are shown for comparison of T3 *vs* T0. Paired t-test was used on log transformed data. Total cholesterol (TC), ratio reduced/oxidized glutathione forms (GSH/GSSG), 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$).

5.5.3. Gut microbiota: TMAO pathway

5.5.3.1. MD and LFD groups

Considering the whole population, baseline levels of carnitine, choline, TMA and TMAO did not differed between MD and LFD groups (carnitine *p*=0.150; choline *p*=0.625; TMA *p*=0.955; TMAO *p*=0.056). Betaine baseline levels were significantly higher in MD group (*p*=0.038). The concentration levels of metabolites related to gut microbiota are reported in Table 21. No modifications in carnitine, choline, TMA and TMAO levels after both dietary interventions, have been observed. A significant increase in betaine concentrations was detected in both groups at T3. The relevance of betaine increment, between 8-9% of average increase, is similar between MD and LFD group (+0.4±1.0 *vs* +0.2±1.3 µg/mL, respectively, *p*=0.690). The relationship between betaine and MD diet was confirmed by the positive correlation between MEDAS and betaine in the whole population (r= 0.133, *p*=0.040, n=240) (Figure 20).

Variable	MD group T0 (n=58)	MD group T3 (n=58)	*p value*	LFD group T0 (n=62)	LFD group T3 (n=62)	*p value*
Carnitine - (µg/mL)	3.47±0.57	3.42±0.55	0.653	3.31±0.93	3.31±0.75	0.538
Choline - (µg/mL)	0.46±0.12	0.46±0.11	0.573	0.44±0.13	0.44±0.13	0.625
Betaine - (µg/mL)	4.27±1.45	4.67±1.48	*0.005*	3.74±1.42	4.05±1.34	*0.032*
TMA - (µg/mL)	0.22±0.07	0.22±0.07	0.843	0.22±0.13	0.22±0.07	0.955
TMAO - (µg/mL)	0.34±0.40	0.39±0.47	0.425	0.22±0.19	0.27±0.34	0.603

Tab. 21 – Variation of carnitine, choline, betaine, trimethylamine (TMA) and trimethylamine-N-oxide (TMAO) between T0 (baseline) and T3 (three months after diet) in MD and LFD groups. Variables are expressed as mean±SD. *p*-values are shown for comparison of T3 *vs* T0. Paired t-test was used on log transformed data.

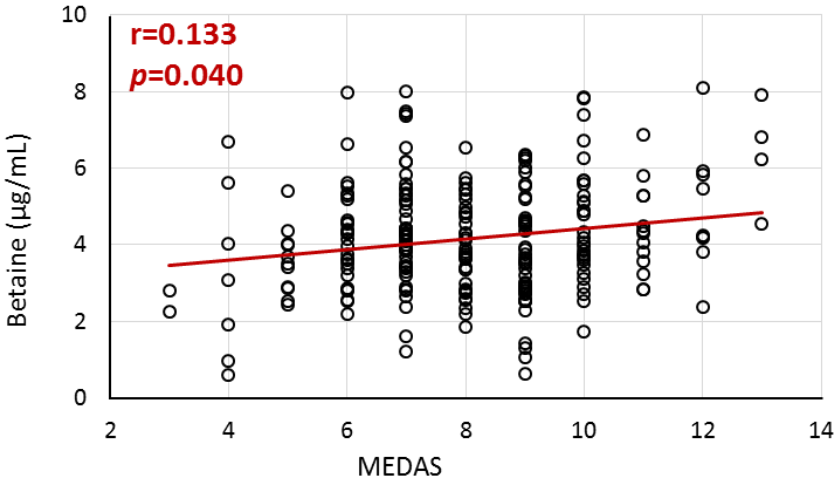


Fig. 20 – Correlations between betaine and MEDAS in the whole population both at baseline (T0) and three months after diet intervention (T3) (n=240). Spearman correlation was used.

Furthermore betaine positively correlated with choline (r= 0.442, $p<0.000$; n=240), and TMAO correlated with its precursors, choline and carnitine (r= 0.264, $p<0.000$; r= 0.177, $p<0.006$, respectively; n=240).

5.5.3.2. MD and LFD subgroups

Baseline levels of carnitine, choline, bataine, TMA and TMAO did not differed between MD and LFD subgroups (carnitine *p*=0.644; choline *p*=0.863; betaine *p*=0.444; TMA *p*=0.787; TMAO *p*=0.556).

The result obtained in the subgroups population are stated in Table 22. As highlighted in the whole MD group, betaine levels increase at T3 in the MD subgroup too, compared to baseline. On the contrary no modification was observed in betaine levels of LFD grouop. The positive correlation between MEDAS and betaine is confirmed too (r= 0.373, *p*=0.001; n=76).

Variable	MD subgroup T0 (n=19)	MD subgroup T3 (n=19)	*p value*	LFD subgroup T0 (n=19)	LFD subgroup T3 (n=19)	*p value*
Carnitine - (µg/mL)	3.38±0.63	3.41±0.56	0.789	3.28±0.62	3.33±0.61	0.703
Choline - (µg/mL)	0.50±0.14	0.50±0.11	0.918	0.49±0.14	0.47±0.11	0.577
Betaine - (µg/mL)	4.02±1.34	4.82±1.24	*0.001*	3.81±1.58	3.71±1.25	0.848
TMA - (µg/mL)	0.30±0.02	0.30±0.03	0.951	0.30±0.03	0.30±0.01	0.949
TMAO - (µg/mL)	0.26±0.44	0.36±0.45	0.220	0.18±0.15	0.33±0.55	0.225

Tab. 22 – Variation of carnitine, choline, betaine, trimethylamine (TMA) and trimethylamine-N-oxide (TMAO) between T0 (baseline) and T3 (three months after diet) in MD and LFD subgroups. Variables are expressed as mean±SD. *p*-values are shown for comparison of T3 *vs* T0. Paired t-test was used on log transformed data.

5.5.4. Complex lipid profile

Through a complex lipid profile approach 183 different lipid compounds have been evaluated: 11 LPCs, 58 PCs, 7 phosphatidylserines (PS), 55 PEs, 5 phosphatidic acids (PA), 10 phosphatidylinositols (PI), 2 lac-ceramides (LacCer), 7 Cer, 4 glucosylceramides (GCer), 15 SMs, 6 sulfoglycosphingolipids (Sul) and 3 gangliosides (GM).

No baseline differences between the two subgroups, MD and LFD subgroups, were observed in any of the lipid evaluated (data not shown). Furthermore, to summarize this baseline data, in Figure 21 is possible to observe no separation between groups at baseline. The two PCA components, i.e. the two most relevant indices summarizing the data, explained 19.0% and 14.1% of the total variance.

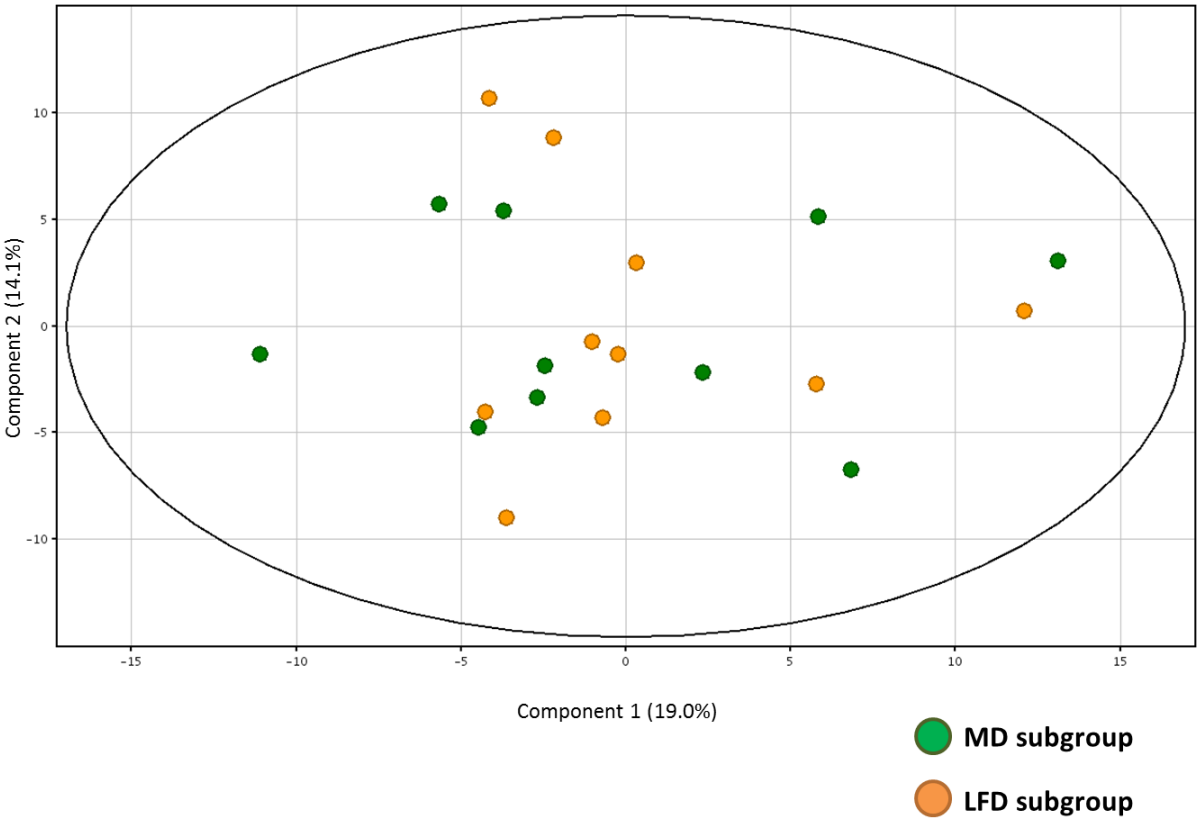


Fig. 21 – Score plot of principal component analysis (PCA) models showing the subgroups, MD (n=19) and LFD (n=19) subgroups, at baseline (T0).

No effect of three months LFD intervention was evidenced on complex lipid profile (Figure 22).

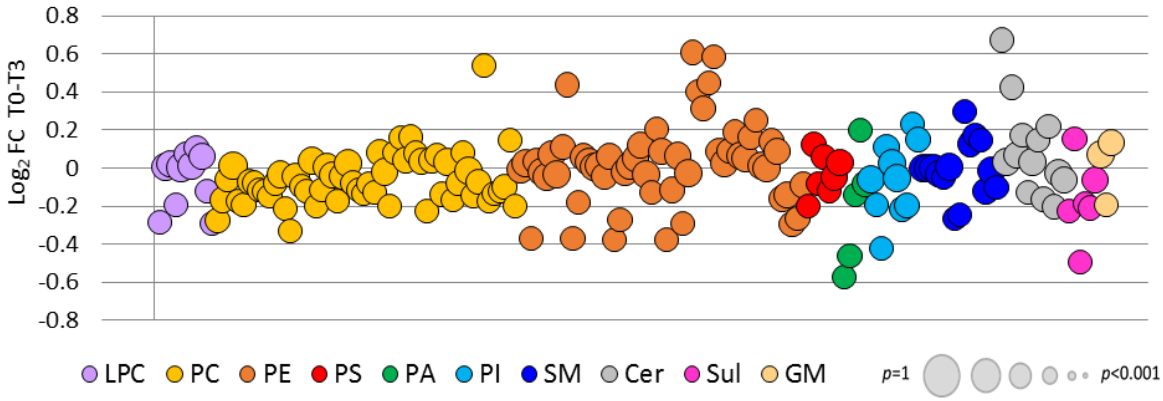


Fig. 22 – Complex lipid profile modification in LFD subgroup (n=19). Log_2 fold change value is reported on the Y-axis and lipid classes on X-axis. Each dot represents a lipid species, color coded *per* lipid class. Dot size indicates significance. Paired t-test with Storey multiple testing correction was used.

Instead, strong effect of MD on complex lipid profile is evidenced. Through the unsupervised PCA was possible to discriminate patients before (T0) and after the MD intervention (T3), with a similar trend for all subjects (Figure 23). The two components explained 21.7% and 17.4% of the total variance.

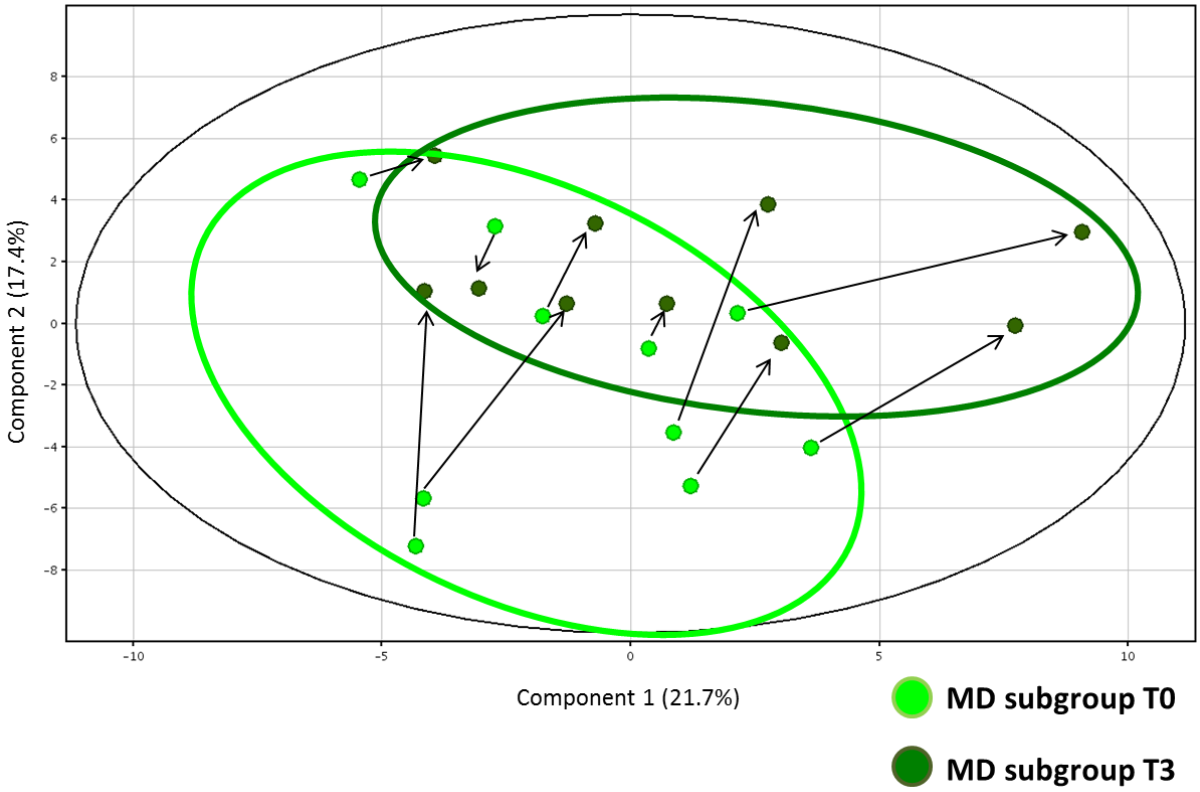


Fig. 23 – Score plot of principal component analysis (PCA) models showing the MD subgroup (n=19) at baseline (T0) and three months after the diet (T3).

The result, is confirmed and better defined in the PLS-DA analysis, a statistical model with higher capability to discriminate between groups (Figure 24). The three components explained 22.5%, 12.2 and 11.9% of the total variance. The explained variation parameter (R^2) was 0.44 and cross-validated predictive ability (Q^2) was 0.10.

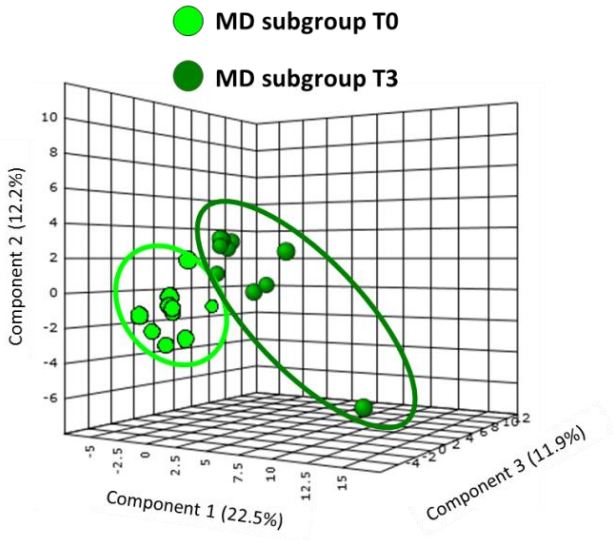


Fig. 24 – Score plot of Partial Least Squares-Discriminant Analysis (PLS-DA) models generated from complex lipid profile of MD subgrop (n=19) before (T0) and after MD intervention (T3).

As depicted in Figure 25, the lipid classes mostly modified were LPCs, PCs and PEs. Among these, many showed an increase of leveles compared to baseline.

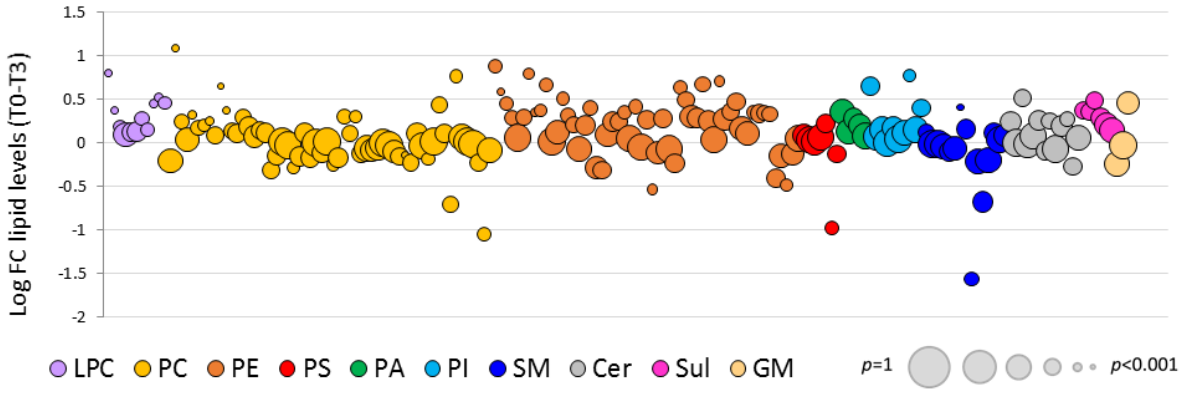
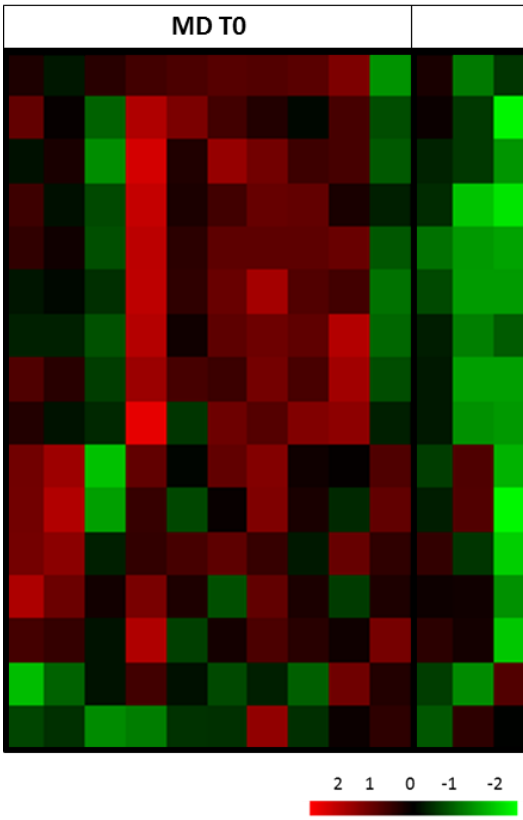


Fig. 25 – Complex lipid profile modification in MD subgroup (n=19). Log_2 fold change value is reported on the Y-axis and lipid classes on X-axis. Each dot represents a lipid species, color coded *per* lipid class. Dot size indicates significance. Paired t-test with Storey multiple testing correction was used.

In Figure 26 is reported the variation of lipids expression significantly altered after MD intervention and the corresponding *p*-value.



Lipid species	*p*-value	Change
lysoPC a 28:1	0.032	up
lysoPC a 16:1	0.022	up
PC aa 32:1	0.032	up
PC aa 34:3	0.022	up
PC aa 34:4	0.020	up
SM 20:2	0.020	up
PC aa 30:0	0.032	up
lysoPC a 14:0	0.020	up
PC aa 26:0	0.022	up
PE aa 34:1	0.020	up
PE aa 36:2	0.032	up
lysoPC a 20:3	0.026	up
PE aa 36:3	0.048	up
PE ae 38:6	0.048	up
PC ae 38:5	0.022	down
PE aa 42:10	0.048	down

Fig. 26 – Heatmap showing a graphic representation of the differences in relative lipids concentrations at T0 and T3 of MD subgroup (n=19). Rows represent lipids and columns the samples. *p*-value is reported. Paired t-test with Storey multiple testing correction was used.

5.6. Untargeted metabolomics analysis

5.6.1. Performance evaluation

To evaluate the metabolic modifications induced by diets, an LC-MS untargeted method for plasma samples analysis was developed. Representative chromatograms, obtained in positive and negative ionization mode, are shown in Figure 27, panel A and B respectively.

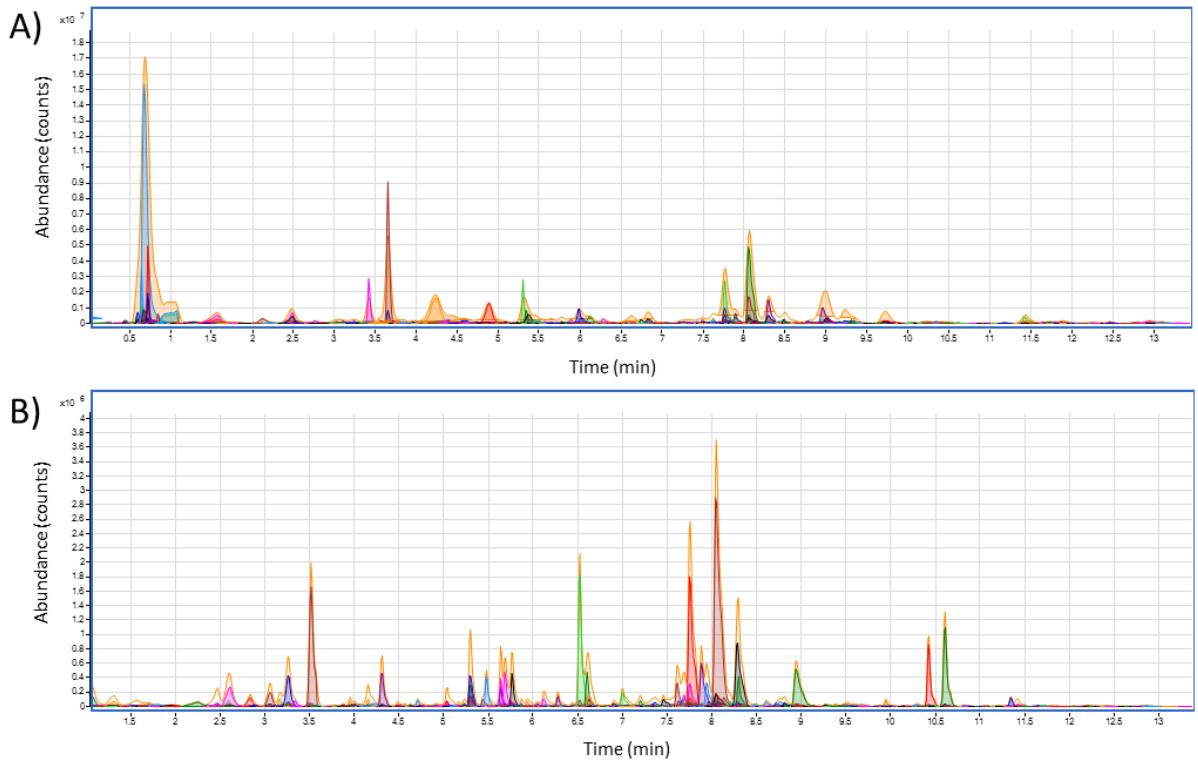


Fig. 27 – Representative exctracted compounds chromatograms obtained from a plasma sample in positive (A) and negative detection mode (B). Abundance, expressed as counts, is reported on the Y-axis and time, expressed as minutes, on X-axis.

To test the reproducibility of sample preparation and the analysis performance, two different approaches based on QC samples and reference standards solution were adopted [409, 410].
As shown in Figure 28, the total ion current (TIC) of QCs versus the order of injection presented, as expected, an abundance decrease due to 40 consecutive hours of analysis for each ionization mode (CV: 15% in positive ionization mode and 3% in negative ionization mode).

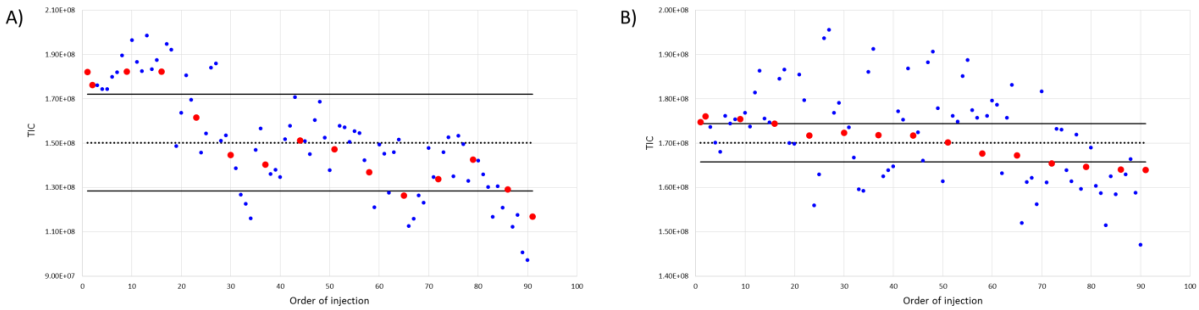


Fig. 28 – Trend plots showing the variation of the total ion current (TIC) mean values over all observations (n=76 samples and n=15 QCs) in positive (A) and negative (B) detection mode. Quality controls (QC) are colored in red and samples in blu. The black lines represents QCs' TIC mean value ± standard deviation.

Furthermore, the intensity of each reference standard, added to the sample before its preparation, was evaluated: CV values were lower than 20 % for all the added standards (Table 23).

Reference standard	**Mass**	**RT**	***Detection mode***	**Mean ± SD**	**CV%**
TMAO-d_9	84.125	0.7	positive	665284±122777	18.8
L-4-tyr-$^{13}C_9$	190.104	0.7	positive	48509±7025	14.5
Methionine-^{13}C	154.068	0.7	positive	67024±9689	14.5
8-OHdG-$^{15}N_5$	288.077	0.7	negative	98565±7648	7.8
			positive	4376±809	18.5
3-nitro-L-tyr-$^{13}C_9$	235.089	0.9	negative	78203±5272	6.7
			positive	126359±21730	17.2
Rivastigmine	250.168	2.7	positive	10269611±1753164	17.1
ASA-d_4	184.068	3.2	negative	26834±4148	15.5
Reserpina	608.273	4.8	negative	88901±6050	6.8
			positive	3165592±438977	13.9
8-iso-$PGF_{2\alpha}$-d_4	358.266	5.1	negative	562742±35859	6.4
11-DH-TXB_2-d_4	372.245	5.2	negative	502737±41733	8.3
			positive	21682±3026	14.0
12-HETE-d_8	328.285	8.1	negative	516995±33507	6.5

Tab. 23 – Performance evaluation through the assessment of reference standards added in the samples (n=76) and quality controls (n=21). In the table are reported reference standard mass, retention time (RT), detection mode, ion intensity mean value ± standard deviation (SD) and coefficient of variation (CV). Trimethylamine-N-oxide-d_9 (TMAO-d_9), L-4-tyrosine-$^{13}C_9$ (L-4-tyr-$^{13}C_9$), 8-hydroxy-2-deoxyguanosine-$^{15}N_5$ (8-OHdG-$^{15}N_5$), 3-nitro-tyrosine-$^{13}C_9$ (3-nitro-tyr-$^{13}C_9$), acetylsalicylic acid-d_4 (ASA- d_4), 8-iso-prostaglandin $F_{2\alpha}$-d_4 (8-iso-$PGF_{2\alpha}$-d_4), 11-dehydro-thromboxane B_2-d_4 (11-DH-TXB_2-d_4) and 12-hydroxyeicosatetraenoic acid-d_8 (12-HETE-d_8).

5.6.2. Data processing

The workflow of the analysis, data processing and compound identification is depicted in Figure 29.

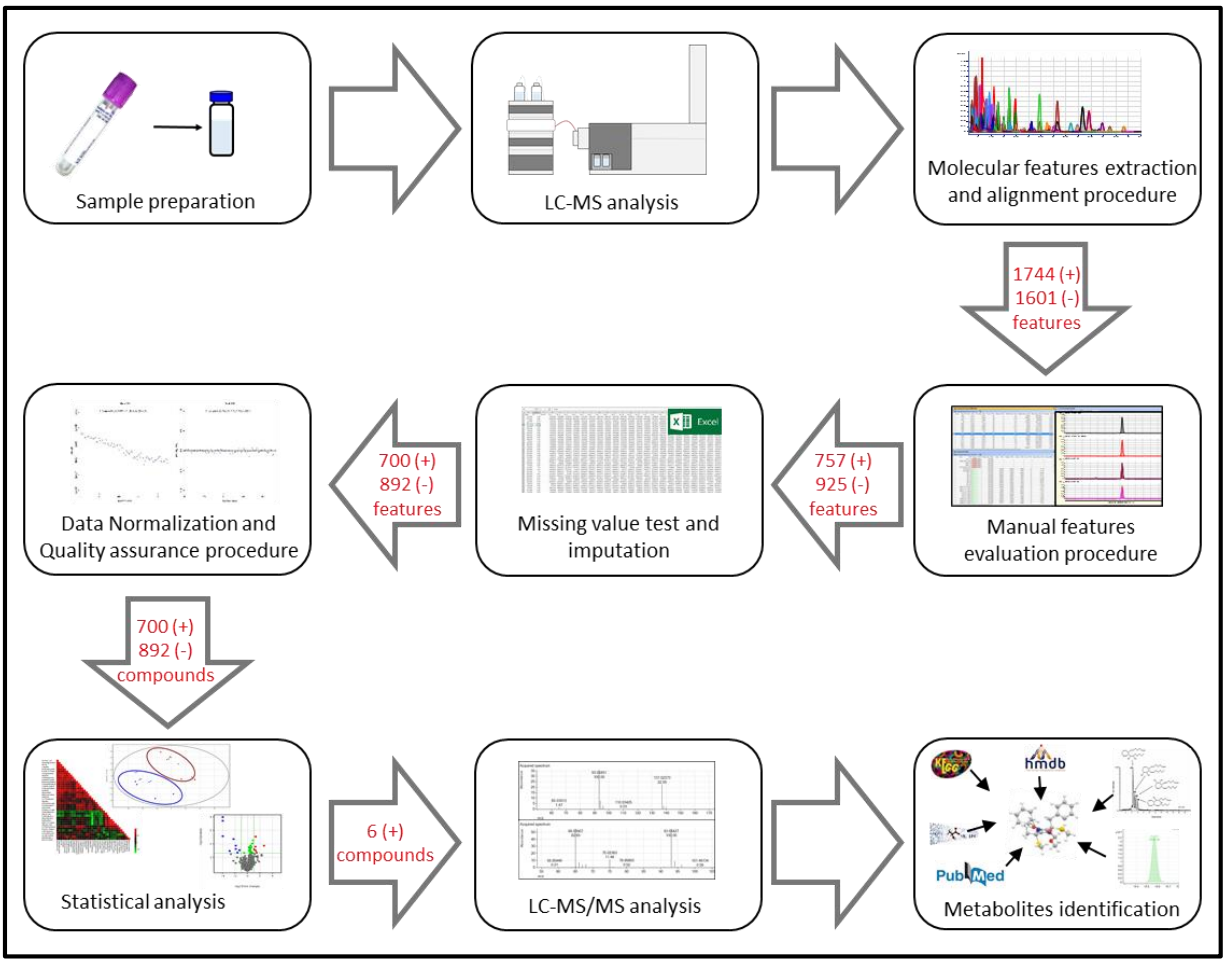


Fig. 29 – Representation of the untargeted workflow applied to analyze plasma samples. In the arrows are reported the number of features or compounds obtained by the different steps, in positive (+) or negative (-) ionization mode.

As regarding to data processing, a total of 3345 features (1744 in positive and 1601 in negative ionization mode) were extracted after data acquisition through the Batch Recursive Feature Extraction algorithm. Following the manual features evaluation procedure, data set was reduced to 1682 (757 and 925, respectively). Features with more than 20% missing values within the same group (MD T0, MD T3, LFD T0 or LFD T3) were removed, thus obtaining a dataset of 1592 features (700 and 892, respectively). Subsequently missing values were imputed by RF algorithm [382] and the dataset were normalized using the support vector regression algorithm [411] which considers

the intensity of the QCs throughout the entire analysis. As shown in the scatter plots (Figure 30) this normalization step allowed to reduce the previously observed variability (positive mode from 15% to 2% CV; negative mode from 3% to 1% CV). Indeed, applying the quality assurance procedure [381], all 1592 compounds were considered reliable compounds.

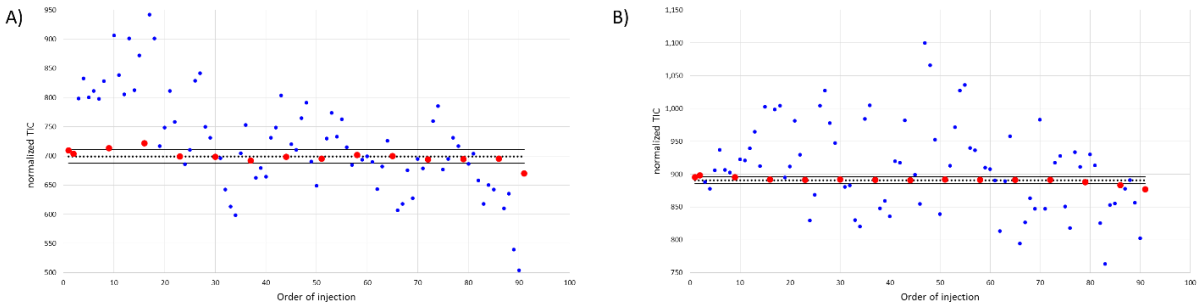


Fig. 30 – Trend plots showing the variation of the total ion current (TIC) mean values over all observations (n=76 samples and n=15 QCs) in positive (A) and negative (B) detection mode after normalization procedure. Quality controls (QC) are colored in red and samples in blu. The black lines represents QCs' TIC mean value ± standard deviation.

5.6.3. Diet metabolic modifications

No differences have been found between groups as baseline through this untargeted metabolomic approach. In Figure 31 are reported all compounds levels at baseline in the MD and LFD subjects. No differences were highlighted in the heatmap.

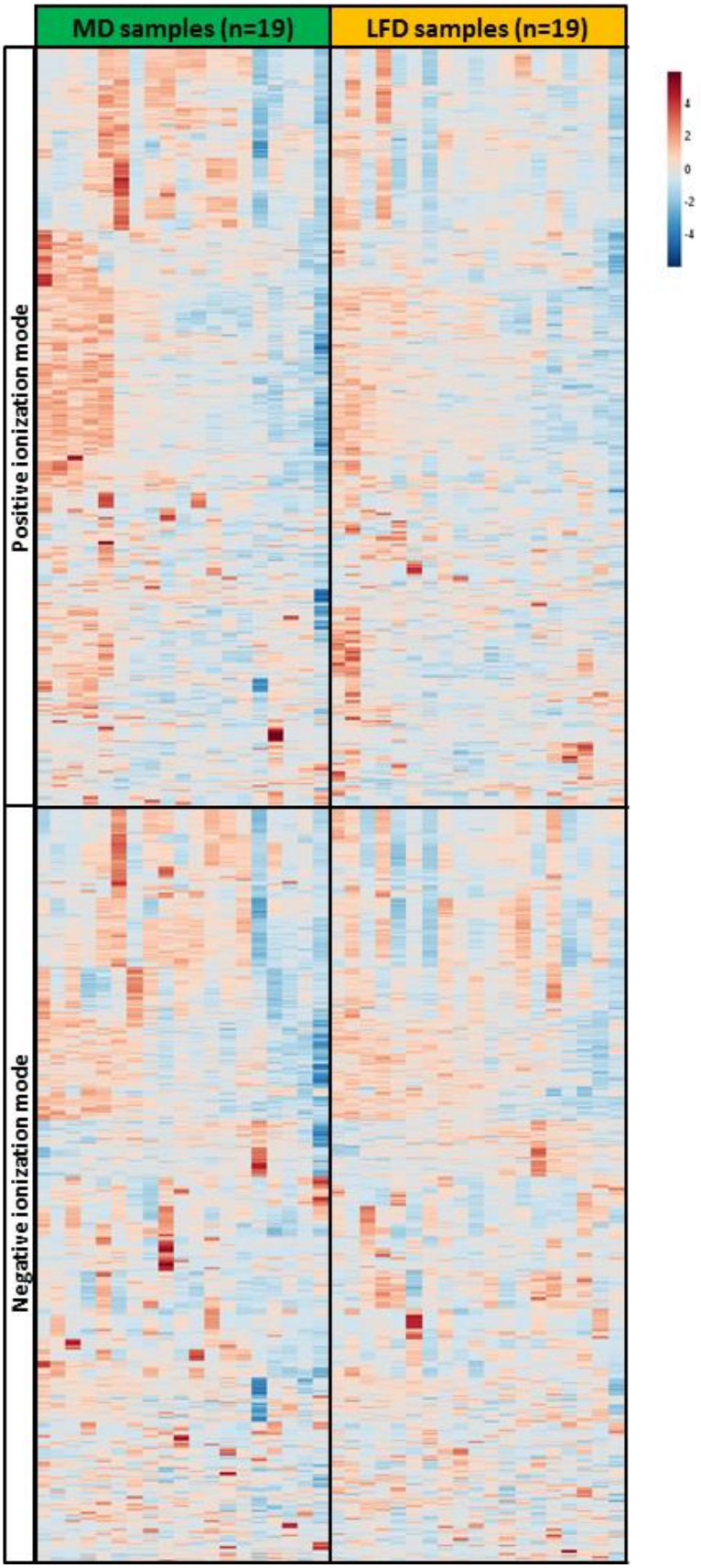


Fig. 31 – Heatmap showing a graphic representation of the differences in relative metabolites concentrations at T0 between MD and LFD group. Rows represent metabolites and columns the samples.

The plot of the first two PCA components is represented in Figure 32. For the MD subgroup the two components explained 23.7% and 1.7% of the total variance for positive ionization mode, 13.8% and 11.9% for negative ionization mode. For the LFD subgroup the two components explained 21.5% and 11.4% of the total variance for positive ionization mode, 14.4% and 13.4% for negative ionization mode. In both subgroups there was no clear separation of the samples at T0 and T3. Supervised PLS-DA confirmed these data: no strong separation between samples before and after dietary interventions was achieved (data not shown).

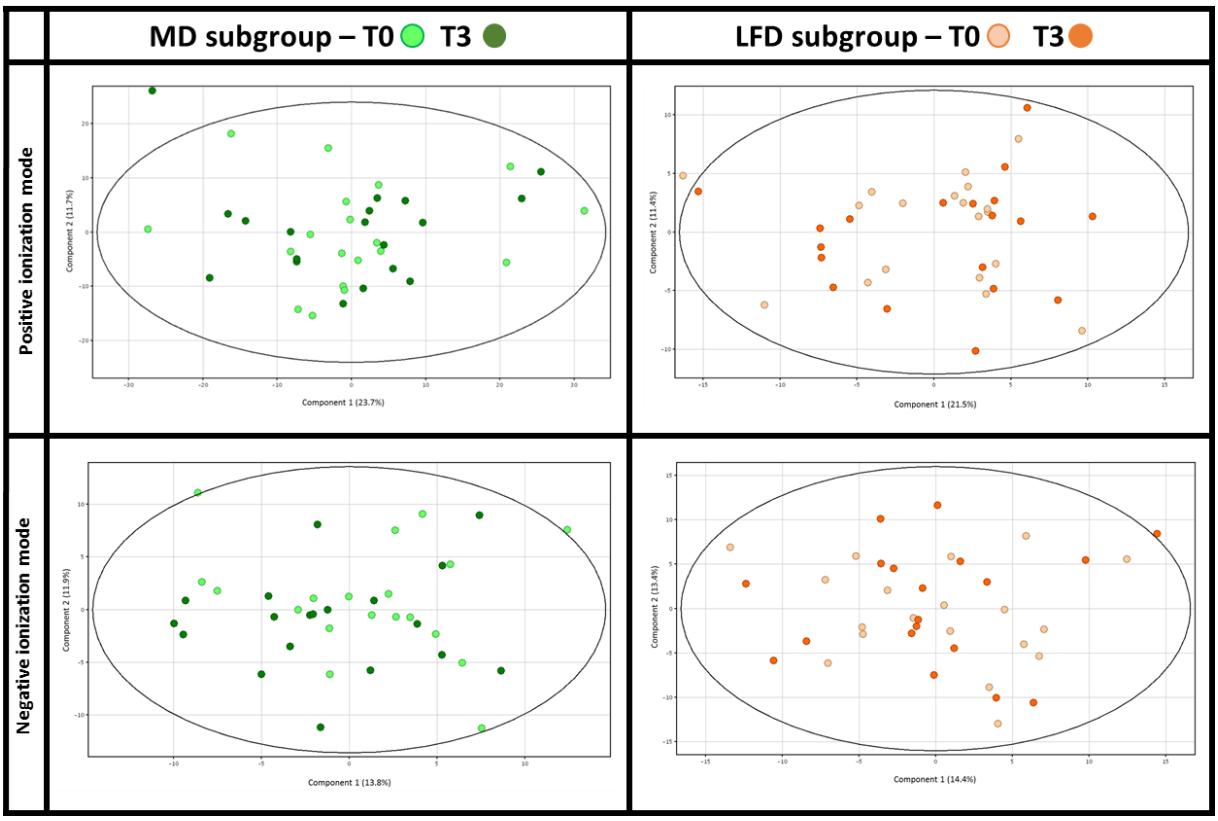


Fig. 32 – Score plot of principal component analysis (PCA) models showing samples at T0 and T3 in the two subgroups, both in positive and negative ionization mode.

To point out which plasma metabolites were modified by dietary interventions, a paired t-test with Storey multiple testing correction between T0 and T3, was performed on the compounds lists of MD and LFD group. The levels of no compound changed after three months of LFD. On the contrary, 6 compound showed a significant modification after MD treatment (p<0.05). The compounds are listed in Figure 33. Most of the compounds showed increasing levels after MD diet.

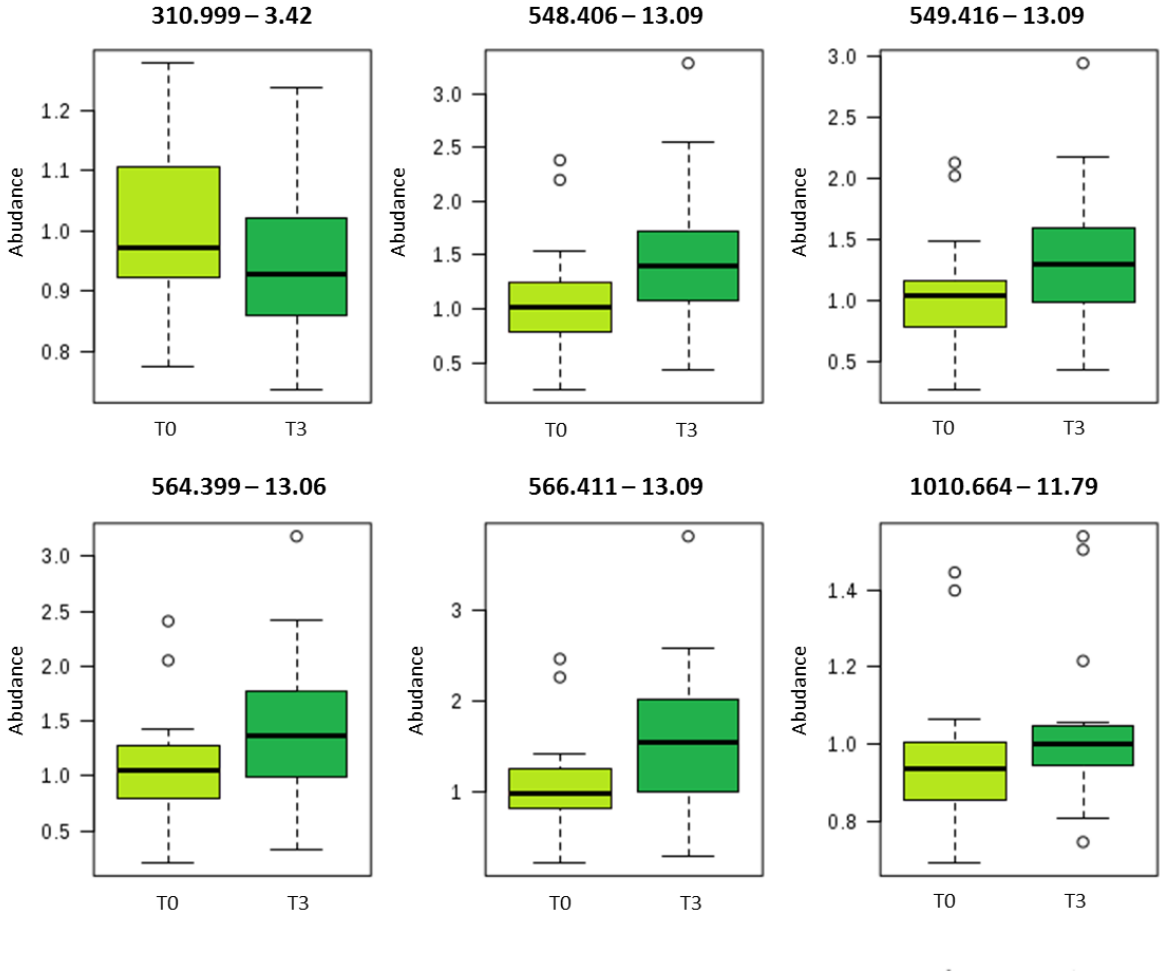


Fig. 33 – Compounds that statistically change between T0 and T3 in MD subgroup. Variables are expressed as median of abundance. Paired t-test with Storey multiple testing correction was used on log transformed data ($p \leq 0.05$). Compounds are defined as measured accurate mass and retention time.

5.6.4. Compounds identification

Not all the significantly different compounds were identified due to the lack of masses and LC-MS/MS spectra on databases.

The compounds with the same RT were strongly related: considering the stable hydrogen isotopic fingerprint, the compound 549.416-13.09 is the stable isotope of the compound 548.406-13.09. Furthermore, the compound 548.406-13.09 is the water neutral loss of 566.4106-13.09. Comparing measured accurate *m/z* values (6 ppm error) and evaluating MS/MS spectra and lipophilic information, the compound 566.4106-13.09 was putatively identified as a ceramide phosphoethanolamines (CPE) (t26:0). Using the same approach the compound 564.399-13.06 was putatively identified as a CPE (t26:1). Therefore, the levels of two different CPE were increased after three month of Mediterranean diet in CAD patients.

6. Discussion

In the RISMeD study, we have evaluated the effects of a Mediterranean dietary pattern, compared to a LFD, in coronary artery disease patients, in order to assess the benefits of MD in secondary prevention. Diets have been administered to patients for three months and the MD adherence was quantified through the MEDAS score, a simple FFQ. Different factors involved in the atherosclerosis have been evaluated, including, classical CAD risk factors, inflammation, oxidative stress, complex lipid profile and gut microbiota.

Study population and Mediterranean diet adherence

Randomized controlled trial, as this one, are usually considered to be the strongest study design in biomedicine providing exact and prescriptive protocols to ensure scientific rigor in the most transparent of ways, by randomly allocating treatment [413]. However, dietary trials reserve several challenges as inadequate adherence to the diet protocol and few approaches to quantitatively control the diet adhesion are available [414].

In order to better understand the effects of MD, two different data analysis strategies have been applied: "***intention to treat***", defined as the analysis of the whole population (n=120, MD=58 and LFD=62), and "***per protocol***", defined as the analysis of an afterwards selected subgroup population (n=38, MD=19 and LFD=19). Indeed, considering the whole population after three months of diet intervention, not only the MD group, but also the LFD one, showed a significant increase in MD adherence, represented by the MEDAS score. The increase of MD adherence in the LFD group, probably due to the overlap of some MD nutritional aspects with the Italian LFD, could hide the results. For this reason, two specific subgroups have been defined: MD subgroup and LFD subgroup. In the former a significant increase in MEDAS score, reflecting MD adherence, have been observed. On the contrary, no modification in LFD subgroup MEDAS score have been observed, indicating no changes in the rate of adherence to a Mediterranean dietary pattern.

The dilemma between *intention to treat* and *per protocol* approaches is a common problem in dietary trials. The first one preserves the advantages of randomization and proves the effects of a treatment strategy. On the other hand, the *per protocol* approach is more appropriate to assess the effect of Mediterranean dietary pattern.

In both cases, there were no differences at baseline between MD and LFD group and subgroups, in terms of demographic characteristics, laboratory parameters, clinical and pharmacological features, except for LDL cholesterol, which was higher in the MD group.

Modifiable CAD risk factors and Mediterranean diet

There are several CAD risk factors, but only some of them are modifiable through diet intervention. Obesity, hypertension, dyslipidemia and hyperglycemia are very common clinical disorders that promotes functional and structural vascular wall injury leading to augmented risk of atherosclerosis. Their treatment and control drastically reduce the risk of secondary CAD event.

In our study, concerning the *intention to treat* analysis (MD=58 and LFD=62), both diet interventions were associated with statistically significant reductions in BMI, waist circumference, blood pressure and plasma lipid profile modifications. Total cholesterol, LDL cholesterol and TGs concentrations decreased after three months of diet. On the contrary, no modifications have been observed in HDL cholesterol and glycaemia levels. These results probably reflect the high adherence to MD of both groups and are in accordance with the observed negative correlation between MEDAS score variation and risk factors values variation. Higher is the increase in MEDAS score during the treatment period, higher is the decrease in BMI, waist circumference and plasma lipid features, regardless group randomization.

Evaluating the subgroups population, both MD and LFD subgroups decreased in BMI and waist circumference, but only the MD subgroup showed lower levels of blood pressure, total and LDL cholesterol, and TGs concentrations compared to baseline. As in the MD and LFD groups, even in the subgroups, none of the diets was able to increase HDL and decrease fasting glycaemia levels.

Considering these results all together, we can assume that both MD and LFD are able to act on obesity/overweight, but only the MD can significantly reduce blood pressure, even if salt intake was not restricted in the RISMeD study, and improve lipid profile in patients who were already receiving antihypertensive medication and lipid lowering drugs. None of the dietary intervention reduces glycaemia concentrations, but, it in this study no diabetic patients have been enrolled and glycaemia levels were close to acceptable range already at baseline.

These data are in accordance with a previous study on CAD patients where was reported a reduction of BMI, waist circumference and blood pressure after three month of both MD or a low-fat therapeutic lifestyle changes diet and no modification in HDL and glycemic levels [415]. Furthermore, they observed a not significant decrease of TGs in both diets. Ambring et al. described a decrement of LDL, TAG and TC, but no changes on HDL and glycaemia by a MD inspired diet on healthy population [416]. In a study on non-alcoholic fatty liver disease patients, 6 month of MD and physical activity significantly increased HDL cholesterol levels [417]. Gomez-Huelgas et al. reported a significant increase in HDL cholesterol after 3-year lifestyle intervention (including

MD) in patients with metabolic syndrome [418]. Our results of no HDL variation might be explained by the short period of treatment, i.e. three months, that could not be sufficient to modulate HDL cholesterol levels.

Observational studies [419, 420] and interventional trials [421, 422] have suggested that increased olive oil intake, typical of MD, produces a decrease in blood pressure [423]. To replace carbohydrate with dietary fat induces TGs levels decrement, while to exchange MUFAs with saturated ones lowers LDL cholesterol levels [224, 424]. Despite the recognized MD benefits, often this diet is incorrectly associated to promoting gain weight because of its high-fat food intake such as olive oil and dried fruit. Despite this belief, previously studies and this study confirmed that MD does not induce increased weight. On the contrary, it lead to a decrease BMI and waist circumference. These results can be explained by the fact that high vegetables, olive oil and dried fruit consumption induce thermogenesis, increases satiety and decrease energy intake from other sources by food compensation [425, 426].

Inflammation and Mediterranean diet

It is already known that inflammation is crucial in the development of instability and rupture of atheromatous plaque and the subsequent appearance of ischemic events [26]. In this study, considering the whole population (MD=58 and LFD=62), we have evaluated different inflammatory markers (leukocytes and basophils count and hs-CRP) but no modification have been observed after three months of diet intervention. However a significant negative correlation have been found between MEDAS score variation and hs-CRP variation: higher is the increase of MD adhesion, higher the decrease of hs-CRP level. This data is in accordance with the *per protocol* analysis results. Indeed, considering the MD subgroup (n=19), where the MEDAS score increased 4.5 points average (2 points more than in the MD group, n=58), hs-CRP, and even basophils count, decrease significantly after MD treatment. These results suggest that hs-CRP could be involved or at least represent a marker of the antiatherogenic mechanisms of MD, supporting the hypothesis that the anti-inflammatory effect of MD plays a pivotal role in cardioprotecion. Pharmacological treatment, in particular aspirin and statins, have pleiotropic anti-inflammatory effects [427, 428], but in the two groups, MD and LFD, these drugs are equally represented. The data obtained are in accordance with previously reported results, on different clinical settings, and with the recent meta-analysis published by Schwingshackl and Hoffmann [310]. For example, in the observational INTERCATH study a strong negative correlation between the MD adherence score and hs-CRP was

evidenced in patients undergoing coronary angiography [275]. Similarly, Lahoz et al. pointed out an inverse correlation between MEDAS score and CRP in Spanish healthy population [429]. Furthermore, they found that this relationship was strongly related to a higher consumption of vegetables, fruits, dairy products, and fish. In obese women, two years of lifestyle changes, including MD adherence, showed decrease in CRP, IL-6 and IL-18 serum levels compared to control group [430]. Another study highlighted that for each unit of increase in MD score, there was 3.1% reduction in the average CRP levels when controlling for potential confounders [431]. Regarding inflammation, the PREDIMED study on primary CAD prevention, showed that three month of MD, compared to LFD, modify the expression of soluble adhesion molecules (CAM-1 and VCAM-1) and cytokines related to atherosclerosis (CRP and IL-6) [261]. In its sub-analysis carried out on the PREDIMED population, it has been addressed also the role of immune cell activation and of soluble inflammatory biomarkers (Mena et al., 2009). The authors observed that the expression of CD49 (an adhesion molecule crucial for leucocyte homing), and of CD40 (a pro-inflammatory ligand) decreased in the MD groups. These inhibitions of both cell-mediated and humoral inflammatory pathways can be viewed as a possible molecular mechanism for the anti-atherosclerotic effect of the MD.

In contrast to these data, few studies and a recent meta-analysis questioned the MD anti-inflammatory role as they did not evidenced significant decrease of inflammatory markers (CRP and IL-6) in CAD patients after MD treatment [271, 316, 432]. Furthermore, preliminary results from the CARDIOPREV study demonstrated, in a CAD cohort, that the impact of diet on CRP is dependent on genotyping of metabolic genes [270]. Genotyping could therefore identify individuals at higher risk of inflammation and thus most likely to benefit from dietary intervention.

To corroborate the MD anti-inflammatory effect observed in this study there are the anti-inflammatory properties of several MD nutrients, such as MUFA, PUFA (e.g. ALA), fiber, alcohol, vitamins (e.g. α-tocopherol, ascorbic acid and β-carotene) and polyphenols through multiple mechanisms. Olive oil, nuts, vegetables, fruits, herbs, oily fish and red wine are particularly rich of these nutrients.

Olive oil, the main culinary fat consumed in a traditional MD [242], is rich of polyphenol and ALA, and it was reported to down-regulate VCAM-1, intercellular adhesion molecule-1 and E-selectin expression in the endothelium [433] and to decrease plasma levels of soluble intercellular adhesion molecule-1 [434]. Several studies have demonstrated that ALA supplementation lowers inflammatory markers concentrations [267, 435]. In addition, the intake of whole grains (Nettleton

et al., 2006), fiber (Ma et al., 2008), or red wine (Chiva-Blanch et al., 2013) has also been associated with an inflammation improvement reducing biomarkers such as hs-CRP, IL-6, IL-1_{α}, TNF_{α}-R2, MCP-1, sICAM-1, or sVCAM-1. Fish, rich in omega-3 fatty acids, can contribute as omega-3 fatty acids induce reduction of cell membrane arachidonic acid content resulting in a decrease of eicosanoids pro-inflammatory synthesis [436].

Another relevant aspect is the relationship between inflammation, diet and weight. Adipose tissue is currently considered to secrete a large number of physiologically active peptides that have similar properties to cytokines [437]. The observed weight loss could contribute to the anti-inflammatory effect of MD but could not be the exhaustive mechanism as both groups, MD and LFD, showed decreased BMI after the diets (considering both *intention to treat* or *per protocol* analysis).

The synergism of all these mechanism produces an anti-inflammatory effect, reflected by the decrease of CRP and basophils levels after three months of strong MD adherence. Considering the pivotal role of inflammation in the pathogenesis and development of atherosclerosis, this result, obtained even with a short diet period intervention, is of considerable significance.

Oxidative stress and Mediterranean diet

ROS influence on endothelial underlying molecules that promote apoptosis, necrosis and therefore thrombosis of atherosclerotic plaques makes oxidative stress a crucial hallmark of CAD and one of its early causative factor.

As ROS cannot be directly measured due to their high reactivity, in the RISMeD study we have evaluated oxidative stress through the measurement of oxidative stress biomarkers (8-iso-$PGF_{2\alpha}$, 8-OHdG and GSH/GSSG) and antioxidant molecules (ɣ-tocopherol and α-tocopherol). Regarding 8-OHdG, as stated by the European Standards Committee on Urinary (DNA) Lesion Analysis (ESCULA), a fully recognized method to determine 8-OHdG is not defined so far [438] even if some methods have been already proposed including enzyme-linked immunosorbent assays (ELISA) [439], HPLC or capillary electrophoresis combined with electrochemical detection [440, 441] and GC or HPLC coupled with MS [442, 443]. Therefore, in the first part of this project, a simple, sensitive and reliable LC-MS method for the quantification of 8-OHdG have been developed and validated [134]. LC-MS is the gold standard technique for quantification, and the validation through the FDA guidelines ensures the highest level of reliability to the developed method.

8-iso-$PGF_{2\alpha}$ concentration represents the most accurate measure of oxidative damage to lipids in humans as it is produced *in vivo* by a non-enzymatic free radical-catalyzed peroxidation of arachidonic acid [141]. Furthermore, higher levels have been evidence in CAD patients compared to healthy subjects [147]. In our study, a significant reduction from baseline of lipid peroxidation in both groups (MD and LFD) and in both subgroups (MD subgroup and LFD subgroup), was observed. These data suggested that a reduction in lipid oxidation damage is achievable not necessary by a MD diet, but even by a LFD. This effect can be, probably, attributed to the low-fat content of the control diet. Indeed, in different experimental studies, diets reduced in fat nutrients have been related to low levels of oxidative stress and of 8-iso-$PGF_{2\alpha}$ [444, 445]. A study on patients with metabolic syndrome showed that both MD and LFD can induce a significant decrease on urinary F2-isoprostanes after 1 year treatment [334]. In addition, a recent study, on older Australian subjects, showed a reduction of F2-isoprostanes blood levels after 3 and 6 month of MD diet, but not in a habitual diet [446]. According to these results, *in vitro* and *in vivo* experiments showed that hydroxytyrosol, contained in olive oil, or olive oil itself can decrease isoprostanes [447, 448]. Similar results have been obtained with a Mediterranean vegetable soup or red wine [449, 450]. On the contrary, 8-OHdG concentrations and GSH/GSSG ratio were unchanged in the two groups and subgroups. 8-OHdG, the final product of guanine oxidation and biomarker of oxidative DNA damage, have been found decremented after 1-year of MD or LFD diet in the PREDIMED study [334]. Hoverer, no variation was evidenced in young students adhering a MD for 3 months [451]. The same study pointed out no changes even in the plasma levels of GSH, the major intra- and extracellular thiol. These data were confirmed in another study on CAD patients treated for 3 months with a MD [415]. Nevertheless, two observational studies demonstrated that adherence to the Mediterranean diet, evaluated through MD scores, is associated with higher GSH/GSSG ratio [452, 453]. Thus, probably a longer diet period is necessary in order to evidence benefits on DNA oxidative damage and modification of GSH/GSSG ratio. The different results, obtained about the different oxidative stress markers, support the need to use more than a single oxidative stress marker to validate the antioxidant efficiency of dietary patterns.

Regarding antioxidant molecules, from baseline to the end of the diet intervention period no changes in ɣ-tocopherol levels have been observed, in any group or subgroup, but unexpectedly a decrease of α-tocopherol plasma concentration was evidenced only in the MD group and MD subgroup. This is in line with two previously studies on a 3 months MD intervention on patients with rheumatoid arthritis or young volunteers that pointed out a decrease of α-tocopherol [451,

454]. However, tocopherols circulate in blood with lipoproteins, thus, the plasma levels are often influenced by the blood lipid concentrations. During the course of this study, the TC decreased significantly in both groups, and particularly in the MD subgroup, and probably influenced tocopherols levels. Thus, ɣ-tocopherol and α-tocopherol were corrected for TC concentration as proposed by Turnham et al [455]. The ratio between ɣ-tocopherol or α-tocopherol and TC did not change in any group.

Taken together, these results provide a strong rationale for recommending dietary pattern changes, such as the Mediterranean diet or LFD, to improve oxidative stress, particularly lipid peroxidation. This MD effect could be explained through the high intake of antioxidant rich food, e.g. olive oil is rich in MUFAs and polyphenols that have high antioxidant activity. Polyphenols are able to down regulate the expression of pro-atherosclerosis genes and related inflammatory and lipid oxidative markers [456]. In addition, antioxidant vitamins (β-carotene, the vitamin A precursor, vitamins C and vitamin E) are introduced through fruit and vegetable consumption.

Gut microbiota and Mediterranean diet

Gut microbiota has been recently implicated as a novel endocrine organ that plays a relevant role in cardiovascular disease. TMAO pathway evaluation, is a subrogate way to estimate diet and gut microbiota. Furthermore, TMAO itself is an independent predictor of CVD risk.

For these reasons, a LC-MS method have been developed and validated during my PhD course, in order to assess not only TMAO plasma levels, but even TMAO precursors (choline, carnitine and betaine) and TMAO intermediate (TMA). Indeed up to now, few LC-MS methods have been published to evaluate TMAO plasma levels but none of them is able to quantify all these compounds in a single analysis [457-460]. The method developed in this study is, to our knowledge, the first one able to determine plasmatic concentration of TMAO, choline, carnitine, betaine and TMA in a single LC-MS analysis. Furthermore, sample preparation is extremely simple, a small amount of plasma is required (50 µL) and the use of specific internal standard for each compound and the validation according to the FDA guidelines ensure strong reliability to the developed method.

Three months of MD or LFD seem not to be enough to modify the gut microbiota, specifically, to change microbiota related to TMAO synthesis, as no modification of plasma TMAO levels have been observed between baseline and the end of dietary treatment. Neither TMA, choline and

carnitine showed alterations. The results obtained in the group analysis (MD *vs* LFD group) were reflected in the subgroup analysis (MD *vs* LFD subgroup).

Considering the *intention to treat* analysis (MD *vs* LFD group) increased betaine levels have been observed after both MD and LFD treatments. However, through the *per protocol* analysis this increase was statistically significant only for the MD subgroup. These results indicates that betaine raise is related to MD. Furthermore, this association is confirmed by the significant correlation between MEDAS score and betaine levels.

Very few interventional dietary trials have been published on these gut microbiota related metabolites. A lifestyle intervention (regarding diet intervention to reduce saturated fat intake and to increase fiber intake) on healthy subject showed no changes in serum TMAO after 9 months [347]. Previously studies demonstrated that betaine supplementation attenuated atherosclerotic lesions in apolipoprotein E-/- mice [461]. On the contrary, dietary supplementation with PC, choline, carnitine, and TMA, in mice, promoted atherosclerosis, that can be prevented by the antibiotic suppression of the microbiota [165]. Furthermore, betaine/choline ratio was inversely associated with CVD at high cardiovascular risk [462], suggesting that betaine could have a relevant role in cardiovascular events prevention. A study on older subjects associated higher betaine plasma concentrations to a favorable cardiometabolic risk profile and lower risk of diabetes mellitus [463]. In a cross-sectional study, on a subgroup population of the PREDIMED study before dietary treatments, lower betaine levels have been found in sample urine of high MD adherence subjects (MEDAS score > 10) compared to low MD adherence subjects (MEDAS score <7) [397]. However, in another PREDIMED study on a different subgroup, a 1 SD increment of plasma betaine after 1 year MD treatment was associated with lower risk of CVD [462].

The results obtained in our RISMeD study, no modification to gut microbiota but increased betaine levels, should be considered very interestingly as no other studies investigating the MD effect on these metabolites have been previously published. The increase on betaine levels can be considered a positive MD effect on the basis of published prospective studies even if the mechanism involves on betaine benefits are not yet clearly understood.

Complex lipid profile and Mediterranean diet

Altered lipid metabolism is among the main culprits in atherosclerosis and thus represent a prime target in cardiovascular research. For this reason, we analyzed the effects of the RISMeD diet interventions on a lipid profile that includes more than 180 lipids as LPCs, PCs, PSs, phosphatidylethanolamine, PAs, PIs, LacCer, Cer, GCer, SMs, Sul and GMs. If no modification have been observed after three months of LFD, a strong effect of Mediterranean diet was evidenced. These changes are highlighted through the built PCA by which is possible to clearly discriminate in the MD subgroup before and after treatment samples. Furthermore, this analysis pointed out a similar "migration" of subjects indicating a comparable effect of MD on all patients. Among the evaluated lipids, MD induced an increase of several PCs with diacyl residues (PC 26:0, PC 30:0, PC 32:1, PC 34:3 and PC 34:4), LPCs with acyl residue (LPC 14:0, LPC 16:1, LPC 20:3 and LPC 28:1) and PEs with diacyl (PE 34:1, PE 36:2 and PE 36:3) or acyl-alkyl (PE 38:6) residues.

Phosphatidylcholines, LPCs and PE are members of the glycerophospholipid family. The PCs and PEs are a major constituent of cellular membranes, blood lipoproteins and natural surfactants, and the reservoir of fatty acids, phosphate and glycerol. The partial hydrolysis of PCs produces LPCs, that are bioactive compounds involved in monocyte recruitment, vascular SMCs proliferation and endothelial dysfunction [464]. Up to now, their role is CAD is still unclear, they seem to have both pro- and anti-atherogenic properties [465, 466] and less is known about individual lipid function. Obviously, PCs and LPCs are related to choline accumulation. However, no modification in choline plasma levels have been observed in our study. This result could be explained by choline absorption regulation: choline is absorbed from the small intestine via mediated transport, which is saturable [467].

Most of the modified lipids seem to have long fatty acid chain, even if the exact fatty acids composition of these molecules was not determined in this study. It was reported by different studies that the longer the chain the lower was the CVD risk [180, 350] and that the substitution of SFAs intake with lower number of carbon atoms exerted a more negative effect on lipids and CVD than those with higher number of carbon atoms [468]. In addition, PCs are the most abundant membrane lipids and long chain PCs confer more fluidity to the cell membranes. Lower levels of PCs (including PC aa 30:0 and 34:3) and LPCs (including LPC 20:3) have been reported to be related to increased arterial stiffness, increased resting heart rate and worse endothelial dysfunction in CAD and peripheral arterial disease patients compared to healthy subjects. Moreover, PC aa 30:0 and LPC 14:0 were higher in healthy subjects than CAD patients [184, 186].

Lysophosphatidylcholines showed an inverse correlation with CVD risk in the Bruneck study too [469].Taken together, we can speculate that the increase of PCs and LPCs after MD intervention could have positive effects.

Regarding MD intervention, a lipidome plasma analysis was performed on a subgroup of the PREDIMED study. No changes in lipids scores (created on lipid families associated to CVD risk: group A composed by lipids inversely associated to CVD; score B composed by lipids directly associated to CVD) have been reported after 1-year MD treatment [349]. However, PC and LPC family were associated to a lower CVD risk and PE family to a higher risk. Evaluating the single lipid, instead of the whole family, only CE 20:3 increased after MD supplemented with nuts treatment [350]. Another study, on patients with metabolic syndrome, reported plasma lipid profile changes after 2 months of RESMENA diet, a diet based on Mediterranean dietary pattern [398]. The most discriminant markers of dietary treatment were PCs and LPCs. Several PCs and LPCs increased after the RESMENA diet compared to a diet based on the AHA guidelines as reported in our results.

Untargeted metabolomics approach and Mediterranean diet

An innovative untargeted metabolomics approach was applied in order to evidence metabolites affected by the Mediterranean dietary pattern and their possible role in MD cardioprotective effect. More than 1500 metabolites have been evaluated but only 6 were modified in the MD subgroup. Not all the significant different compounds were identified due to the lack of masses and LC-MS/MS spectra available on databases. CPE 26:0 and CPE 26:1 were identified through the comparison of their acquired LC-QTOF-MS/MS spectra with those available on databases and through the evaluation of the agreement between RT and their polarity. Probably, the use of other techniques, such as multi-dimensional MS and NMR could be useful to elucidate the unknown compounds.

Increased levels of these two CPEs, CPE 26:0 and CPE 26:1 have been evidenced. These lipid compounds are produced from Cer through a sphingomyelin synthase related to protein in the endoplasmic reticulum (ER) [470]. The CPE functions are not known, probably, they prevent Cer accumulation in endoplasmic reticulum [471]. Up to know, little is known about CPEs, thus, we can only speculate that the increase of CPE levels could reflect the increase of Cer even if no significant modification in Cer plasma levels have been found.

Two limits of our untargeted metabolomics study have to be highlighted as they could have determined the little changes we have found. It is possible that 3 months interventions is too short

to induce appreciable changes in endogenous metabolites or the number of patients had not enough statistical power to detect differences due to the huge number of considered metabolites. Indeed, applying a lower restrictive statistical analysis (without correction for multiple comparison) more than 100 compounds showed altered levels, but this is not considered a reliable approach.

7. Conclusions

The present study has several strengths: it involves a plenty number of patients; the randomized design with the presence of an active comparator (low-fat diet) avoids main confounding factors (sex, age, drugs, etc.); good adherence of patients to diets has been obtained; the capability to record several aspects involved in coronary artery disease allows a more comprehensive evaluation of the diet effects. However, some potential limitations should be highlighted: the RISMeD study was performed only in Italy, in a population with a diet that already partly includes foods of the Mediterranean diet; the dietary assessment was based on a simple food frequency questionnaire and not biologically verified.

However, the strength and the consistency of the data presented here suggests that the beneficial effect of Mediterranean diet on secondary prevention, observed in epidemiologic studies, could be mediated in several ways by:

1) lowering BMI and waist circumference
2) decreasing systolic and diastolic blood pressure
3) reducing total cholesterol, LDL cholesterol and triglycerides levels
4) downregulating proinflammatory biomarkers
5) limiting lipid peroxidation
6) incrementing compounds correlated to lower CVD risk such as betaine, phosphatidylcholines and lysophosphatidylcholines

In conclusion, our results suggest a positive effect of Mediterranean diet on coronary artery disease patients that can be the results of improving different antiatherogenic features. Their synergy could be the most important determinant of the MD positive effect. This study suggests that, a wider dissemination of Mediterranean diet should be advised as lifestyle change parallel to drug therapy in secondary CAD prevention.

8. References

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