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DIET, OXIDATIVE STRESS STATUS AND INFLAMMATION IN PATIENTS WITH CARDIOVASCULAR DISEASE: IN SEARCH FOR A LINK

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Contents

Abstract

BACKGROUND. Previous studies have provided a rationale for understanding the health benefits of Mediterranean Diet (MD) against a variety of chronic diseases.
OBJECTIVES AND EVIDENCE ANALYZED. We have used new data from the IMPROVE Study -a multicenter, longitudinal, observational study, carried out in five European countries- and fresh information from the RISMED, a study carried out in our Institution in subjects with a recent history of coronary revascularization to address: 1) the relationship between MD adherence and carotid intima-media thickness (c-IMT) progression; 2) whether such relationship is similar in populations with different nutritional patterns, and 3) whether the relationship between MD, baseline carotid IMT, and clinical events involves changes in inflammation, oxidative stress, and fatty acid [FA] composition.
FINDINGS. In the analysis of the IMPROVE Study we have found: 1) a significant negative trend between most of the c-IMT-progression variables and a new relatively simple food frequency questionnaire to evaluate a Mediterranean-like dietary pattern (MLPD) score, validated *a priori* vs VEs in different European Countries; 2) that the significant association between MLDP adherence and VEs is independent of baseline c-IMT and c-IMT progression, and 3) that MLDP score adherence is associated to changes both in inflammatory markers and white blood cells counts. Information from the RISMED Study documented that: 1) MD adherence significantly affects indices of inflammation and oxidative stress balance; 2) blood FA profile is an index of the quality of food intake which might be used to estimate diet quality and compliance with nutritional advices; 3) it is possible to favorably modify patients' plasma FA profiles by consuming a MD, and 4) a relationship exists between inflammatory markers/oxidative stress status and modifications of the blood FA pattern.
CONCLUSIONS AND RELEVANCE. MD adherence is key to reduce oxidative stress, inflammation and VEs in subjects at risk of cardiovascular events. Since common approaches that simultaneously target old and new risk factors for cardiovascular

disease enhance cardio-protection and longevity, the need for evaluating MD as a strategy for a better health at older ages should be explored thoroughly.

Riassunto.

STATO DELL'ARTE. Studi precedenti hanno fornito una spiegazione degli effetti benefici della dieta mediterranea (MD) nei confronti di varie malattie croniche.

OBIETTIVI E DATI ANALIZZATI. Abbiamo utilizzato nuovi dati dallo studio IMPROVE - uno studio multicentrico, longitudinale e osservazionale, svolto in cinque paesi europei - e informazioni preliminari dal RISMED, uno studio condotto presso il nostro Istituto in soggetti con una recente storia di rivascolarizzazione coronarica per valutare : 1) la correlazione: aderenza ad una MD e progressione dello spessore medio-intimale della carotide (c-IMT); 2) se tale correlazione sia simile in popolazioni con differenti modelli nutrizionali, e 3) se la relazione tra MD, c-IMT al basale ed eventi clinici (VEs), si accompagni a cambiamenti negli indici di infiammazione, di stress ossidativo e di composizione ematica in acidi grassi [FA].

RISULTATI. Dalle nuove analisi dello studio IMPROVE è emerso: 1) un significativo trend negativo di associazione tra la maggior parte delle variabili di progressione del c-IMT e il punteggio derivante da un nuovo score nutrizionale (MLPD), generato da un questionario relativamente semplice di frequenza di assunzione di 7 alimenti tipici di una dieta mediterranea. Tale score è stato in precedenza validato contro l'insorgenza di eventi vascolari (VEs) nelle diverse popolazioni europee coinvolte nello studio IMPROVE; 2) che l'associazione significativa tra l'aderenza allo score MLDP e i VEs è indipendente dalla progressione del c-IMT e dal c-IMT al basale, e 3) che l'aderenza allo score MLDP è associata a cambiamenti sia nei marcatori di infiammazione che nella conta dei globuli bianchi. I risultati dello studio RISMED documentarono che: 1) l'aderenza alla DM influenza in maniera significativa gli indici di infiammazione e di stress ossidativo; 2) che il profilo dei FA del sangue è un indice della qualità del cibo assunto e che tale indice può servire a stimare la qualità degli alimenti ingeriti e la conformità dei pazienti ai consigli nutrizionali; 3) che il consumo di una DM può modificare favorevolmente il profilo in FA del plasma dei pazienti, e 4) che esiste una correlazione tra marcatori dello stato infiammatorio e di stress ossidativo e modifiche del pattern di FA.

CONCLUSIONI E RILEVANZA. In soggetti a rischio di eventi cardiovascolari, l'adesione ad una MD è fondamentale per ridurre lo stress ossidativo, l'infiammazione e gli VEs. Poiché gli approcci che contemporaneamente modificano vecchi e nuovi fattori di rischio per la malattia cardiovascolare aumentano la protezione cardiaca e la longevità, occorre esplorare in modo approfondito la MD come strategia per una migliore salute nell'anziano.

Introduction

In 2013, UNESCO recognized the Mediterranean Diet (MD) as an "Intangible Cultural Heritage of Italy, Portugal, Spain, Morocco, Greece, Cyprus and Croatia". Despite different habits in the area, common characteristics prevail in the dietary patterns in the Mediterranean basin: high consumption of olive oil, legumes, unrefined cereals, fruits, and vegetables, moderate/high fish consumption, moderate consumption of dairy products (mostly as cheese and yogurt), moderate wine consumption (especially during meals), and low consumption of meat and meat products. Olive oil –that has a central position in all such dietary patterns-, is important not only for its own health benefits, but also because it is often consumed with large 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 (**Figure 1**). The Italian variant of MD is characterized by high pasta consumption, whereas fish consumption is particularly high in Spain. [1, 2] Total fat content is generally ≥40% (Greece), or ≥30% (Italy) of total energy intake. [1] However, the monounsaturated to saturated fats ratio of MD in Southern European countries is much higher than in other regions of the world, including northern Europe and North America. Thus, it is appropriate to consider these 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. [3]

Figure 1 Mediterranean Diet: geographical distribution and major components

Historically, it might be convenient to define MD 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 invaded the basin.[1]

History

In the Greek medical tradition, first 'rational' medical treatises were written in the fifth century BC by a series of authors associated with Hippocrates and his thought over a period of more than 150 years. In the thought of the Hippocratic doctors, food was absorbed into the body to be broken down into nutritional substances to sustain the body and to guarantee the body's heat. How these products acted within the body, however, was not clear.

In his adaptation of the Hippocratic system, Galen (second century AD) extended and refined the Hippocratic system and identified nutrition, drug therapy and surgery as three branches of medicine. Accordingly, many doctors, including Galen wrote cookery books.[4] Galen wrote extensive treatises on nutrition, and clarified that diet might also have drug-based outcomes. Whether and to what extent diet was an integral entity or the sum of identifiable components (e.g., olive oil, fruits, vegetables) to be evaluated separately, or whether the beneficial effects of a diet (e.g. the MD) or of its major components were reproduced in different populations (e.g. in people living far from the Mediterranean region) was not addressed by Galen.[1]

Ancel Keys' initiative.

Ancel Keys developed the concept that, at the population level, "rich" diets might be linked to higher plasma cholesterol and to a higher than normal tendency to coronary artery disease (CAD).[5] This observation was pursued during his long-stay in Italy (Pioppi), where he highlighted that the rate of ischemic heart disease was lower in Mediterranean regions.

This was tested in the "Seven Countries Study", a longitudinal survey of population samples from Finland, Greece, Italy, Japan, The Netherlands, U.S. and Yugoslavia, with at least 5-year follow-up. Medical records; ECGs at rest and after exercise, and a blood sample were obtained from population-based men 40-59 years old. The basic concept of a 3-way relationship between dietary lipids, serum cholesterol, and CAD emerged from the results of the Seven Countries Study[6, 7]. In cross-population analyses, 10-yr coronary mortality rate was strongly related to average dietary saturated fatty acid intake. Higher values were found in Finnish, Dutch, and American samples, intermediate intake levels in the Mediterranean population samples, and low values in Japanese men. Similar findings were found for serum cholesterol: the highest the sample average cholesterol level, the highest the coronary mortality rate. In addition, dietary saturated fatty acids were closely related to serum cholesterol. Overall, these data argued for serum cholesterol as a major CAD risk factor, and for the need of a population-wide prevention of epidemic cardiovascular (CV) disease (CVD)[8].

Studies supporting Ancel Keys' observation

The Lyon Diet Heart Study is a prospective, randomized single-blinded secondary prevention multicenter trial -death and nonfatal AMI being its primary endpoints-, designed to test the hypothesis that a MD enriched with α-linoleic acid (ALA) might improve the prognosis of survivors of a first acute myocardial infarction (AMI).[9-12] Of the 600 survivors enlisted, half adopted a prudential diet and as many adopted a Mediterranean-type of diet, with no more butter, cream and delicatessen (which were replaced by poultry); less meat (beef, lamb, pork), and more bread, more cereals, more legumes and beans, more fresh vegetables and fruits, more fish, and more olive oil. Olive oil was recommended for salad and food preparations. For subjects who did not like it, olive oil was replaced by an experimental canola oil-based margarine (erucid acid free rapeseed oil). All dietary instructions were detailed and customized to each patient.[9-12] This strategy was well accepted by the patients who, at the end of the trial, were still following dietary patterns superimposable to those of the protocol.[9-12]

The whole protocol stemmed from the lowest rates in the world of cardiovascular diseases (and of vascular complications in patients with established CAD) in populations either following a MD or a diet low in n-6 fatty acids and rich in n-3 fatty acids (a major component of fish and fish oil).[9-12] In addition to a low n-6/ n-3 fatty acid ratio, and to the high oleic acid intake (and the low saturated fat intake) of the traditional MD, patients were instructed to lower animal fat consumption and to use vegetable oils such as olive oil (that does not contain ALA) and/or canola oil (that contains ALA and moderate amounts of LA). [12] The experimental MD tested in the trial supplied less than 30% of energy from fats and less than 8% of energy from saturated fats. As to essential fatty acids, the intake of LA, the main n-6 fatty acid, was restricted to 4% of energy and the intake of ALA, the main n-3 fatty acid, made up more than 0.6% of energy.[13]

The subjects were followed up for 5 years starting 6 months after the ischemic event.

At the 2-year follow-up, there were 16 CV deaths in the control group and 3 in the experimental one. After correction for other factors, a surprisingly high (76%) reduction in the risk of cardiac death was found during the observation period.[12] Consistent with the protective effect of the diet, a striking 50 to 70% reduction of the risk of recurrence was reported after four years of follow-up.[9-12] Such reduction was associated with enhanced plasma levels of eicosapentaenoic acid (EPA) and its parent fatty acid, ALA. In keeping with this, plasma arachidonic acid (the fatty acid precursor of the 2 series of prostaglandins and thromboxane), was significantly ($p<0.005$) reduced in subjects who had received the experimental diet. In addition, vitamin E, which has been previously reported to reduce the risk of CAD[14], was significantly ($p<0.005$) increased while granulocyte count was lowered. On the other hand, high-normal white cells counts have been related with cerebral thrombosis and re-infarction, maximal association being found with granulocyte counts. [15-17] The involvement of white cells in blood rheology, endothelial cell injury (free radical generation), and blood coagulation (generation of tissue factor) provides a biological plausibility for this association. Thus, mechanisms other than polyunsaturated fatty acids changes should be considered in the cardio-protective effects of this diet.[18-20]

European Prospective Investigation into Cancer and Nutrition (EPIC). This is an ongoing multi-center prospective cohort study designed to investigate the relationship between nutrition and cancer, with the potential for studying other diseases.[21] EPIC is the largest single resource available today world-wide for prospective investigations on the etiology of cancers (and other diseases) that can integrate questionnaire data on lifestyle and diet, biomarkers of diet and of endogenous metabolism (e.g. hormones and growth factors) and genetic polymorphisms.[21] The study currently includes 519, 978 participants (366, 521 women and 153, 457 men, mostly aged 35–70 years) in 23 centers located in 10 European countries, to be followed for cancer incidence and cause-specific mortality for several decades. At enrolment, which took place between 1992 and

2000 at each of the different centers, information addressing usual diet was collected through a dietary questionnaire and information on lifestyle variables through a non-dietary questionnaire. Anthropometric measurements were performed and blood samples taken; plasma, serum, red cells and buffy coat fractions were separated and aliquoted were stored mostly in liquid nitrogen for long-term evaluations.[21] The usual dietary intake during the year preceding enrollment was assessed with the use of a semi-quantitative validated food-frequency questionnaire including approximately 150 commonly consumed foods and beverages and the questionnaire was administered by specially trained interviewers.[22, 23] For each of the items, individuals were asked to report their frequency of consumption and portion size, the latter being calculated based on information provided on household units and 76 photographs of usual portion sizes. Standard portion sizes were used for the estimation of consumed quantities, [23] and nutrient intakes were calculated by means of a food-composition data base modified to accommodate dietary specificities of individual Countries.[24]
Eventually, 14 all-inclusive food groups or nutrients were considered: meat, fish, eggs, dairy products, potatoes, vegetables, legumes, fruits and nuts, cereals, sugar and sweets, nonalcoholic beverages, saturated lipids and margarines, and monounsaturated (mainly olive oil), and polyunsaturated lipids (vegetable-seed oils). For each participant, intake (g/day) of each of the indicated groups and total energy intake were calculated.[22] To calibrate dietary measurements, a standardized, computer-assisted 24-hour dietary recall was implemented at each center on stratified random samples of the participants, for a total of 36,900 subjects.
On 22,043 Greek adults from the Greek cohort, Trichopoulou et al. carried out an observational prospective study to investigate the capacity of a 10-point scale Mediterranean (MD) score to predict the overall and CAD mortality. During a median of 44 months of follow-up, there were 275 deaths. A high adherence to the MD was associated with a lower total mortality (adjusted hazard ratio [HR] for death associated with a two-point increment in the MD score, 0.75 [95 %

confidence interval [CI], 0.64 to 0.87]). An inverse association with high adherence to MD diet was true for both death due to CAD (HR, 0.67 [95% CI, 0.47 to 0.94]) and death due to cancer (HR, 0.76 [95% CI, 0.59 to 0.98]).[a] The association with the MD score appeared to be slightly stronger for mortality from CAD than for mortality from cancer, although mortality from cancer was reduced significantly. The inverse association between the Mediterranean-diet score and total mortality was independent of sex, body-mass index (BMI), waist-to-hip ratio, smoking, level of education and of physical activity. In analyses stratified according to age, the relation between the MD score and mortality was significant among participants 55 years of age or older but not among participants younger than 55 years of age (P=0.34).[24] In analyses adjusted for age, sex, BMI, smoking habits, waist to-hip ratio, energy-expenditure score, total energy intake, and years of education, the only individual measures that were predictive of total mortality were the intake of fruits and nuts and the ratio of monounsaturated/saturated lipids.

PREDIMED Study was a multicenter nutritional primary prevention randomized clinical trial (RCT) carried out in Spain between 2003 and 2011 which tested the long-term effects of the MD on incident CVD in men and women at high CVD risk (but no history of previous CVD episodes at enrolment) aged 55-75 years (men) or 60- 80 years (women). Participants were selected from >200 primary care facilities affiliated with 11 recruiting sites. The presence of either type 2 diabetes mellitus (T2DM) or ≥3 risk factors (smoking, overweight or obesity, hypertension/HTN, dyslipidemia/DLP, and family history of early-onset CVD) were major criteria for recruitment. Participants were randomized into one of three diets: 1) MD supplemented with extra-virgin olive oil (EVOO); 2) MD supplemented with nuts; and 3) control diet (advice on a low-fat diet). Trained dietitians delivered the intervention. Throughout the study, participants attended individual visits and group sessions in which they were instructed to follow their diets. Participants also

[a] An example of dietary changes resulting in such an increment would be a substantial increase in the intake of monounsaturated lipids relative to saturated lipids and a substantial reduction in the intake of meat.

attended quarterly group sessions where they received written material with information on key Mediterranean foods and seasonal shopping lists, menus and specific recipes for a typical week. of EVOO (1 L per week, including a minimum of 50 mL/day for participants and the rest for family needs) or mixed nuts (30 g/day: 15 g walnuts, 7.5 g almonds and 7.5 g hazelnuts plus extra allocations for the family) were supplied at no cost to each participant randomly assigned to the MD groups on a quarterly basis during the group sessions with dietitians. Participants in the control diet group attended similar quarterly sessions with explanations and written material on the low-fat diet and they received non-food gifts in these sessions. The three diets were energy-unrestricted and no intervention on physical activity was carried out. Validated food frequency questionnaires covering 137 foods were collected yearly by the dietitians. [25] Fasting blood and spot urine were obtained and serum, plasma and DNA samples were stored. Urinary hydroxytirosol and plasma ALA measurements were determined in random sub-samples and were employed as objective biomarkers of adherence to EVOO and walnut consumption, respectively. The pre-specified primary end-point of the trial was incident CVD (a composite of non-fatal myocardial infarction/MI, non-fatal stroke or CVD death). The trial was neither powered nor designed to independently assess each of the three components of the combined end-point. Secondary outcomes included total mortality, T2DM, metabolic syndrome (MetS), peripheral arterial disease (PAD), atrial fibrillation (AF), neurodegenerative diseases and major cancers. An event adjudication committee, whose members were blinded to group allocation, was responsible for event ascertainment. As many as 7,447 participants were randomized into the three intervention groups that were well balanced with respect to their baseline characteristics and pharmacologic treatments. The mean age of participants was 67 years, 57% were women and the mean body mass index was 30 kg/m2. The baseline prevalence of diabetes was nearly 50% and the prevalence of DLP and HTN was higher than 70% and 80%, respectively. Compliance with the intervention in the two MD groups was adequate.[26] There were no between-group differences in physical activity during

the study. The effect of baseline adherence to the 14-point score with respect to the subsequent incidence of the primary CVD end-point during follow-up was assessed.[27] The multivariable-adjusted HR for participants with a baseline 14-item screener in the 2nd-3rd quintile who scored between 8-9 points was 0.72 (95% CI: 0.55-0.94), and for those with the highest adherence (two upper quintiles, scoring 10-14 points), it was 0.47 (CI: 0.35-0.65). The observed rates per 1,000 person-years for the primary end-point were 8.1, 8.0, and 11.2 in the MD+EVOO, MD+nuts, and control groups, respectively. The unadjusted HRs were 0.70 (CI, 0.53-0.91) for the MD+EVOO and 0.70 (CI, 0.53-0.94) for the MD+nuts. The pre-specified primary end-point of the trial occurred in 288 participants during a median follow-up of 4.8 years. No effect on all-cause mortality was apparent. Significant disease risk reductions were observed for incident T2DM (in the subset of participants initially free of T2DM) [28, 29] and for other CVD outcomes, such as PAD[30] and AF[31]. In comparison with the control group, participants randomized to either MD were more likely to show reversion of MetS, with HR 1.35 (CI 1.15–1.58) for the MD+EVOO, and HR 1.28 (CI 1.08–1.51) for the MD+nuts. Similarly, the MD interventions reduced blood pressure (BP) and the risk of HTN[32, 33], and slowed the progression of subclinical atherosclerosis, as determined by changes in ultrasound-assessed carotid intima-media thickness (c-IMT) and plaque measurements.[34, 35]

MD scores

The need to measure dietary intake without error, propose food frequency questionnaires (FFQs) as the gold-standard technique to reach this goal being FFQs low-cost and easy-to-use tools.[36, 37] 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, moreover to better characterize they risk to develop different pathology (e.g. cancer, CVD). Among them 2 are the most widely used and

accepted FFQs: European Prospective Investigation into Cancer and Nutrition (EPIC)[24] and the Prevención con Dieta Mediterránea (PREDIMED).[38]

EPIC Score. The Mediterranean food pattern of EPIC score was evaluated by developing an a priori 10-point score. [24, 39] A value of 0 or 1 was assigned to each of nine components, with the sex-specific medians used as the cut-off points. For beneficial components (vegetables, legumes, fruits and nuts, cereal, and fish), persons whose consumption was below the median were assigned a value of 0, and all others were assigned a value of 1. For detrimental components (meat and dairy products), persons whose consumption was below the median were assigned a value of 1, and all others were assigned a value of 0. A value of 1 was given to men consuming 10–<50 g of alcohol per day and to women consuming 5–25 g. For lipid intake, the ratio of monounsaturated fatty acids [MUFAs] to saturated fatty acids [SFA] was calculated. Those above the sex-specific median in the MUFA/SFA ratio were given 1 point. Thus, the total Mediterranean diet score ranged from 0 (minimal adherence to the traditional MD) to 9 (maximal adherence)

PREDIMED Score. The PREDIMED SCORE is a validated 14-point MD screener [40] used by dietitians as a tool to both assess actual adherence to the MD and enhance future adherence. These 14 items are:

1. Use of olive oil as the main culinary fat
2. Consumption of ≥4 tablespoons/d of olive oil (including oil used for frying, salads, out-of-house meals, etc.)
3. Consumption of ≥ 2 servings/d of vegetables
4. Consumption of ≥ 3 servings/d of fruits
5. Consumption of <1 serving/d of red meat, hamburger or meat products (ham, sausage, etc.)
6. Consumption of <1 serving/d of butter, margarine, or cream
7. Consumption of <1 serving/d of sweetened and/or carbonated beverages

8. Consumption of ≥1 serving/d of wine

9. Consumption of ≥ 3 servings/week of legumes

10. Consumption of ≥ 3 servings/week of fish or shellfish

11. Consumption of <3 servings/week of commercial sweets or pastries (not homemade), such as cakes, cookies, biscuits or custard

12. Consumption of ≥ 3 servings/week of nuts (including peanuts)

13. Preferential consumption of chicken, turkey or rabbit meat instead of veal, pork, hamburger or sausage

14. Consumption of ≥ 2 servings/week of sofrito, a sauce made with tomato and onion, leek or garlic and simmered with olive oil.

Dietitians collected yearly food frequency questionnaires covering 137 foods.[41]

The protective role of the individual components of the MD: current evidence.

Biochemical, clinical, and epidemiologic research during the last 15 years has provided a rationale for the health benefits of the MD against a wide range of chronic diseases. [42, 43] Although the responsible compounds/mechanisms of such protection have not been established, an inverse relation of vegetables and fruits consumption with cancer having been shown in Italy, France, Spain, and Greece.[44-51] Four recently reviewed epidemiologic studies[52, 53] show that olive oil conveys some form of protection against breast cancer, and limited data suggest that olive oil may reduce the risk of endometrial and ovarian cancer.[46, 54] Vitamin E, which exists in abundance in olive oil, is associated with a statistically significant decrease in the risk of sporadic colorectal cancer in carriers of wild-type ki-ras genotype.[55] Moreover, an olive oil compound, 2-(3,4-dihydroxyphenyl) ethanol (DPE), is a potent specific inhibitor of lipoxygenase activities.[56] MD is associated with high intakes of Ca^{2+} Mg^{2+} and K^{1+}, which exert major beneficial effects on a wide range of physiologic processes.[57] Moderate daily consumption of wine during meals is a characteristic of the MD.[58] Moderate drinking of alcoholic beverages reduces the risk of CAD probably by increasing levels of serum high-density lipoprotein (HDL) cholesterol. The latter levels are probably as important for the prevention of CAD as low levels of serum low-density lipoprotein (LDL) and total cholesterol.[59, 60] Moreover, monounsaturated fat, including olive oil, increases HDL cholesterol more than does polyunsaturated fat[61] and carbohydrates [61-63]making it an optimal energy-generating nutrient.[42, 43] Olive oil and complex carbohydrates derived from legumes, whole-wheat bread, and cereals, prevent/modulate postprandial hyperglycemia, which could be important in MetS. Finally, consumption of olive oil-derived monounsaturated fat may increase bone mineral density thus reducing the risk of osteoporosis.[3] Details of individual components of the MD will be provided below.

Single food components

Fats. Both the AHA/ACC and ESC guidelines strongly endorse replacing saturated and trans-fatty acids with monounsaturated and polyunsaturated fats for both primary and secondary prevention.[64, 65] In addition to recent RCT data, [38] early meta-analyses primarily evaluating the effects of fatty acids on surrogate markers of CV disease, indicated that MUFA intake increases lipid levels, including HDL.[66] A large meta-analysis suggests an inverse correlation between blood pressure and MUFAs intake.[67] The cardioprotective effects of olive oil are accounted for by the presence of its phenolic compounds obtained from the water-soluble fraction and include mostly the low-molecular-weight molecule hydroxytyrosol and oleuropein, which are both potent antioxidants, free radical scavengers, and enzyme modulators.[68] Two randomized, blinded crossover trials have assessed the antioxidant effect of dietary supplementation of extra virgin olive oil in humans: EUROLIVE[69] and the Virgin Olive Oil Study (VOLOS).[70] The Italian VOLOS trial [70] studied the inflammatory protective potential of olive oil in 22 mildly dyslipidemic patients. After a 7-week treatment period, no change in overall serum lipid profiles were found. In contrast, total antioxidant capacity of plasma and circulating levels of serum thromboxane B_2 (an index of maximal platelet activation) were both reduced by the administration of olive oil. In the Effect of Olive Oil on Oxidative Damage in European Populations (EUROLIVE) study, [69] a randomized, crossover controlled trial performed at 6 research centers across 5 European Countries, patients received olive oil with low, medium, or high phenolic content for 3 weeks, with intervening 2-week washout periods. Markers of oxidative stress, (e.g. conjugated dienes, hydroxy fatty acids, and circulating oxidized LDL) linearly decreased with increasing phenolic content of the diet. In contrast to butter and walnut-rich meals, no effect of olive oil-rich meal does not influence postprandial activation of NF (nuclear factor)-kB pathway in monocytes of healthy volunteers,[71] thus arguing for an anti-inflammatory effect of olive oil.

Finally, some data indicate a beneficial effect of olive oil supplementation on endothelial function in low- to moderate-risk patients.[72] In keeping with this, a randomized crossover trial in a small group of healthy patients showed MD (rich with olive oil) improves endothelial function; reduces systemic inflammation and lowers the endothelial progenitor cell numbers, a marker of increased endothelial repair.[73]

Fruits and Vegetables. The ESC [64] and the AHA [65] strongly endorse the use of fruits and vegetables to reduce CV disease risk. In addition, the AHA has strongly recommended the intake of a variety of phytochemicals very abundant in fruits and vegetables.[74] These recommendations are based upon a broad base of observational studies, and subsequent meta-analyses. Although the results were slightly skewed by heterogeneity and publication bias, a meta-analysis of nearly 200,000 patients showed a 4% relative risk reduction in CV disease with each serving of vegetables, with a 7% relative risk reduction in CV disease with each daily increase in servings of fruit.[75] Another large (over 200,000 patients) meta-analysis of observational studies showed a 17% reduction in CV disease events with 3-5 servings of fruits and vegetables daily as the primary endpoint.[76]

A 3.0-mm Hg decrease in systolic blood pressure among women (with no difference in men) who consumed a higher amount of fruits, vegetables, or vitamin C has been reported.[77] A cross-sectional analysis of the SUN prospective cohort [78] showed that fruit and vegetable consumption is inversely associated with blood pressure in a Mediterranean population with a high vegetable-fat intake. Increased fruit and vegetable intake has also been linked with a low BMI in a cross-sectional study.[79] Finally, the prospective Chicago Western Electric Study showed a slight benefit on blood pressure, over a 7-year period of time, in middle-aged men with an increased fruit and vegetable intake compared with those with a higher red meat intake.[80] Population-based evidence from the EPIC-Heart study showed, after an 8-year follow up of 313,074 patients without overt atherosclerosis, a 22% lower risk of coronary death in those consuming 8 portions of fruits and vegetables a day, as compared with 3 portions or less.[81] RCT data only

address the CV disease benefits of fruits and vegetables by using CV disease surrogates as endpoints. A 2001 RCT showed a strong trend for increased fruits and vegetables consumption as to lowering in lipid or blood pressure profiles.[82] A subsequent RCT showed a significant effect of fruit and vegetable consumption on both plasma antioxidant concentrations and blood pressure.[83] In addition to the evidence relating the antioxidant properties [84] and the health benefits of an high flavonol intake,[85] effects on nitric oxide (NO) species and/or on weight loss are also associated with the beneficial effects of diets high in fruits and vegetables [86].

Whole Grain. The AHA guidelines indicate that diets high in fiber (e.g. whole grains, oats, and barley), reduce CV disease morbidity and mortality through lipid lowering, and recommend a total dietary fiber intake of 25-30 g per day from whole foods.[87] Likewise, the ESC endorses intake of foods high in dietary fiber to promote CV disease health.[64] RCTs show data on surrogate markers of CV disease such as blood pressure, cholesterol, and markers of inflammation. However, no information is available on hard CV disease endpoints. A small RCT showed beneficial effects on BMI and waist circumference in the group randomized to hypocaloric whole grains as compared to that receiving hypocaloric refined grains.[88] Cholesterol level lowering, the group obtaining all carbohydrates from 12-week whole grains had a significant improvement in C-reactive protein (CRP). A larger, subsequent, RCT confirmed these results in 230 participants randomized to 3 daily servings of whole grains ± whole oats vs refined carbohydrates, the former serving exhibiting a significant reduction in blood pressure and lipid profiles.[89] However, in over 300 overweight individuals in whom normal carbohydrate intake was randomly replaced with control, intermediate whole grain, or high whole grain diets for 4 months, no significant differences in surrogate markers of CV were observed after 4 months.[90] In spite of this, a 21% reduction in CV events and mortality in prospective cohort studies has been documented in a meta-analysis devoted to whole grains merits.[91] Larger observational studies such as the Iowa Women's Study and the Nurse Health Study[92, 93] show a reduction in CV disease

morbidity with increased intake of whole grain sources. Finally, observational data show improved all-cause and CV disease mortality in women with T2DM who consume whole grains.[94] Mechanisms [95] of the benefit of whole grains on CV disease include reduced inflammation, lower reactive oxidation species generation and improved antioxidant capabilities.[96] High-fiber whole grain diets inhibit absorption of fats, simple carbohydrates, or toxins. improved lipid profiles, and improved blood pressure control,[97] and improved glucose metabolism[98, 99], and weight loss [100] have also been reported. Yet the recommendation promoting whole grains has no RCT data behind it to show a reduction in CV disease morbidity or mortality. As such, increased whole grain intake for CV disease prevention should be pursued in the frame of a MD to maximize its cardioprotective potential.

Nuts. An early evaluation of observational studies showed that replacing walnuts, peanuts, almonds, or other nuts for a serving of carbohydrates or saturated fats reduced blood lipids as well as the risk for CV disease by 30% and 45%, respectively.[101, 102] Almost 10 years later, it has been argued that nut consumption leads to a 0.67 risk reduction on CV disease.[103] Furthermore, a 2009 meta-analysis showed a significant reduction in LDL cholesterol, inflammatory and oxidants mediators with increased walnut consumption,[104] without any evidence of reduction in risk factors or CV disease mortality. A more recent meta-analysis, however, pointed out benefits of nuts[105] on weight loss. A meta-analysis of 4 observational studies, showed an inverse relationship between nut consumption and CV disease (primary endpoint), and documented ≈40% decrease in the incidence of primary CV disease with consumption of at least 4 nut servings per week and up to 10% reduction with a single serving per week.[106] Due to the small sample size of the studies and the large variety of nuts studied, old RCT data are less convincing with respect to this issue. Recent RCT data provide evidence that the MD supplemented with nuts provides primary CV disease-prevention benefits.[38] Being these benefits seen in the frame of a MD, it would be

inappropriate to ascribe the benefit solely to nuts. In keeping with their ability to improving CV morbidity and mortality, several cohort studies and few small RCTs have shown a benefit of nuts as to improving lipid profiles and reducing blood pressure and reactive oxygen species (ROS) generation, and improving vascular function. Such evidence on CV disease prevention argues for increased nut consumption as being able *per se* to provide CV benefits, greater benefits being observed when added to the MD.[107]

Legumes. Legumes, the seeds of plants that contain roots which use nitrogen-fixing bacteria, are considered a low-glycemic useful dietary source of protein and fiber. Initial observational studies showed legume and soy intake to be beneficial to prevent CV disease (11% reduction in CV disease in women who consumed legumes 4 or more times weekly over those who consumed legumes one or fewer times weekly).[108] Similar observational data have been reported in Japan[109] and China.[110] However, RCT data seldom address hard CV disease endpoints, and the grouped data show only marginal benefits as to lipid levels, blood pressure, or endothelial function. Small RCTs in low- to moderate-risk patients show little change in lipid levels [111] [112, 113]. On the other hand, one meta-analysis shows a 5% reduction in LDL levels in patients randomized to high legume intake,[114] documenting that this reduction had no effect on hard CV disease endpoints. RCTs have shown a benefit on blood pressure in patients with moderate hypertension.[115] In keeping with this, a large sample of RCTs studies[116] and an earlier meta-analysis[117] show no benefit from soy supplementation on normotensive patients. Consistent with prior meta-analyses[114] a recent RCT shows no benefit of soy on NO bioavailability and BP in moderately hypertensive, postmenopausal women.[118] However, subsequent meta-analyses have challenged this statement. Due to a potential anti-inflammatory and vaso-protective benefit, studies has examined the effect of soy on endothelial function. A recent meta-analysis of RCTs shows no benefit of soy on endothelial function,[119] a moderate benefit of soy supplementation being only documented when age-related results

are adjusted for baseline endothelial function. Thus, presently there is little direct evidence that legume intake alone has direct CV disease benefits. It should thus be hypothesized that legume intake must be part of the MD, and cannot be supplemented in isolation for CV disease protection.

Plants and polyphenols. An international panel of experts reviewed the effects of a moderate consumption of beer on human health has been carried out by.[120] Low-moderate (up to 1 drink per day in women, up to 2 in men), non-bingeing beer consumption, reduces the risk of CV disease. This effect is comparable to that of wine, at similar alcohol amounts. Moderate consumption of either beer or wine may confer greater CV protection than spirits. Although specific data on beer are inconclusive, observational studies indicate that low-moderate alcohol consumption is associated with a reduced risk of developing neurodegenerative disease. There is no evidence that beer drinking differs from other alcoholic beverages as to the risk of some cancers. Besides the obvious exceptions (people engaged in actions that require concentration, skill or coordination; children; adolescents; pregnant women, individuals at risk for alcoholism, and those with cardiomyopathy, cardiac arrhythmias, depression, and/or liver/pancreatic diseases), evidence suggests a J-shaped relationship between alcohol consumption and all-cause mortality, lower risks for moderate alcohol consumers than for abstainers (or heavy drinkers) being found. [120]
Studies in model organisms (e.g., C. elegans, D. melanogaster), and in laboratory rodents suggest that red wine constituents (e.g., resveratrol) may positively affect both health and lifespan.[121, 122] In a mouse model, resveratrol activates transcriptional factor Nrf2 and lowers oxidative stress, hence protecting the endothelium [123] and inhibiting inflammation in macrophages (by downregulating the proinflammatory NFκB expression).[124] Resveratrol induces Sirt1 [125-127], and it is conceivable that the endothelium protection and NFκB inhibition appears to be connected to Sirt1 expression. Olive oil phenols induce proteosomal activity and Sirt1 signalling. [128, 129] The role of polyphenols in gene regulation via sirtuins and

transcription factors Nrf2 and NFκB is now clear.[130] Quercetin (a polyphenol found in onions) also induces Sirt1.[127] Mice consuming diets rich in olive oil phenolics (e.g., hydroxytyrosol) exhibit decreased oxidative damage markers (e.g., lipid peroxides, protein carbonyls) and improved expression of Nrf2-dependent genes encoding antioxidant (γGCS, NQO1) and cardioprotective proteins (e.g. paraoxonase).

n-3 Polyunsaturated fatty acids. The theory that eating fish, a major component of the MD may protect against cardiac death was first derived from the results of a secondary prevention trial, the Diet And Reinfarction Trial (DART), which showed a significant ≈30% reduction both in total and CV mortality in patients who consumed at least two servings of fatty fish per week.[131] The authors suggested that the protective effect of fish might result from a preventive effect on ventricular fibrillation (VF), since no benefit was observed on the incidence of non-fatal AMI. The hypothesis was consistent with the evidence that the very-long-chain n-3 fatty acids, the dominant fatty acids in fish oil and fatty fish, have major effects on the occurrence of VF in the setting of myocardial ischemia/reperfusion in several animal models.[132] In dogs, using an in vivo model of sudden cardiac death (SCD), Billman et al showed a marked reduction of VF after intravenous administration of pure n-3 fatty acids, including the very-long-chain fatty acids present in fish oil and ALA.[133]

A second aspect of the involvement of n-3 fatty acids in SCD is related to their role in the metabolism of eicosanoids. In competition with n-6 fatty acids, precursors of prostaglandins and thromboxane with potent pro-aggregatory and vaso-constrictive effects, n-3 fatty acids are the precursors of a broad array of structurally diverse and potent bioactive lipids (including eicosanoids, prostaglandins and thromboxane), which play a major role in the prevention of VF during myocardial ischemia/reperfusion.[134] Suppression (≥70%) of ventricular premature complexes in middle-aged patients with frequent ventricular extra systoles randomly assigned to take either fish oil or placebo has been

documented.[135] Survivors of AMI[136] and healthy men[137] improved their measurements of heart rate variability while receiving fish oil. This is in keeping with the fact that parasympathetic cardiac tone provides protection against VF.[138] Epidemiological studies support the hypothesis of a clinically significant antiarrhythmic effect of n-3 fatty acids in secondary prevention of CAD.[139] In a large prospective study (more than 20 000 participants with a follow-up of 11 years), Albert et al found that the risk of SCD was 50% lower in men who consumed fish at least once a week than in those who had fish less than once a month.[140] In that study, the main protective effect of fish consumption (or of very-long-chain n-3 fatty acids) was related to a reduction of arrhythmia. In a double blind trial in patients with implantable cardiac defibrillator, Leaf reported a 40% reduction of recurrent ventricular arrhythmias following fish oil capsules[141], but this hypothesis was challenged[142] Presently, there is no clear explanation for that discrepancy between these two trials. Dietary confounders (e.g. saturated fatty acid intake; background marine/plant n-3 vs n-6 fatty acid intake, trans fatty acid intake) which could interfere with the n-3 fatty acids given with the capsules, were not measured in these trials nor were they included for adjustments in the calculations of the risks. The GISSI-Prevenzione trial addressed the question of the health benefits of vitamin E and very-long-chain n-3 fatty acids (EPA+ DHA). Patients (n = 11,324) surviving a recent AMI (≈3 months) were randomly assigned to supplements of n-3 fatty acids (1 g daily), vitamin E (300mg daily), both or none (control) for 3.5 years. The primary efficacy endpoint was the combination of death and non-fatal AMI and stroke. Secondary analyses included overall mortality, CV mortality and SCD. Treatment with n-3 fatty acids significantly lowered the risk of the primary endpoint (15% decrease of the relative risk). Overall mortality was reduced by 20% and CV mortality by 30%. However, the effect on SCD (45% lower) accounted for most of the benefits seen in the primary combined endpoint and both overall and CV mortality. There was no difference across the treatment groups for non-fatal CV events, a result comparable to that of DART.[131] Two central questions remain to be addressed with respect to these

data. 1) In the GISSI- Prevenzione trial, all patients were advised (before randomization) to follow a MD type of diet after their AMI. In their report, the GISSI investigators confirmed that the patients of both groups actually did so, > 80% of them reporting daily olive oil consumption.[143] Whether such patients would have been protected while consuming a non-MD is unknown so far. However, similar to the Lyon Diet Heart Study, [9, 12] pooling together the randomized groups and adjusting for age, sex, smoking, concomitant drug therapy and randomized treatment, the GISSI investigators found that the higher the MD adherence (as evaluated by an MD score) the lower the risk of dying (from any cause), a 50% difference in such risk being found between the best and the worst MD adherence.[144] 2) Unpublished data (the European IMMIDIET Project) suggest that compared with British (South of London) and Belgian (Flemish) populations, the Italian population is relatively deficient in n-3 fatty acids. It is not sure whether the results with such a low dose of n-3 fatty acids would have been similar in populations with higher intakes in n-6 and trans fatty acids.

Al in all, large case-control studies and prospective intervention trials consistently showed that dietary and non-dietary intake of ω-3 fatty acids directly or indirectly affects cardiac electrophysiology and that n-3 fatty acids supplementation lowers AMI and SCD in patients with CAD. [145].[146-148] However, experimental data also suggest newer directions to be pursued in the area, the anti-atherogeneic and anti-inflammatory properties of n-3 FA having been largely investigated in *in vitro*, animal and human studies. Cellular models of early atherogenesis based on cultured endothelial cells challenged with various pro-atherogenic stimuli show that both DHA and EPA significantly affect major critical events leading to endothelial activation, including the expression of VCAM-1. Such regulatory effects occur in a range of DHA concentrations compatible with nutritional supplementation to a normal Western diet and are strictly related in magnitude to the extent of incorporation into total cell lipids.[149-151] In addition to the expression of transmembrane molecules involved in leukocyte recruitment, effects of DHA and EPA also involve the pro-inflammatory and chemoattractant soluble proteins

IL-6,[152] IL-8,[151] macrophage-colony stimulating factor (M-CSF),[153] and monocyte chemoattractant protein (MCP)-1.[154] Such effects are associated by reduced monocyte adhesion to cytokine-activated endothelium.[150, 151] *An antioxidant effect* can also be documented in cultured endothelial cells supplemented with DHA.[153, 155, 156] A reduced membrane assembly and activation of the ROS-producing enzyme complex NADPH-oxidase, has been reported as a likely consequence of plasma membrane changes occurring upon DHA incorporation.[155] On the other hand, when supplemented to macrophages in culture, EPA and DHA significantly reduce the stimulated release of MMPs,[154, 157] thus likely contributing to plaque-stabilizing effects of PUFA in humans.[158] In addition, fish oils prevent serotonin-induced smooth muscle cells proliferation,[159] an effect that is involved in preventing restenosis after endovascular interventions.

Clinical Vascular Diseases

Athrothrombosis.

Atherothrombosis -the substrate of the large majority of complications of atherosclerosis leading to clinical vascular disease and the leading cause of death worldwide,[160]- is an unpredictable, sudden rupture or erosion/fissure of the atherosclerotic plaque,[161] leading to platelet activation and thrombus formation in the vessels.[162] Thrombus formation is a dynamic process in which platelets aggregate but also spontaneously disaggregate, leading to embolization of platelet aggregates from an evolving thrombus.[163] In the case of an occlusive thrombus, depending on the localization of the atherosclerotic plaque, there will be an acute ischemic event in the coronary, cerebral or peripheral vascular territory, potentially leading to permanent tissue damage. In the case of a non-occlusive thrombus, ischemic symptoms are temporary. Occlusive thrombosis in atherosclerotic coronary arteries may give rise to major clinical events such as AMI, cardiac death and acute coronary syndrome (ACS). Atherothrombosis of the cerebral arteries may result in transient ischemic attacks (TIAs) or ischemic stroke. In the peripheral arteries, thrombosis superimposed on atherosclerotic plaques contributes to the progression of peripheral arterial disease (PAD), producing intermittent claudication (leg pain on walking that is relieved by rest); ischemic necrosis and, potentially, loss of the limb. Hence, the same underlying vascular process cause ACS, TIA, stroke, and PAD.[164] Because of the generalized nature of atherosclerosis, symptoms in one vascular bed, for example, AMI, are highly predictive of risk of further ischemic atherothrombotic events elsewhere.[165]

Cardiovascular Disease (CVD)

CVD, the major complication of atherothrombosis, is the number one cause of death worldwide, with yearly deaths expected to increase from 17.3 to 23.6 million by 2030.[166] Today CV deaths accounts for approximately 1/3 of all deaths globally. Medical expenses and medication costs associated with the treatment of

CVD are huge, totalling $126 billion in 2010 in the US. [167] The attention devoted to the CVD is due to the rapid increment of CV deaths during the last decades (12.5% increment).[168] In reality, in the 2005/2015 decade there was a 15.6% reduction of age-specific deaths.[169-171] However, this was relevant in High-income countries (HICs) than in Middle-income countries (MICs) and Low-income countries (LICs).[170, 172] Accordingly, the vast majority of CVD deaths now occur in LICs and MICs.
In 2013, the World Health Organization, launched the "25x25 Global Action Plan", a project aimed at reducing the mortality by 25% by 2025. This project focuses on correction of 4 mains health-related behaviors: tobacco use, diet, physical activity, and alcohol.[173] The occurrence of CVD is multifactorial and major risk factors are T2DM, hypertension, smoking habit, overweight, and dyslipidemia. Early recognition and treatment of patients at high risk of atherosclerosis is a major goal to reduce the incidence of atherothrombotic events and CV disease.

Ischemic Heart Disease

IHD is the leading component of the CVD burden. Both the prevalence and the mortality of IHD increase dramatically with age.[169] During the past 25 years, both the incidence and mortality (age standardized) have decreased globally, the greatest decline being found in HICs.[174, 175] Age-standardized mortality also remains high in South Asia, North Africa, and the Middle East.[169] On the other hand, the large increases documented during the 1990s in Eastern Europe and Central Asia are now reversing.[169, 174] Years of life lost because of IHD are highest in South Asia, mostly reflecting early onset of IHD. [174, 175]

Congestive Heart Failure

Congestive Heart Failure (CHF) due to ischemic heart disease (IHD), hypertensive heart disease, or cardiomyopathy/ myocarditis contributes significantly to disease burden.[169] Both the prevalence of CHF and the associated mortality increase with age.[169] Despite advances in CHF management during the past 3 decades, data from HICs suggest that 5-year survival after the diagnosis of CHF is still only 50-

60%, mortality rates being substantially higher in LICs and MICs.[176, 177]Population based studies in North America and Europe estimate a prevalence of CHF of 1% to 2%, with 80% of new cases occurring in those >65 years of age.[176, 178] Limited data from Asia suggest that the prevalence of CHF may range there between 1% and 7%. However such estimates predominantly rely on either single-center or hospital based studies, which may not truly reflect community rates.[179]

Atrial Fibrillation

Atrial fibrillation (AF) is the sixth leading cause of CVD-related mortality and the eighth leading cause of disability among CV conditions.[168, 169] The prevalence of AF increases dramatically in older age groups as does the associated mortality.[169] The age-adjusted prevalence and incidence of AF have increased during the past decades.[180] The highest prevalence of AF has been reported in North America and the lowest in the Asia-Pacific region.[180] However, these data also may not be reliable because few large community-based studies have examined AF epidemiology outside of Western countries, and methods used to detect AF vary.

Rheumatic Heart Disease

Rheumatic heart disease (RHD) is the fifth leading cause of CVD-related mortality and ranks sixth in disability from CV conditions. The disease burden is concentrated in poor countries, where overcrowding and reduced access to care coexist with an inadequate management of valvular disease.[169] Current estimates suggest that the highest prevalence of RHD now occur in countries of Oceania, South Asia, South East Asia, Central Asia, Sub-Saharan Africa, parts of the Middle East, and in the Caribbean.[181] Since 1990, the prevalence of RHD appears to have increased in these endemic regions while incident acute rheumatic fever has decreased.[182] Although these differing trends may be related to longer survival of patients with RHD, this may also be the result of more RHD cases being identified earlier with wider use of echocardiography.[181]

Cerebrovascular disease

Stroke is the second leading contributor to CVD disease burden. The prevalence of stroke increases with age, peaking in those between 74 and 79 years of age.[169] Stroke-related mortality also increases with age.[169] Approximately 63% of ischemic and 80% of hemorrhagic stroke now occur in LICs and MICs. Ischemic stroke is more common than hemorrhagic stroke although hemorrhagic stroke is associated with higher mortality and contributes more to disability.[183, 184] On average, populations in MICs and LICs suffer strokes 6 years younger than in HICs.[185] Countries with the highest age-standardized prevalence of stroke currently are in Oceania, Eastern Europe, Central Asia, and South- East Asia while mortality from stoke is highest in Oceania and Sub-Saharan Africa.[169, 185]

In a world of magnetic resonance imaging the historical definition of TIA as "a transient episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia without acute infarction"[187] lasting less than 24 hours is no longer appropriate. Although most patients are asymptomatic at presentation and have normal physical examination, [188] the incidence of acute ischemic stroke within 48 hours of an emergency visit for TIA is 4.8% (182/3,814 patients; 95% CI 4.0% to 5.6%).[189] Secondary stroke prevention—the rapid implementation of multiple interventions—reduces the outcome of stroke by 80%.[190-193] Because accurate diagnosis, rapid testing, and implementation of treatments can prevent disabling and fatal strokes, TIA has enormous public health importance.[188]

Subarachnoid hemorrhage

Subarachnoid hemorrhage accounts for 5 to 10% of all strokes in the United States,[194] and affected patients tend to be younger than those affected by other subtypes of stroke, which results in a greater loss of productive life.[195] Subarachnoid hemorrhage without a preceding trauma is due to an intracranial aneurysm rupture in 80% of cases. Among the patients with aneurysmal subarachnoid hemorrhage who survive, half suffer long-term neuropsychological

effects and decreased quality of life.[196] Early identification and treatment of the aneurysm can prevent aneurysm re-rupture and address sequelae from the initial rupture. Intervention may be appropriate in cases of subarachnoid hemorrhage that are not caused by aneurysms (e.g., cases involving arteriovenous malformation), but up to 10% of cases of non-aneurysmal subarachnoid hemorrhage involve no vascular abnormality, and surgical or endovascular treatment is not necessary.[197]

Peripheral vascular disease

PAD affects more than 5% of population older than 60.[198] In the US, PAD affects about 8 – 10 million Americans, and every year it causes 500,000 hospitalizations and 100,000 angiograms.[199] Significant CAD (in at least one coronary artery) has been documented in 60% – 80% of patients with PAD, and hemodynamically significant carotid artery stenosis (by duplex ultrasound) has been found in 12% – 25% of cases.[200] The risk of AMI is increased by 20% – 60%, whereas the risk of coronary death is increased 2 – 6-fold in PAD patients.[160] For this reason, early diagnosis of PAD may help identify patients at risk for CV events.[201] PAD is associated with a 40% increase in the risk of stroke, and PAD severity is positively associated with the incidence of TIAs and stroke.[199] The clinical spectrum of PAD is widely variable: patients may either be asymptomatic or have pain as a result of a minimal walking.[202] Most asymptomatic patients with PAD will be identified through ankle-brachial index (ABI) screening.[203] ABI (i.e. the ratio of the highest systolic blood pressure in the lower limb to that of the arm) is an easy, reliable means for evaluating PAD severity.[204] PAD with intermittent claudication (IC) is often undiagnosed and, in turn, undertreated. The low percentage of diagnosis (~ 30%) among those with a PAD is of concern because of the high risk of adverse outcomes related to the worsening of PAD. [205-207] Among those with ABI 0.9, ~ 25% will experience worsening claudication necessitating surgical repair or amputation. In a 10-year follow-up [208], about 55% of PAD (ABI 0.9) patients died of CV disease, 10% of cerebrovascular disease, and 25% of non-vascular reasons. Less

than 10% died of other vascular events (mostly, aortic aneurysms in the abdomen). In another follow-up[209], 10-year mortality was 61.8% in males with symptomatic PAD (ABI 0.9); in comparison, in males without PAD (ABI 0.9) mortality was 16.9%. Mortality rates in females were 33.3% and 11.6%, respectively. Less than 25% of patients with PAD (ABI 0.9) survived for 10 y, vascular mortality being the dominant cause of death in that setting. After correction for established risk factors, PAD severity was an independent predictor of death.

Critical limb ischemia (CLI; ABI 0.4), is observed in 12% of the PAD population.[210] In most cases, CLI is an advanced thrombotic complication of PAD, due to inadequate resting blood flow to the lower limbs, and is marked by rest pain, ulceration, and eventually gangrene and loss of the limb.[211] CLI is seldom the result of an acute event (e.g. embolism, thrombosis, or trauma). Patients with CLI are candidates for prompt revascularization. CLI increases mortality: in the first year after the diagnosis 25% of patients (45% with amputation) will die, and 30% of them will have amputations, whereas only 45% will survive with both legs. After 5 years more than 60% of patients have died. [212]

Subclinical Vascular Diseases: Methods of Detection

Carotid Intima media thickness (c-IMT)

c-IMT assessment is a non-invasive imaging test for subclinical atherosclerosis.[213, 214] This test has been widely accepted as one of the strongest predictors of major CV events (stroke, AMI, CHF, CV death).[215, 216] The presence of carotid plaques is an even more reliable predictor of CV events than c-IMT.[217] Thus, this surrogate marker of subclinical atherosclerosis provides important prognostic information over and above traditional CV risk factors.

High resolution B-Mode Eco-tomography

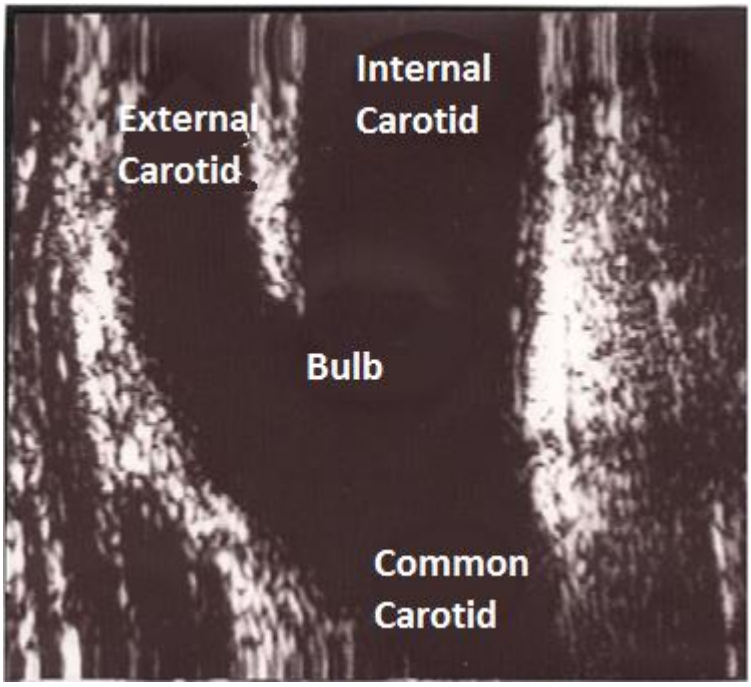
B-Mode ultrasonography is a non-invasive technique that allows to study subclinical atherosclerosis of the major surface vascular districts. Morphological information relating both to the lumen and the blood vessel are achieved by using this technique. High Resolution B-Mode Eco-tomography uses high frequency ultrasound (7-13 Mhz) and provides real-time bi-dimensional images of the major unshielded surfaces of blood vessels. This technique also provides detailed information on the size and characteristics of early small injuries of the wall.[218-222] B-Mode ultrasonography shows a great sensitivity, specificity, accuracy and reproducibility.[223] It is cheaper than invasive techniques and allows for carefully performing studies on the evolution of atherosclerosis and the effect of treatments. Limits of B-Mode Eco-tomography include: the inability to display anechogenous structures (e.g. lipid areas) or structures with blood-like acoustic impedance (hemorrhage inside a plaque or recent thrombotic occlusions).[224] Eco-colordoppler and angiography should be preferred when measuring calcific stenosis.

Ultrasound anatomy of carotid arteries

In a typical ultrasonographic image, the common carotid, the carotid bifurcation and the internal and external carotid arteries are easily detectable **(Figure 2)**. In ultrasonographic images, the beginning of the dilation of the bifurcation is the distal

limit of the common carotid and is recognized by the presence of a medium-intensive physiological thickening, the "bifurcation crest/peak". The bifurcation, separates the internal carotid, -which discharges the frontal parts of the brain and the vision organs-, and the external carotid that disrupts the neck, face, and cranial walls. The Carotid bifurcation has two key anatomical reference points that allow it to be identified. The lower margin of the carotid bifurcation is defined by the proximal part of the bifurcation dilation, the upper limit is given by the so-called "flow-divider", which separates the origin of the internal and external arteries. The internal carotid is delimited proximally by the flow-divider and differs from the outer carotid for the larger caliber and for the origin with a typically bulbous morphology. The branch of the thyroid artery from the outer carotid artery further differentiates the two arteries.[225]

Figure 2. Carotid Bifurcation



Thickness of the intramammary complex (or extracranial carotids IMT)

IMT of the extracranial carotids is the most important parameter that can be evaluated using B-Mode ultrasonography. The anatomical correspondence between the ultrasonic image and the intimal average complex has been

documented more than 30 years ago[220] in a study where a real estimate of the mean intimal thickness was obtain by measuring the distance between the "blood-intima" and "media-adventitia" interfaces of the abdominal aorta and of common carotid arteries. This method is now standardized and is used as a reference standard for studying carotid atherosclerosis and for evaluating CV risk. The major benefit of the c-IMT analysis is that it allows the evaluation of individual subjects to develop early atherosclerotic disease, before the occurrence of atherosclerotic lesions (plaques). In addition to being an index of local atherosclerosis,[226, 227] c-IMT also predicts similar lesions in other districts e.g. the coronary vasculature.[215, 223, 228-232] c-IMT is also directly related with major risk factors for atherosclerosis.[74, 233-236] Current epidemiological data indicate that ≥1 mm c-IMT increase in asymptomatic adults is a risk factor for AMI and/or cerebrovascular disease.[237] c-IMT thus provides a comprehensive view of over time injuries on the arterial wall due to risk factors, [237] and has been proposed to be included in algorithms for refining CV risk estimates[215, 238] The predictive value of c-IMT is further strengthened by the identification of high-risk patients for cardio- and cerebro-vascular events.[215, 239] Accordingly, this technique is currently employed in clinical and research centers to assist physicians to prescribe pharmacological treatment in primary prevention in patients at high CV risk.[240]

MD and subclinical disease

MD and c-IMT

Few epidemiological studies have addressed the relationships between MD and c-IMT. [34, 35, 241, 242] Two studies have investigated the relationship between MD and c-IMT progression in cohorts of children or elderly individuals, [242-245] and a single interventional study, carried out in a Mediterranean population (PREDIMED), has addressed the effect of MD on c-IMT progression. To date there are no large observational studies addressing the relationship between MD adherence and c-IMT progression in populations from different European countries with different nutritional patterns/habits.

In view of its capacity to predict incident coronary events, c-IMT has been proposed as a surrogate marker of coronary atherosclerosis. [213, 246] Analysis of baseline data of the IMPROVE study has identified latitude as the strongest independent determinant of c-IMT. [247] The association between latitude and carotid atherosclerosis profile remained statistically significant over and above traditional and new vascular risk factors (VRFs) and baseline Framingham risk score (FRS). [248, 249] In addition, a significant association between c-IMT and c-IMT progression and future vascular events has been shown in the IMPROVE study. [247, 249]

MD and Carotid Plaques

The association between MD adherence and carotid plaque thickness and area was first evaluated in an observational study that included 1,374 participants of the population-based Northern Manhattan Study (60% female, 60% Hispanic, 18% White, 19% Black, mean age 66 ± 9 years).[250] c-IMT and plaque were measured using B-mode ultrasound. A MD adherence score (range = 0–9, 9 representing maximal adherence) was employed as a continuous variable and as quintiles (3/4/5/6 –9 vs. 0–2). There was no association between MD and c-IMT or plaque presence. MD adherence was inversely associated with the 75th percentile of

plaque thickness and median of plaque area in quantile regression analyses. These associations persisted after controlling for demographics, smoking habit, physical activity, and total energy consumption (effect of a 1-point increase in MD score on the 75th percentile of plaque thickness = −0.049 mm, p = 0.03; median of plaque area = −0.371 mm2, p = 0.03), and when also controlling for vascular disease biomarkers, medication use, BMI, and previous cardiac disease. The protective associations were strongest for a MD score of 5 (4th quintile) vs. 0–2 (bottom quintile). Differential effects of a MD on plaque thickness and area across race/ethnic groups was suggested.[250] In the same cohort, no association with clinical stroke was found. However, larger studies have found an association of MD with clinical stroke[251] and associations of MD with imaging biomarkers of cerebrovascular damage.[252, 253] Studies have also shown that the top quartile of carotid plaque area (vs. the lowest quartile) is associated with a 3–4-fold increased risk of stroke, AMI or death.[254, 255] Accordingly, plaque area may be a better measure of atherosclerosis than plaque thickness,[254-257] plaques progressing along the carotid artery 2–3 times faster than for thickening.[258] Despite the relationship between carotid plaque burden and risk of clinical events, few studies have examined the role of vascular risk factors in relation to this novel marker of disease risk. Likewise, whether the association between MD and carotid plaque burden is consistent with a dose–response relationship or a threshold effect is unknown so far.

MD and Vascular Stiffness

Whether adherence to the MD, obesity, and BP are related to indices of AS has been evaluated in childhood. Two hundred and seventy-seven children aged 12 years were measured with the R6.5 Pulsecor® monitor, which performs measurements using an upper arm BP cuff held at above systolic pressure for a short time.[259] Of such children, 43% were overweight or obese. The AI in the brachial artery, the peripheral pulse pressure to central pulse pressure (PPP/CPP) ratio, and the reflected wave transit time to height ratio were used as indices of

AS. The degree of adherence to the MD was assessed by the KIDMED index, which includes 16 questions on specific dietary habits. In multivariate regression models, the KIDMED index had a negative correlation with AI (β=-0.114; p=0.026) ant this relation was independent of obesity. In contrast, indices of AS were related to mean peripheral BP (β=0.110; p=0.063), heart rate (β=-0.508; p=0.000), and height (β=-0.370; p=0.000), while BMI had an independent correlation to PPP/CPP (β=-0.182; p=0.039).[259]

Information in adults only pertain to acute postprandial conditions in subjects simultaneously receiving wine and olive oil.[260] Fifteen healthy subjects consumed four standard meals on different days, containing 50 g of olive oil and 250 ml of wine, in a randomized cross-over study design. Two types of wine [red (R) and white (W)] and two types of olive oil [green (G) and refined (O) (rich and poor in antioxidants, respectively)] were used in all possible combinations. Applanation tonometry and aortic pulse wave analysis were performed when fasting and 1, 2 and 3 hours post-prandially. A second group of 15 healthy individuals matched for age, gender and body mass index served as controls. All meals decreased AIx (RO and RG, P<0.001; WO, PU0.007; and WG, PU0.039). The AIx reduction after RG, RO, WO and WG was significantly different from the AIx response of the control group. No difference was observed in the reduction of AIx between sessions, but a significantly earlier peak decrease in AIx, as well as a prolonged decreasing effect, was observed after RG and RO consumption compared to WO and WG. Central systolic and diastolic pressure were diminished after all four combinations of wine and olive oil (P<0.05), an information consistent with the concept that a combined consumption of wine and olive oil provides beneficial postprandial effects on hemodynamics. [260]

MD and FMD.

The relationship between diet and endothelial dysfunction has been examined in epidemiological and interventional studies. The prospective cohort Nurse's Health Study compared endothelial function as related to two dietary patterns[261]: a food

pattern characterized by a high intake of fruits, vegetables, whole grains, fish and poultry, and a Western food pattern characterized by higher intake of red meat, processed meat, refined cereals and fries. Regardless of risk factors such as smoking and age, and after controlling for body weight, women who consumed a "prudent" diet had significantly lower levels of E-selectin -a biological marker of endothelial dysfunction-, than women who they consumed a Western diet.

MD and Conventional Vascular Risk Factors for CAD

Blood pressure

The Dietary Approaches to Stop Hypertension (DASH) clinical trial[18] enrolled 459 adults with systolic BPs of <160 mm Hg and diastolic BPs 80–95 mm Hg. Initially, the subjects were fed a control diet that was low in fruits, vegetables, and dairy products, and a diet that has a fat content typical of the average US diet. Then they were randomly assigned to receive: 1) the control diet; 2) a diet rich in fruits and vegetables; or 3) a "combination" diet rich in fruits, vegetables, and low-fat dairy products with reduced saturated and total fat. The "combination" diet was associated with 5.5 mm Hg and 3.0 mm Hg lower systolic and diastolic BPs, respectively, than the control diet ($p<0.001$), while the fruits-and vegetables diet reduced systolic BP by 2.8 mm Hg more ($p<0.001$) than the control diet. Among the 133 subjects with hypertension, the combination diet reduced systolic and diastolic BP by 11.4 mm Hg and 5.5 mm Hg more, respectively, than the control diet ($p<0.001$ for each). Among the 326 subjects without hypertension, the corresponding reductions were 3.5 mm Hg ($p<0.001$) and 2.1 mm Hg ($p=0.003$).[18] These findings argue for not limiting to single nutrients the focus of dietary changes for BP control. Blood pressure variability (BPV) is a novel risk factor for CVD. It is unknown whether dietary pattern plays a role in modulating BPV.[262] As many as 274 consecutive patients with stable CAD were followed-up. The MD score (MDS) was derived for all individuals upon recruitment, blood pressure (BP) was measured during each subsequent clinic visit and the visit-to-visit BPV was calculated. The occurrence of major adverse CV events (MACEs) and all-cause mortality were monitored. After a mean follow-up of 77 ± 12 months, 16.1% of the study population developed MACEs. About 11.3% died from all causes. Patients who developed MACEs or all-cause mortality had a greater systolic BPV compared to those who did not develop an adverse event. Patients who developed a MACE had a lower MDS and further analysis revealed those who developed a stroke had a lower MDS compared with those who did not, but there were no significant

differences in MDS between CAD patients with or without subsequent acute coronary syndrome, CV, or all-cause mortality. After adjusting for confounding variables, a high MDS was an independent predictor for low systolic BPV (B −0.74, 95% confidence interval −1.27 to −0.21, P < 0.01) and was protective against a subsequent stroke (hazards ratio 0.48, 95% confidence interval 0.24 to 0.94, P = 0.03).[262]

Physical activity

It is known that the age-dependent decline in endothelial and microvascular integrity may be reversed when combining an 8-week exercise with an MD intervention. Whether the risk-reduction improvement in microcirculatory and cardiorespiratory functions are sustained in this age-group after a 1-year follow-up has been also investigated.[263] Twenty sedentary healthy participants (age, 55 ± 4 years) underwent cardiopulmonary exercise tolerance test and were assessed for their upper- and lower-limb vascular endothelial cutaneous vascular conductance (CVC) using laser Doppler fluximetry (LDF) with endothelium-dependent [ACh (acetylcholine chloride)] and endothelium-independent [sodium nitroprusside] vasodilation, 1 year after completing the intervention. With respect to ACh, compared to baseline, both MD and exercise groups improved their microvascular responses, a stronger improvement in the MD group in comparison to the exercise group, for ACh (p = 0.04, d = 0.41) being found. In the upper body, the time point and group interaction for ACh, indicated a non-statistically significant (p= 0.07, d= 0.24) improvement for MD. Cardiorespiratory improvement in ventilatory threshold was maintained 1 year after (12.2 ±3.0 vs. 13.2±3.2ml · kg−1 · min−1, p b 0.05). Since the original improvements were still evident, particularly in the microcirculatory and cardiorespiratory assessments, 1 year after the initial study, the data were taken to suggest that a brief intervention combining MD with exercise in this high-risk group is a promising long-term health benefit strategy.[263]

Glucose control

Some studies in T2DM patients reported a positive effect of the MD on glycemic control, [264-269] others did not.[270-272] A recent meta-analysis has addressed some of the unmet issues in this area.[273] The control diets comprised low-fat diet, usual dietary habits, non-restricted calorie low-carbohydrate diet, the 2003 American Diabetes Association (ADA) diet and high-carbohydrate diet. Although the MD diet varied across studies, all the included diets comprised the basic characteristics of an MD diet. However, two studies compared three separate diets, one study[268] compared a MD versus two types of control diets, and the other one compared two versions of MD versus a control diet. Finally, two trials[270, 272] used a high-monounsaturated fatty acid-enriched intervention diet, that was comparable to the traditional MD. These different 11 arms were evaluated separately.[273] Nine studies with 11 arms (n = 1,178 patients) contributed data for the change in HbA_{1c} from baseline. Compared with control diets, the MD was associated with a significant reduction in HbA1c (mean difference, − 0.30; 95% CI, − 0.46 to − 0.14) by using a random effects model. Evidence of between-study heterogeneity was found in this analysis (I^2 = 67.2%; P = 0.001). Changes in fasting plasma glucose (FPG) levels were pooled from six studies with seven arms (n = 580 patients). Subjects who consumed MD had decreased FPG levels (−0.72 mmol/l; CI, − 1.24 to − 0.21) compared with subjects who consumed control diets. Heterogeneity of the effect measures on FPG was detected (I^2 = 66.1%; P = 0.007). Five studies with six arms (n = 531 patients) measured fasting insulin. Compared with control diets, there was a minimal but statistically significant decline in fasting insulin levels (−0.55 µU/ml; CI, − 0.81 to − 0.29) in subjects who used MD. No heterogeneity was observed (I^2 = 0.0%; P = 0.46). As to insulin resistance, although the beneficial effect of MD compared with control diet was not statistically significant (mean difference, − 0.55; CI, − 1.53 to 0.42), the MD had greater probability on improving insulin resistance. Moderate heterogeneity was present between the studies (I2 = 45.8%; P = 0.137).[273] Six studies with seven arms reported changes in BMI from baseline. A random effects meta-analysis comprising 520 patients randomly

assigned to the MD and 500 assigned to the control diets were employed for this study. The MD was more effective in decreasing BMI compared with the control diets (mean difference, − 0.29 kg/m2; 95% CI, − 0.46 to − 0.12; I^2 = 0.0%; P = 0.976). As to body weight, six studies involving 835 patients showed that MD was associated with a significant weight loss as compared with control diets (0.29 kg; CI, − 0.55 to − 0.04; I^2 = 0.0%; P = 0.924). In addition, compared with control diets, MD also significantly decreased total cholesterol (mean difference, − 0.14 mmol/l; 95% CI, − 0.19 to − 0.09) and triglyceride (−0.29 mmol/l; CI, − 0.47 to − 0.10) concentrations and increased HDL-cholesterol (0.06 mmol/l; CI, 0.02 to 0.10), without affecting LDL cholesterol (−0.11 mmol/l; CI, − 0.24 to 0.01). There was moderate heterogeneity for the analyses of triglyceride (I^2 = 58.0%; P = 0.03) and HDL (I^2 = 53.6%; P = 0.04) cholesterol. Finally, a trend towards reduction in systolic (−1.45mmHg; CI, − 1.97 to − 0.94; I^2 = 0.0%; P = 0.58) and diastolic (−1.41 mmHg; CI, − 1.84 to − 0.97; I^2 = 0.0%; P = 0.95) blood pressure was found in the MD groups.[273]

Total cholesterol

The relationship between serum total cholesterol and long-term mortality from (CAD) in different cultures was first analyzed in the Seven Countries Study. [274] Total cholesterol was measured at baseline and at 5- and 10-year follow-up in 12,467 men aged 40 through 59 years in 16 cohorts located in seven countries: five European countries, the United States, and Japan. To increase statistical power six cohorts were formed, based on similarities in culture and cholesterol changes during the first 10 years of follow-up. The age-standardized CAD mortality rates in the six cohorts ranged from 3% to 20%. The RRs for the highest compared with the lowest cholesterol quartile ranged from 1.5 to 2.3, except for Japan (RR of 1.1). For a cholesterol level of ≈5.45 mmol/L (210 mg/dL), CAD mortality rates varied from 4% to 5% in Japan and Mediterranean Southern Europe to ≈15% in Northern Europe. Except for Japan, the relative increase in CAD mortality due to a given cholesterol increase was similar in all cultures. Using a linear approximation,

a 0.50-mmol/L (20-mg/dL) increase in total cholesterol corresponded to an increase in CAD mortality risk of 12%.[274]

LDL-cholesterol

Lowering LDL-cholesterol concentration is the primary target of therapy for the prevention of CV disease (CVD).[275-277] However, in addition to LDL-Cholesterol concentrations, it has been shown that a more detailed analysis of LDL physico-chemical properties (e.g., size and oxidation) provides further insight into individual CV risk,[278, 279] individuals with a predominance of small, dense LDL particles (sdLDL) being at increased risk of CAD compared to those with larger, buoyant LDL particles.[280-282] Compared with large LDL, sdLDL possess a lower affinity for the LDL receptor and a longer half-life in plasma[283], bind more tightly to arterial proteoglycans[284], penetrate the arterial sub-endothelium more easily[285] and are more susceptible to oxidation.[286] LDL oxidation contributes to atherosclerotic plaque formation by causing endothelial dysfunction, the release of inflammatory cytokines and macrophage transformation into foam cells.[279] Accordingly, oxidized LDL (oxLDL) concentrations have been identified as an important marker of atherosclerotic lesions.[279, 287] Sex disparities have been reported for LDL physico-chemical properties, men being characterized by a higher proportion of sdLDL and greater concentrations of oxLDL than premenopausal women.[288-293] A specific study was carried out to verify whether the impact of MD on LDL size distribution, as well as on oxLDL concentrations differs between men and women. It appeared that adhering to the MD, in addition to a clinically-relevant reduction in LDL-cholesterol concentrations, has additional positive effects on LDL particle size phenotype, leading to a favorable redistribution from smaller to larger LDL in men, but not in women.[294]

HDL-cholesterol

Dietary supplementation of unsaturated FA protects against atherosclerosis and CV events.[295] The effects of dietary FA on the lipid metabolism have been widely

investigated: mono- and poly-unsaturated FA (MUFA/PUFA) supplemented to the diet are effective in lowering total cholesterol (TC) and LDL cholesterol, while saturated FA (SFA) increase TC and LDL-cholesterol.[61, 296] HDL cholesterol shows a slight decrease with PUFA dietary supplementation.[61] However this concept has been challenged.[296] Plasma levels of PUFA, but not SFA, are associated with lower apolipoprotein AI (apo-AI) and HDL-C levels in the Framingham study, but with different features in men and women.[297] How plasma non-esterified fatty acids (NEFA) composition correlates with plasma HDL-C levels in a free living population of Southern Italy that follows a "modern" version of the MD with a lower content of fibers and an excess of introduced calories in comparison with the "traditional" diet has been addressed in one study.[298] Olive oil constitutes the majority of dietary fat, in this study. The study population was characterized by a high prevalence of obesity[298, 299] and MetS.[299] On average, HDL-C levels of this population were slightly lower than the Italian levels.[298, 299] HDL-C levels of this population correlated positively with the proportion of plasma PUFA and negatively with SFA. Among individual NEFA, HDL-C levels strongly and negatively correlated with C14:0, C18:1n9 and C20:1n9, and positively with C16:1. An "in vitro" model of cultured hepatoma cells was then conditioned with NEFA and those FA that best correlated with plasma HDL-C levels in the population, or that resulted very abundant in the plasma, as C18:0. This "in vitro" study documented that myristic acid modified the HDL binding to liver cultured cells. C14:0 increased the nonspecific binding of HDL to cell surface and it did not affect the saturable receptor-mediated binding. C14:0. Thus, myristic acid is negatively correlated with plasma levels of HDL-C in a MD characterized by an excess of obesity and MetS, and part of this correlation might be explained by an increase of HDL binding to hepatic HSPG and subsequent cholesteryl esters stripping by proteoglycans-bound lipases.[300]

Triglycerides

Observational studies have found an association between elevated triglyceride (TG) levels and increased risk for CV disease morbidity and mortality. In the 1990s, the lipid research clinics follow-up study[301], the physicians' health study[302], and a meta-analysis of 17 population-based prospective studies [303] consistently found that increased concentrations of TGs are associated with an increased risk of AMI, CAD, and mortality, independent of HDL concentrations. [304-306] In recent years, a genetic predisposition to elevated TG levels has also been documented.[307] TGs have also independently contribute in the causal pathway of atherosclerosis. The effect of the MD on TGs has been thoroughly investigated. In a 3-mo intervention, a MD supplemented with walnuts reduced TGs by 0.15 mmol/L compared with a low-fat control diet.[308] One 3-mo intervention trial compared a low-fat diet and a MD for effects on TG-rich lipoprotein (TRL), TGs, and TRL-TGs.[309] In both groups, TGs were 0.2 mmol/L lower after adjustment for BMI; TRL-TGs were also lower by 0.16 mmol/L in the MD group.

Smoking habit

Vardavas et al.[310] performed a literature search to investigate whether adherence to MD might have a role to modify the deleterious effects on human health of active and passive smoking. They found that both epidemiological and laboratory studies show that MD has a protective effect against biochemical and molecular processes that lead to cancer, CV disease and respiratory illness. Based on the high daily intake of vitamins and antioxidants, MD provides a variety of compounds that positively affect certain outcomes related to smoking. Moreover, some diseases attributable to smoking, such as lung cancer, asthma and CV disease, are inversely associated with the use of certain antioxidants and lipids.

Obesity

Traditional nutritional advice for the treatment of obesity and associated disorders has emphasized avoiding animal fat and, preferably, all types of dietary fat, and replacing them with carbohydrate (CHO).[311] The central arguments against animal fats consumption and of fatty foods in general, have been a high content in cholesterol-rising saturated fatty acids (SFA) and excess energy, thought to promote obesity. Scientific evidence has accumulated concerning the beneficial role of diets with a relatively high MUFA content on CV risk factor outcomes, including diabetes.[312] The debate on what is the best nutrient to replace energy sources from SFA in the diet, CHO or MUFA, has indirectly been solved by the Women's Health Initiative study[313] in which the lack of protective effect of a high-CHO diet against CV disease has been documented. Recent results from a 20-year follow-up of the prospective Nurses' Health Study[314] suggest that a low-CHO diet (high-fat and/or high-protein) does not promote CAD and might actually reduce its incidence when the diet is high in unsaturated fat and vegetable proteins. The results of observational studies in Mediterranean countries, where people complying with the MD consume significant amounts of olive oil, have shown that increasing adherence to such high-fat, high-MUFA dietary pattern is associated with decreasing obesity rates.[315-317] Accordingly, a prospective study from a Mediterranean country has shown that increasing olive oil use is not associated with weight gain.[318] Moreover, a recent cohort study (n=613 individuals) has 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.[319] Another randomized trial with ad libitum MD enriched with olive oil or mixed nuts, the PREDIMED study, showed no 3-month weight gain with these high-fat diets.[308] Restricted energy diets that were relatively high in fat because they incorporated olive oil were effective alternatives to the traditional low-fat diet for initial weight loss and maintenance in feeding studies in obese persons.[320, 321] A satiating effect of olive oil intake with ensuing food compensation might explain its lack of a fattening effect. In this regard, experimental evidence has been provided that

mobilization of intestinally-derived oleoylethanolamide, a lipid messenger of satiety, is enabled by uptake of dietary oleic acid.[322] These positive effects accounted for by the MD have also been observed in other European region. In a recent study including a sample size of almost 500,000 individuals from 10 Mediterranean, Central, and Northern European countries, the adherence to a MD, modified to apply across Europe, was associated with lower waist circumference in men and women after controlling for BMI, total energy intake, and other potential confounders. Interestingly, the inverse association between MD Score and waist circumference was stronger in men and women from Northern European regions who were overweight or obese.[323] In another prospective cohort study (EPIC-PANACEA) in 373,803 individuals from 10 European Countries,[324] individuals with a high adherence to the MD showed a 5-y weight change of -0.16 kg (95% CI: -0.24, -0.07 kg) and were 10% (95% CI: 4%, 18%) less likely to develop obesity than individuals with a low adherence to the MD pattern.

Sex

Sex differences in the impact of the MD on glucose/insulin homeostasis have been documented (see above). Whether these sex-related effects were associated with changes in NEFA has been verified in 38 men and 32 premenopausal women (24–53 y).[325] Variables were measured during a 180 min Oral Glucose Tolerance Test (OGTT) before and after the MD. A sex-by-time interaction for plasma insulin iAUC was found (men: −17.8%, P = 0.02; women: +9.4%, P = 0.63; P for sex-by-time interaction = 0.005). A sex-by-time interaction was also observed for insulin sensitivity (Cederholm index, P = 0.03), for which only men experienced improvements (men: +8.1%, P = 0.047; women: −5.9%, P = 0.94). In contrast, no sex difference was observed for glucose and C-peptide responses. Trends toward a decrease in NEFA AUC (P = 0.06) and an increase in NEFA suppression rate (P = 0.06) did not show any sex difference. Changes in NEFA were not associated with change in insulin sensitivity. Compared to women, more favorable changes in

glucose/insulin homeostasis were observed in men. These differences in response to the MD are not explained by sex differences in NEFA response.

Long-term adherence to the MD following a nutritional intervention promoting the Mediterranean food pattern in Canadian men and women presenting CV risk factors has also been evaluated. [326] The 12-week nutritional program used a motivational interviewing approach and included individual and group sessions. A food frequency questionnaire was administered to evaluate dietary intakes from which a Mediterranean score (Medscore) was derived and the Three-Factor Eating Questionnaire allowed for assessing eating behaviors. Measurements were performed at baseline and after the 12-week nutritional intervention, and then at 3 and 6-month post intervention. No gender difference was observed in changes in the Medscore during the nutritional intervention and follow-up. However, the Medscore returned towards baseline values during follow-up in men and women ($P < 0.0001$). Men reported larger decreases in red and processed meat and larger increases in whole fruit intakes than women ($P = 0.03$ and $P = 0.04$, respectively). Men showed a greater decrease in habitual susceptibility to disinhibition than women ($P = 0.03$). A gender by time interaction was found for waist circumference, i.e. men having lower waist circumference at the end of the intervention as well as at follow-up than at baseline. Women's waist circumference decreased in response to the intervention only ($P = 0.05$). Changes observed in total-cholesterol (C) to HDL-C ratio, triglyceride levels and triglycerides to HDL-C ratio were more pronounced in men than in women after the intervention as well as at follow-up ($P \leq 0.03$).

Aging

Dietary manipulation might be a major tool for partially modifying the structure and consequently the features of biological membranes.[327] The possibility of supporting cell membranes with specific membrane components that are characterized by an elevated turnover allows to ameliorate and/or counteract modifications induced by the activity of free radicals. If the new fatty acids to be

used in this process are provided in the form of virgin olive oil, a variety of benefits are likely to occur. With its very high content of MUFA (mainly oleic acid), olive oil greatly enriches all biological membranes so that it partially and gradually replaces the other fatty acids, mainly PUFA. This process produces membranes that are less susceptible to oxidative injury because of the increased concentration of oleic acid. It is important to emphasize that the propagation of lipid peroxidation phenomena requires the fatty acids to possess two or more double bonds.[327] On the other hand, virgin olive oil also contains large amounts of free-radical scavengers (e.g. α-tocopherol, several different phenolic compounds and even coenzyme Q) that neutralize the toxic species and sometimes even prevent the early steps of their formation.[328-330] The efficacy of dietary interventions based on virgin olive oil to modify the fatty acid pattern of biological membranes making them less prone to oxidative modifications, has been widely demonstrated.[61, 331-333] In addition, MUFA lead to favorable changes in the lipid profile of lipoproteins, generating LDL particles more resistant to oxidative modifications.[334] Therefore, it has been suggested that MD might be a new, promising direction in the treatment (and in the prevention) of age-related diseases, as a useful adjuvant to the corresponding pharmacological therapy.[327]

MD and Emerging Vascular Risk Factors

Fatty acids profile

The beneficial effect of MD is thought to be related to its ability to affect several key risk factors.[335] Through the intake of antioxidants, fiber, and polyunsaturated fats, MD lowers blood pressure and plasma homocysteine concentrations, improves endothelial function, and insulin resistance, and decreases the risk of thrombosis and of ventricular arrhythmias.[32, 259, 336-340] On the other hand, vis-à-vis the seminal concept in the Seven Countries Study that 10-yr coronary mortality rate was significantly related to sample average saturated fatty acid intake in the habitual diet, several authors have emphasized reductions in CV risk -in primary

and secondary prevention studies-, when replacing saturated fatty acid intake with MUFA and/or PUFA [341-343]. FA influence plasma levels of atherogenic lipoproteins[344] and affect several aspects of inflammation,[345] a key mechanism in atherothrombosis.[346]

Presently, among a variety of debated dietary recommendations to decrease the risk of CV disease, reducing the intake of saturated fatty acid (SFA) (or replacing it with monounsaturated or polyunsaturated fatty acids) is a priority in CV prevention guidelines.[347, 348] However, the results of two meta-analyses have questioned the link between saturated fat intake and risk.[349, 350] Even though the FA profile of individuals is influenced by dietary intake - as is the case of LA and ALA- it is also endogenously modulated by the activity of specific enzymes.[351] Studies [36, 352-354] indicate that, compared with healthy subjects, patients with CAD exhibit higher blood levels of trans fatty acids and SFA and lower levels of PUFA, with controversial data about n-6 PUFA, especially AA.[355] Blood levels of palmitic acid are strongly associated with CAD. [356] Whether, however, blood FA: 1) is an index of the quality of food intake; 2) it might be used as a tool to estimate diet quality and compliance with a nutritional advice, and 3) the adverse plasma fatty acid profile in patients with CAD can be favorably modified by consuming a MD, has not been established in previous reports.

Inflammation

So far, the beneficial effect of MD against CAD has been accounted for by the ability of this dietary pattern to modify traditional risk factors of atherosclerosis. Recently, some authors have suggested that the anti-inflammatory effect–at the level of the vessel wall- may be key to explain the link between MD adherence and low CV mortality.[357] There is compelling evidence that inflammation is crucial at all stages of the atherosclerotic process.[358] Early phases of atherosclerosis (fatty streak formation) involve the recruitment of inflammatory cells from the circulation, their adhesion to endothelium and migration to sub-endothelial space, a complex process which involves adhesion molecules (e.g. interleukin [(IL])

production and up-regulation on endothelial cells and leucocytes.[359] Adhesion molecule expression on circulating peripheral blood mononuclear cells is a crucial step for the firm adhesion of such cells to the endothelium during the inflammatory reaction linked to atherosclerosis development.[358, 359]. Thereafter, resident macrophages and lymphocytes become activated and secrete cytokines that in turn activate other cell types leading to a self-perpetuating inflammatory process in the vascular wall that is instrumental in plaque formation, vulnerability and rupture leading to thrombosis.[358, 359] Thus, ongoing inflammation is also crucial in the development of instability and rupture of atheromatous plaques and the development of ischaemic events in most advanced stages of the disease.[358, 359]

Adipose tissue is both a dynamic endocrine organ, and a highly active metabolic tissue. Fat produces and secretes adipokines, which play an important role in the induction of insulin resistance and in the atherosclerotic process. Visceral adiposity is more strongly associated with insulin resistance than subcutaneous fat. However, the precise chain of events linking over-nutrition, obesity, activation of the innate immune system and reduced insulin sensitivity in peripheral tissues remains incompletely understood.[360] In a randomized controlled study in 120 obese women, a multidisciplinary program aimed at reducing weight through lifestyle changes (diet and exercise) was associated with reduced levels of CRP and IL-6, and increased levels of adiponectin, associated with improved insulin sensitivity.[361] Mechanisms underlying a dietary intervention in patients at increased cardiac risk were also explored[357] in 180 patients (99 men and 81 women) with the MetS randomized to receive a MD-style diet (instructions about increasing daily consumption of whole grains, vegetables, fruits, nuts and olive oil) vs. a cardiac-prudent diet (less than 30% fat intake). After 2 years, while endothelial function improved, body weight decreased more in the intervention group than in the control group, but even after controlling for weight loss, inflammatory markers, such as IL-6, IL-7, IL-18 and CRP, and insulin resistance declined more in the MD intervention group than in the control group. Only 40

patients in the intervention group still had MetS after 2 years when compared with 78 patients on the control diet.

Oxidative stress.

It is long been known that ROS produced by activated leukocytes and macrophages are crucial to control invading microorganisms. Now it is clear that these intermediary metabolites produced under physiological conditions during oxygen metabolism, also play a critical role as signal molecules in health and disease. Mitochondria are the most important source of intracellular ROS. However, ROS can be generated by a variety of enzymes including oxidases, cyclooxygenases and lipoxygenases. The primary ROS produced in the body is superoxide anion (O^{2-}), which is generated from a 1-electron reduction of molecular oxygen. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the main source of O^{2-} in mammalian cells.[362] NADPH oxidase is a multicomponent enzyme that has a membrane portion known as cytochrome b_{558} (that comprises a large gp91phox, and a smaller p22phox subunit), which is inactive until it is associated with the cytosolic component (p47phox, p67phox and p40phox). Under physiological conditions, reactive and incompletely reduced forms of oxygen are detoxified into water by anti-oxidant defence and repair enzymes to maintain a low steady state of toxic oxidants. [363] Steady-state levels of O^{2-} depend on both their rate of production and the activity of a wide array of dietary antioxidants and endogenous antioxidant enzymes such as glutathione (GSH, reduced form), catalase, and superoxide dismutase (SOD). [364] In plasma, glutathione (GSH, reduced form) and glutathione disulphide (GSSG, oxidized form) are transported from tissues by concentration-dependent transport systems.[365] Results from animal experiments have shown that the of GSH to GSSG ratio (GSH/GSSG) in plasma decreases in response to tissue oxidative stress.[366] Therefore, GSH/GSSG may be preferable to either GSH or GSSG alone as an overall indicator of the redox status and to be used as a marker of oxidative stress.[367] In mammals, there are 3 isoforms of SOD: a cytosolic or copper/zinc (Cu/Zn- SOD), a

manganese (MnSOD) localized in mitochondria, and an extracellular form of Cu/ZnSOD. Although produced by distinct genes, these 3 SODs catalyze the same reaction: dismutation of O^{2-} into hydrogen peroxide plus molecular oxygen.[368]
While an imbalance in the mitochondrial respiratory chain is the main source for the generation of abnormally high levels of ROS, an impaired antioxidant capacity leads to an excess in the production of ROS as well, and in turn to oxidative stress, i.e. to the disruption of the redox signalling and control [369] with subsequent oxidation of macromolecules, and tissue damage and dysfunction.[370] In particular, oxidative stress is involved in major events related to CV disease such as DNA damage, tissue remodelling, and alterations in gene expression.[371]
Biochemically, GSH/GSSG redox decreases lipid hydroperoxides by reducing these peroxides into alcohols and suppressing their generation.[372, 373] Decreased lipid hydroperoxides, in turn, lower oxidized LDL and slow the atherosclerotic process.[372, 373] Additionally, a lower GSH/GSSG may result in protein glutathionylation and oxidatively altered GSH-GSSG redox signalling[367] and associated gene expression and apoptosis, which may contribute to atherosclerosis. Clinically, an unfavourable GSH/GSSG has been found in patients with AMI [374] and has been related to the progression of atherosclerotic lesions after percutaneous coronary interventions.[375]
Among multiple key factors in the physiology of the endothelium, oxidative stress has been identified as a major regulatory mechanism. Current evidence is that MD leads to improved redox balance, increased nitric oxide bioavailability and decreased availability of pro-inflammatory and pro-oxidant molecules.[339, 357, 376, 377]
Old studies from this Institution have reported that phenols -natural antioxidants present in virgin olive oil-, affect endothelial function in a dose-dependent manner.[378] The possibility that key components of MD act synergistically on endothelial function through their antioxidant properties has been also addressed. The combination of high-phenol virgin olive oil and red wine (rich in antioxidants) induce favorable effects on postprandial flow-mediated dilatation in healthy young men.[379] Although the exact molecular routes underlying these effects are

still matter of investigation, hydroxytyrosol (a key phenol antioxidant of virgin olive oil) and resveratrol –a major antioxidant of red wine-, both exert their vaso-protective effects in response to oxidative injury by the activation of the Nrf2 pathway, which, in turn, promotes the expression of antioxidants, e.g. NAD(P)H:quinone oxidoreductase 1.[123, 380] Moreover, polyphenols cause nitric oxide, and endothelium-derived hyperpolarizing factor mediated relaxation.[381] Compelling evidence suggests an association between oxidative stress and obesity/insulin resistance. In nearly 3,000 patients from the Framingham Heart Study, an association between increasing body mass index and increasing systemic oxidant stress has been reported.[382] Data from 2,002 nondiabetic subjects of the community-based Framingham Offspring Study indicate that, even after correcting for BMI, systemic oxidative stress is associated with insulin resistance in individuals at average or high risk of diabetes. (metabolic syndrome or IFG)[383] Short-term (up to six months) caloric restriction in overweight men and women produced significant body weight reduction associated with decreased DNA damage, another surrogate marker of oxidative stress.[384] Finally, individuals at high CV risk who improved their diet toward a MD pattern show significant reductions in cellular lipid levels and LDL oxidation.[385] Adiponectin -a protective factor against atherosclerosis and insulin resistance-, plays an important role in modulating both insulin sensitivity and concentrations of circulating plasma glucose and NEFA, a lower concentration of circulating plasma of NEFA being a good predictor of reduced insulin sensitivity and increased risk of T2DM. [386] Accordingly, a MD-type of diet reduces the need for glucose-lowering drugs and improves short-term glucose control more than a low-fat diet in overweight patients with newly diagnosed T2DM. [103]. Regardless of caloric restriction, a prolonged adherence to MD in overweight men results in a rise in circulating level of adiponectin, and lowers several markers of oxidative stress.[387] In 987 diabetic women from the Nurses' Health Study with no history of CV, the closer the adherence to a MD the higher the adiponectin concentration. [388] Of the components of the MD pattern, alcohol, nuts, and whole grains show the

strongest association with adiponectin concentrations. In insulin-resistant offspring of obese T2DM patients, a MD rich in MUFA (23%) improves insulin sensitivity, and this is associated with increased postprandial adiponectin mRNA gene expression in peripheral adipose tissue.[389] All in all, in addition to lowering blood pressure, and total cholesterol and triglycerides; increasing HDL-cholesterol, and improving insulin sensitivity, a prolonged adherence to MD, with or without caloric restriction in overweight or obese men, is associated with reduced oxidative stress. The latter changes are amplified when MD is associated with calorie restriction and increased physical activity.[390] This information is particularly important for individuals who carry additional risk factors, such as T2DM, obesity, and the metabolic syndrome, and fail, as most do, to have a consistent and long-term weight loss.

Rationale

Since the first data from the Seven Countries Study,[391] several studies carried out in different populations have established a beneficial role for the main components of MD as to the occurrence of all-cause cardiovascular (CV) disease, including CVD and chronic degenerative diseases, malignancy, lipid metabolism, blood pressure, and pathophysiological settings such as endothelial dysfunction and overweight.[392]

A systematic review of prospective cohort studies that have analysed the relation between the adherence to several MD scores and the risk of CV mortality in primary prevention settings (404,491 subjects), confirmed these findings showing that a two point increase in the adherence to MD scores was significantly associated with 9% reduction of the risk of cardiovascular mortality, largely independent of vascular risk factors (VRFs).[393] To this end, a literature-based MD score based on nine items that may predict the CV risk at individual level has been proposed.[394]

Concerning the relationships between MD score and subclinical atherosclerosis (as indexed by carotid intima media thickness; c-IMT) and/or its change over time (cIMT progression), the results reported in the literature are sparse and often inconsistent.[34, 242-245, 250] Epidemiological studies investigating the association between MLDP and c-IMT progression in large cohorts are completely lacking.

As to oxidative stress status evaluation, it is increasingly clear that in different stages during the atherothrombotic process, abnormally high levels of reactive oxygen species (ROS) are generated that can overwhelm antioxidant systems and adversely affect cell function by causing oxidative post-translational injury.[395] A similar altered equilibrium can be found when evaluating both inflammation markers and blood fatty acids profile in patients with CVD.[396, 397] Indeed, blood fatty acids (FA) composition has been implicated in cardiovascular risk, presumably through changes in oxidative stress and inflammation, two mechanisms involved in the pathogenesis of atherothrombosis.[36] Previous data

from our group suggest that patients with CAD adhere less to features of MD and exhibit a different fatty acid profile compared to healthy subjects.[396]

Aims of the present study

Primary objectives of the present study were to evaluate:

1) The relationship between MD adherence and c-IMT progression;

2) Whether such relationship is similar in populations from different European countries with different nutritional patterns/habits (e.g. in people living far from the Mediterranean area);

3) Whether the relationship between MD (or of its components) and baseline c-IMT burden is consistent with a dose–response relationship or a threshold effect;

4) Whether the relationship between MD, baseline c-IMT, and clinical events involves changes in major and emerging (e.g. inflammation, oxidative stress, and FA composition) vascular risk factors.

5) Whether and to what extent MD adherence relates to inflammatory markers, markers of an oxidative stress status, and to FAs profile in males and females with a recent history of coronary revascularization.

Methods

For the objectives of the present report, data from two major studies carried out in this Institution have been analyzed: the IMPROVE Study and the RISMED Study. Additional information concerning these two studies are reported below.

The IMPROVE Study

Study objectives The primary objective of the IMPROVE study was to evaluate the association between Mediterraneran-like dietary pattern (MLDP) and c-IMT progression over 15 months and to assess whether c-IMT progression was an independent predictor of risk of future vascular events (VEs) (AMI, cardiovascular death, stroke, or any intervention in the carotid, coronary, or peripheral arterial districts) occurring from the 15th to the 36th month in the follow-up. To achieve this goal the 3-year follow-up was split into two time periods: from 0 to 15 months to measure the c-IMT changes over time and from 15 to 36 months to collect VEs.[249]

Study design.

The design and the major results of the IMPROVE studies have been published elsewhere.[249, 398] Briefly, the IMPROVE study is a multicenter, longitudinal, observational study, funded by European Union (EU) within the Vth framework program, which involves seven recruiting Centers in five European countries: Finland (two Centers), France, Italy (two Centers), the Netherlands, and Sweden. The study was designed according to the rules of Good Clinical Practice (GCP), and the ethical principles established in the Declaration of Helsinki. Each participant provided two different informed consents; one for general participation in the study and one for genotyping.[247] The protocol was designed for a study duration of 36 months. Recruitment of a total of 3,598 patients (514 per center) was targeted. About 21,000 subjects were screened: 3,400 in Milan, 1,450 in the first Kuopio center and 2,354 in the second, 4,239 in Stockholm, 4,050 in the

Netherlands, 3,804 in Perugia, and 1,800 in Paris. Men and women, aged from 55 to 79 years, with at least three VRFs, asymptomatic for cardiovascular diseases and free from any conditions that might limit longevity or c-IMT visualization were considered as eligible for the study. Individuals who met the eligibility criteria and who signed both informed consents were enrolled in the study. Due to its observational nature, participation did not require any change in medication(s).[247] A total of 3,711 individuals were enrolled, aged 54-79 years were enrolled. All participants never had a cardio- or cerebro-vascular event before enrolment and all have at least three VRFs. Subjects were considered to be exposed to a VRF when one of the following criteria was satisfied: male sex or at least 5 years after menopause for women; hypercholesterolemia (mean calculated LDL-C blood levels > 160 mg/DL or treatment with lipid lowering drugs); hypertriglyceridemia (triglycerides levels > 200 mg/DL after diet or treatment with triglycerides lowering drugs); hypoalphalipoproteinemia (HDL-C < 40 mg/DL); hypertension (diastolic blood pressure > 90 mmHg and/or systolic blood pressure >140 mmHg or treatment with anti-hypertensive drugs); diabetes or impaired fasting plasma glucose (blood glucose level > 110 mg/DL or treatment with insulin or oral hypoglycemic drugs);[399] smoking habits (at least 10 cigarettes/day for at least thirty months); family history of cardiovascular diseases. *Exclusion criteria were*: age under 54 or over 79 years; abnormal anatomical configuration of neck and muscles, marked tortuosity and/or depth of the carotid vessels and/or uncommon location of arterial branches; personal history of AMI, angina pectoris, stroke, TIA, aortic aneurysm, intermittent claudication, surgical intervention of revascularization in carotid, coronary or peripheral arterial territories, CHF (III-IV NYHA Class); and history of serious medical conditions that might limit longevity (e.g. cancer).[249]

Ultra sonographic protocol.

Carotid ultrasound was performed using seven identical ultrasonic machines (Technos system, ESAOTE), equipped with 5-10 MHz linear array probes. All

machines were identically calibrated. Calibration was checked with a phantom before the start of the study and after 1 year. Sonographers and readers were trained and certified by the coordinating Center (Department of Pharmacological and Biomolecular Sciences) in Milan. The far walls of the left and right common carotid (CC), the bifurcation (BIF) and the internal carotid artery (ICA) were visualized in anterior, lateral and posterior angles and recorded on sVHS videotapes. Images were acquired with the subject's head rotated approximately 45° opposite to the side being imaged and the three angles were acquired with the transducer held approximately at 45°, 20° and 60° from the vertical. Each selected image of the CCs (in their entire length), the BIFs, and the first proximal centimeter of the ICAs were measured in at least three different frames randomly distributed within the cardiac cycle. In order to explore the influence of the cardiac cycle on IMT measurements, in a subsample of 100 subjects, CC-IMT_{mean} was re-measured using a dedicated software, [400] which allows continuous measurements of IMT within 3 to 5 beats, thus allowing recording of the kinetics of IMT changes associated to cardiac cycle. Peak systolic and end-diastolic IMT measurements, as well as their average, were then compared with IMT measurements obtained according to the IMPROVE protocol without electrocardiographic synchronization. Preliminary data showed that IMT measurements obtained with the IMPROVE protocol (0.667±0.172 mm) well fitted with the mean value (0.665±0.172) of IMT measurements obtained in peak systolic (0.631±0.164 mm) and end-diastolic (0.699±0.172 mm) phases, with a very good correlation (r = 0.9993) and a minimal bias. In each carotid segment (1 cm length), both mean and maximal IMT were evaluated. Plaques were incorporated into these measurements. Eleven C-IMT variables were considered: 1^{st}CC-IMT_{mean}, 1^{st}CC-IMT_{max}, CC-IMT_{mean}, CC-IMT_{max}, BIF-IMT_{mean}, BIF-IMT_{max}, ICA-IMT_{mean}, ICA-IMT_{max}, IMT_{mean}, IMT_{max} and $IMT_{mean\text{-}max}$. 1^{st}CC-IMT_{mean} is the average of six mean IMT values obtained by measuring both left and right CC (1 cm length proximal to the bifurcation) in the 3 angles (anterior, lateral and posterior). The greatest among all the maximal IMT values measured in each one of these six 1^{st}CC

segments (two carotids x three angles) was defined 1st$CC\text{-}IMT_{max}$. $CC\text{-}IMT_{mean}$ is the average of all mean IMT values obtained from left and right CC visualized in their entire length (excluding the 1st cm) with sequential probe movements of 1 cm length, according to the 3 scan angles (anterior, lateral and posterior). The total number of segments visualized ranged from 6 to 24 according to the subject's length of the neck. In each segment, the software automatically provided also the maximal IMT value. The greatest among all the maximal IMT values detected in each one of the 6 to 24 CC segments was defined as $CC\text{-}IMT_{max}$. $BIF\text{-}IMT_{mean}$ is the average of six mean IMT values obtained by measuring both left and right BIF (1 cm length) in the 3 angles (anterior, lateral and posterior). The greatest among all the maximal IMT values measured in each one of these 6 BIF segments (left and right Bif x three angles) was defined $BIF\text{-}IMT_{max}$. $ICA\text{-}IMT_{mean}$ is the average of six mean IMT values obtained by measuring both left and right ICAs (the 1st cm proximal to bifurcations) in the 3 angles (anterior, lateral and posterior). The greatest among all the maximal IMT values measured in each one of these 6 ICA segments (left and right ICA x three angles) was defined $ICA\text{-}IMT_{max}$. Composite variables (IMT_{mean}, IMT_{max} and $IMT_{mean\text{-}max}$) refer to the whole carotid tree:
IMT_{mean} is the average of 1st$CC\text{-}IMT_{mean}$, $CC\text{-}IMT_{mean}$, $BIF\text{-}IMT_{mean}$ and $ICA\text{-}IMT_{mean}$.
IMT_{max} is the greatest value among 1st$CC\text{-}IMT_{max}$, $CC\text{-}IMT_{max}$, $BIF\text{-}IMT_{max}$ and $ICA\text{-}IMT_{max}$.
$IMT_{mean\text{-}max}$ is the average of 1st$CC\text{-}IMT_{max}$, $CC\text{-}IMT_{max}$, $BIF\text{-}IMT_{max}$ and $ICA\text{-}IMT_{max}$.
About 62% of participants were followed throughout the study by the same sonographer, and all scans for each subject were assigned to a single reader after coding and were read blindly in the centralized laboratory of Milan using a dedicated software (M'Ath, Metris SRL France).[401] These measurements were not reported to patients. The total number of carotid plaques (IMT_{max} >1.5 mm) in each carotid segment and in the whole carotid tree (also normalized according to the number of segments actually visualized) and the number of non-visualized carotid segments were also recorded. To evaluate changes of C-IMT over time, ultrasonographic measurements were repeated at 15 months using the same

ultrasonographic protocol (positions and angles of ultrasound transducer with respect to the neck) used at baseline. C-IMT change for each ultrasonographic variable, expressed in mm/year, was calculated as the difference between the 15-month measurement and the corresponding baseline value divided by the length of the time of intervention.

The variables selected for the statistical analyses were the changes per year of mean and maximum IMT values of the first centimeter of CC proximal to the bifurcation (1^{st}CC-$IMT_{mean\text{-}progr}$ and 1^{st}CC-$IMT_{max\text{-}progr}$), of the remaining part of CC (CC-$IMT_{mean\text{-}progr}$ and CC-$IMT_{max\text{-}progr}$), of BIF (Bif-$IMT_{mean\text{-}progr}$ and Bif-$IMT_{max\text{-}progr}$) and of ICA (ICA-$IMT_{mean\text{-}progr}$ and ICA-$IMT_{max\text{-}progr}$) as well as the changes of the mean, maximum and mean-maximum IMT values of the whole carotid tree ($IMT_{mean\text{-}progr}$, $IMT_{max\text{-}progr}$, and $IMT_{mean\text{-}max\text{-}progr}$). To explore hypothesis that vascular risk may be better associated with a focal rather than diffuse progression and that the maximal change may occur in any site regardless of its location and its initial size, the "Fastest-$IMT_{max\text{-}progr}$", i.e. the greatest value chosen among the progressions of IMTmax detected in the eight carotid segments measured was also considered. Examples of the Fastest-$IMT_{max\text{-}progr}$ in three subjects are shown in (Supplemental Figure IV). The precision of the ultrasonographic measurements at baseline has been reported.[247, 398]

Laboratory analyses.

Laboratory analyses were performed at baseline and at month 30. Several biological samples were kept in a biobank. Specifically, the biobank contains 14 aliquots of 0.5 mL EDTA plasma and 8 aliquots of 0.5 mL serum for each subject. In addition, for each subject, 2 x 5 mL whole blood was stored for DNA extraction. DNA was purified (in the Atherosclerosis Research Unit, Karolinska Institute Stockholm, Sweden) from all patients who signed informed consent for genetic studies. Blood sampling for laboratory tests was performed after an overnight fast. Plasma glucose concentration, blood cell count, hematocrit and differential counts were measured locally. Blood for centralized biochemical analyses was kept at

room temperature for a minimum of 30 min to allow clotting to occur. Serum, prepared by centrifugation at 2,000g for 20 min, was dispensed in polypropylene tube and frozen at –80^{o}C prior to shipment for centralized biochemical analyses and biobanking in Stockholm (Karolinska Institute Stockholm, Sweden). Serum concentrations of total, HDL and LDL cholesterol (by Friedewald's formula), triglycerides, uric acid, high-sensitive C-reactive protein (hs-CRP) and creatinine were analyzed in a centralized laboratory in Stockholm (Department of Clinical Chemistry, Karolinska University Hospital Solna, Stockholm, Sweden) with the use of LX Beckman instruments. Cholesterol, triglycerides and uric acid were measured with enzymatic methods, creatinine with colorimetry-alkaline picric and hs-CRP with turbidimetry (NIPIA methods). Additional information concerning methods for laboratory analyses have been reported.[247]

Nutrition variables, physical activity, smoking habits, and psychosocial variables.

Milk, wine, beer, spirits consumption and total amount of alcohol were recorded in terms of “DL/day”. Fruit consumption was recorded in terms of “pieces a day”. The consumption of meat, fish and eggs was recorded in terms of “times a week”. The consumption of tea and coffee was recorded in terms of “times a day”. Although collected in a semi-quantitative way, these variables were used in the analyses in terms of consumers (>0) or not consumers (=0). The main type of fat consumed (lard, butter, olive oil, seed oil, margarine) and the type of milk consumed (not skimmed, semi-skimmed, skimmed) were also recorded.[247]

Quality control.

Standard operating procedures (SOPs) Before the start of the study, investigators were provided with detailed Standard Operating Procedures (SOPs) and were required to abide by them, in conformity with GCP. *Case Report forms (CRFs)* Data were collected using electronic CRFs allowing automatic control of all entered data by logic controls and range checks, and easy identification of missing data. Once compiled, each file was sent to the data management Center (DMC) and included

into the central database without any further typing. *Confidentiality of data* Information about participant identity (name, address, phone number and identity in the study) was retained at each recruitment center. To protect confidentiality, all information transferred to the central DMC (University of Milan) was anonymized. *Monitoring* of data quality was performed by regular site visits, mail and telephone interviews. *Record retention.* Each principal investigator retains copies of all relevant information for a period of 15 years after the completion of the study.[247]

The Mediterranean-Like Dietary Pattern (MLDP) score.

At baseline, dietary intake maintained during the year preceding enrolment was assessed by a semi-quantitative 11 items food-frequency questionnaire, administered by trained personnel. A MLDP score adherence score analogous to the Greek Mediterranean Index [24] and based on only seven nutritional items (fruits, fish, wine, olive oil, meat, milk and eggs) was then constructed. Since the amount and type of vegetables, legumes, nuts and cereals consumed largely differs among populations [402], the items included in the questionnaire were limited to foods well diffused in all the countries involved. For fruit or fish, high consumption (top tertile of their distributions, i.e. fruit ≥3 servings/day and fish >2 times/week) was scored one, other intakes received 0 points; for meat, eggs or milk a low intake (bottom tertile of their respective distributions, i.e. meat <2 times/week, eggs ≤1 times/week, milk ≤3 DL/day) received 1 point. A predominant consumption of olive oil, rather than of other types of fat, and a moderate consumption of wine (1-2 glasses/day) also received one point. Based on the scale obtained, score 0 indicates minimal adherence and score 7, maximal adherence to MLDP. Such MLDP was adopted being its indices inversely associated with the incidence of stroke in a recent Italian study. [403] The predictive performance of the MLDP score was first validated versus the incidence of vascular events and then tested versus primary endpoints (i.e. 15-mo c-IMT progression). Because a

standardized questionnaire and a common clinical and subclinical assessment were used, the results for the entire cohort could be readily compared among IMPROVE centers and among EU. Besides diet, IMPROVE also included a baseline assessment of socio-demographic, anthropometric, lifestyle, clinical and biochemical measures.

The RISMED Study

Study objectives

The RISMED Study was a randomized, parallel groups, open-label, intervention trial in which a 3-mo intensively advised MD was tested vs. an usual low-fat dietary advice to assess: 1) the possibility to favorably modify the adverse plasma fatty acid (FA) profile in patients with CAD by consuming a MD; 2) the effect of a MD on the oxidative balance and on indices of systemic inflammatory activity, and the relationship between these potential changes and the modification of the blood FA pattern, and 3) whether blood FA is an index of the quality of food intake and whether it might be used as a tool to estimate diet quality and compliance with a nutritional advice.

Study design

Participants (n= 75 per arm) were males and females, age 30-75, with a recent history of coronary revascularization, randomized after clinical stabilization (at least 60 days after any coronary procedure or event). Blood parameters (blood FA, total cholesterol, LDL-C, HDL-C, triglycerides, glucose), anthropometric variables (weight, height, waist circumference and BMI), blood pressure and heart rate were measured at randomization ad at the end of the treatment. *Exclusion criteria* were: patients with diabetes mellitus, food intolerance, BMI < 19 or > 33, or those who assume drugs or food supplements with omega-3 FAs or natural or synthetic antioxidants.

Nutritional advice.

MD was personalized in term of total calories, and included: fatty 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; men: 1-2 glasses of red wine a day, women: 1 glass of red wine a day; not more than 150 g red meat a week. Cold cuts, sweets, cakes, butter, fatty cheese were

discouraged. The control group received dietary counselling for mild weight loss (when needed) and low intake of saturated fats (<10% of total energy) and sugar rich foods. Dietary assessment was administered at enrolment by a validated dietary recall tool, the EPIC FFQ (food frequency questionnaire). Compliance to the assigned diet was assessed at the middle of the study using a 7-day food diary. The score proposed by Trichopoulou et al[24] was computed in both groups as a measure of adherence to the MD. A 7- day dietary recall was administered during the second month of the study to assess participants' compliance. Food and macronutrient intake throughout the year before inclusion was also estimated at enrolment using a validated FFQ of the EPIC, administered by a registered nutritionist. To assess whether blood FA is a reliable index of the quality of food intake, data obtained with the FFQ were correlated with the level of classes and specific types of FA in blood. Moreover, the change between baseline and final levels of FA classes and types were correlated with the compliance to the MD as assessed through a 7-day dietary recall performed at the middle of the study, a higher compliance with diet being associated with greater changes in levels of FA.

Laboratory analyses.

Blood and urinary samples were used for routine biochemical determinations. For *blood FA analysis*, whole blood, obtained from a fingertip of fasting subjects, was collected on a special adsorbent embedded with the antioxidant butylhydroxytoluene (Blood Collection kit, Sigma-Aldrich, St. Louis, MO). FA methyl esters, directly prepared by transesterification, were analyzed by gas liquid chromatography at oven temperature from 120 °C to 235 °C.[404] The following indices of oxidative stress and systemic inflammatory activity were determined at randomization and at the end of the study: hs-CRP; whole blood reduced glutathione (GSH) and its oxidized form (GSSG). hs-CRP was measured by immunoturbidimetry. Newly developed methods were used to measure GSH and GSSG. Each will be described in detail below together with the method employed for determining blood FA profile.

FAs profile determination.

Advantage of this method mainly consists in the fact that the profile of FAs reflects both plasma and red blood cell composition. Blood sampling is performed at the beginning and at the end of the study by fingertip puncture with an automatic punctured pen for diabetics with a disposable lancet. Blood drops are collected on an adsorbent paper strip (Sigma-Aldrich) suitable for chromatographic analysis of FA. Such paper contains two areas of 1.4 cm^2 each allowing for the collection of about 50 µl of blood that can be easily removed for extraction of FAs analysis. To avoid the degradation of the FA throughout the storage period, the adsorbent paper is soaked with the antioxidant butylhydroxytoluene (BHT) in ethanol (5 µg/µl) prior to sampling. To prevent degradation of the blood sample, once the blood is dried, the chromatographic strips are packed in individual envelopes (Ziplock), with the addition of a silica scrubber, and the samples are stored at -19° C for ≈two weeks. Preliminary studies documented that: 1) no chromatographic peak attributable to the adsorbent paper is detected in the chromatographic analysis of FAs, and 2) the samples treated according to this method are stable up to a few years. For the determination of the FA, a portion of chromatographic paper (1cm^2) with the blood sample is transferred into vials with a sealed cap and a teflon protective seal. Each vial is added with 0.6 ml of 3N methanol solution in hydrochloric acid (Supelco) and the vial is placed in a dry thermostat bath at 90° C for 1 hour (Liebish Laboratechnick). After FA methylation and cooling in a cold room at 4° C, 1 ml of water; 1 ml of KCl saturated solution, and 400 µl of n-hexane are added to each vial to continue FAs methyl esters (FAME) extraction. After agitation, samples are centrifuged for 8 minutes at 3,250 rpm at a temperature of 6° C to facilitate phase separation and extract the supernatant containing the FA methyl esters. Finally, FAMEs are analyzed by injecting approximately 0.5 µl of each sample into the GC 2010 Shimadzu gas chromatograph equipped with PTV injector and FID detector and connected to a dedicated DDS 1000 software for data collection and processing. The auxiliary gas employed is helium, and the column used is a DB-FFAP (Agilent) with a length of 15 m, an inside diameter of 10

mm and a film of 0.10 µm thick. The temperature of the column chamber is programmed as follows: an increase of 25°C/minute from 150° C to 205° C, isotherm at 205° C for 5 minutes, a further increase of 25° C / minute to 220° C, isotherm at 220° C for 2 minutes and finally an increase of 8°C/min to 248° C which is maintained for the next 9 minutes. Since a rapid gas chromatograph is used, with a run time of 19 minutes and 80 seconds each, the pressure is a higher than expected based on information from usual gas chromatographs (≈7.5 bar). This increment allows for the detection of FAs with a carbon number ranging from 14 to 24. When processed with the appropriate software, the chromatograms obtained allow the data to be expressed as percentage values of the total FAs. Product to precursor ratios of specific FAs are calculated as surrogates of the activity of specific enzymes involved in FA metabolism; C16:1n-7/C16:0 ratio estimated Δ9 stearoyl-CoA desaturase-1 (SCD1) and C18:3n-6/ C18:2n-6 ratio estimated Δ6 desaturase (D6D). [404] *The reproducibility* of this analytical method has been tested by analyzing samples of old methylated FAs from a single subject to which, on the same day, two blood samples were analyzed over three different time periods (the same day, after one week and three weeks after storage) and three blood samples analyzed in the same day but picked up respectively at fasting, before the lunch and two hours after the meal.

Measurement of GSH and GSSG.

Chemicals and reagents: -l-Glutamyl-l-Cysteinyl-Glycine (GSH), -l-glutamyl-lcysteinyl-glycine disulfide (GSSG), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA) and all HPLC grade and LC–MS grade solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Chromatographic columns were purchased from Phenomenex (Torrance, CA, USA). Millex®-GV Syringe filter units (13 mm, 0.22 m) were obtained from Millipore (Bedford, MA, USA).[405]

Method development and validation

GSH and GSSG were measured on whole blood added with 10% trichloroacetic acid (TCA) in 1 mM EDTA solution to precipitate proteins and stored at -80°C until analysis. Levels of GSH and GSSG were assessed by LC-MS/MS method using a TSQ Quantum Access (Thermo Fisher Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer coupled with ESI operated in MRM in positive mode. Separation of analytes was carried out on a Luna PFP analytical column (100x2.0 mm, 3 µm, Phenomenex, Torrance, CA, USA). The LC mobile phases were: (A) ammonium formate 0.75 mM adjusted to pH 3.5 with formic acid and (B) methanol. Separation was performed under isocratic conditions with 99% mobile phase A at flow rate of 200 µL/min and a column temperature of 35°C. The MRM for GSH (m/z 308.1→m/z 76.2 + 84.2 + 161.9) and GSSG (m/z 613.2→m/z 230.5 + 234.6 + 354.8) were performed with collision energy optimized for each compound. Levels of GSH and GSSG were expressed as µmol/g Hb.

Calibration standards and quality controls

Stock solutions of GSH and GSSG were prepared at 1 mM in 10% TCA solution and stored at -80°C. Calibrators containing both GSH and GSSG were prepared daily by diluting the stock solutions with 0,1% formic acid. In-house quality control (QC) samples of 3 different concentrations levels (GSH: 8, 2, 0.5 µM and GSSG: 1, 0.25, 0.625 µM), were prepared daily. A pooled whole blood supernatant (PWBS) was aliquoted and stored at -80°C and used in the validation procedures.

Linearity, calibration, and matrix effects evaluation.

Linearity of the assays was assessed by repeated (n=3) analysis of calibrators with concentrations ranging from 0 to 100 µM. Calibration curves were prepared in the concentration ranges between 0.5-8 µM and 0.0625-1 µM for GSH and GSSG respectively. The curves were constructed by plotting the peak area vs the analyte concentration. Linear regression analysis was used to determine the slope, intercept, and correlation coefficient (r). Calibration curves were prepared both in

0.1% formic acid and in 400-fold diluted PWBS to evaluate potential matrix effects by comparing the slope of the standard calibration curve with the slope of matrix-matched standard curve.

Precision, accuracy and recovery.

Intra-day precision was evaluated by replicate preparations and analysis of quality controls (QCs) (n=6) and of PWBS (n=6) performed on the same day. To assess the inter-day precision, the same analyses were repeated on 3 different days. Intra-day and inter-day precision was expressed as coefficient of variation (CV). Accuracy was evaluated in "standard addition" experiments. The same GSH and GSSG amounts used for QCs were spiked into both PWBS (n=6) and 0.1% formic acid, and then quantified. Accuracy was evaluated by comparing the estimated (measured) with the added (true) concentrations. Accuracy (bias, %) was expressed as: ((Measured value – True value)/ True value) x100. Recovery (%) was evaluated by adding the same GSH and GSSG amounts, used for QCs, into fresh collected whole blood samples (n=6) kept on ice, before the TCA precipitation.

Lower limit of detection (LOD) and quantification (LLOQ)

The LOD of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected, but not necessarily quantified, as an exact value. It was assessed as the smallest detectable signal in diluted whole blood supernatant, above baseline noise (signal to noise ratio, S/N =3). The LLOQ (i.e. the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy) was defined as the concentration of GSH and GSSG when their signal was 10 times that of the blank (S/N=10).

Statistical analyses

The IMPROVE study

Quantitative variables were summarized as mean ± standard deviation (SD), or median (interquartile range) when appropriate. Variables with skewed distributions were log-transformed before analysis. Baseline characteristics of subjects, stratified by MLDP score categories, were compared by linear regression and chi-square for trend, as appropriate. Multivariable linear regression with stepwise selection was employed to identify independent predictors of MLDP. In the validation analysis, the association between MLDP score and VEs was assessed by three Cox models: Model-1: unadjusted. Model-2: adjusted for age, gender and stratified by latitude. Model-3: as model 2 plus lifelong exposure to cigarette smoking (pack-years), body mass index (BMI), education (as indexed by years of school), physical activity, occupation (categorized as: 1, white collars; 2, service workers and 3, manual workers), plasma concentrations of LDL and HDL cholesterol, triglycerides, glucose, hs-CRP, creatinine, pulse pressure, antiplatelet and statin treatments. Kaplan-Meier curves stratified by classes of MLDP score were also computed. The relationship between MLDP score and c-IMT or c-IMT progression was evaluated by linear regression models adjusted for the same set of covariates as Cox models. In the case of c-IMT progression, the analyses were also adjusted for the corresponding c-IMT baseline value. A further Cox model was run including model 3 covariates plus baseline c-IMT and Fastest-IMTmax-progr. All tests were two-sided and carried out by the SAS statistical package v. 9.4 (SAS Institute Inc., Cary, NC, USA).

The RISMED study

Continuous variables are presented as mean±SD, and differences between two groups (LFD and MD) were compared using the t-test for independent samples. Categorical variables were reported as frequency and percentages, and they were compared using the chi-square test. The association between MD and Oxidative

stress status and between oxidative stress and single items of the Mediterranean diet were testing by Pearson correlation. A p value <0.05 was considered statistically significant. All data were collected in an Excel database and analyses were performed using SAS version 9.4 (SAS Institute, Cary, North Carolina).

Results

The IMPROVE Study

Baseline characteristics of participants with and without VEs are shown in **table 1.** Mean age was 64.2 years, and 47.9% of subjects were males. A total of 514 (13.9%) participants were free from relevant pharmacological treatments. Starting from the 15-month visit, at which time changes in C-IMT were assessed, the median follow-up was 21.5 months (interquartile range, 20.8–22.7). Among the 3,482 subjects included in the analysis, 129 (3.7%) developed a VE in the follow-up period: 15 had an AMI (0 fatal), 26 were hospitalized for angina pectoris, 24 underwent angioplasty, and 10 coronary bypass grafting. There were 2 sudden cardiac deaths; 21 ischemic strokes (0 fatal); 22 TIAs, 2 peripheral revascularization, and 7 new diagnoses of intermittent claudication. Twenty-one participants had >1 VE during the follow-up. The baseline characteristics of subjects with and without incident VEs are shown in **Table 1**. Fewer than 10% of all subjects changed their medications during follow-up.

Table 1. Baseline characteristics of participants without and with VEs

	Without events (n = 3353)	With events (n = 129)	P value
Kuopio, n (%)	936 (27.9)	42 (32.6)	
Stockholm, n (%)	497 (14.8)	18 (13.9)	
Groningen, n (%)	443 (13.2)	31 (24.0)	
Paris, n (%)	460 (13.7)	10 (7.8)	
Milan, n (%)	520 (15.5)	14 (10.9)	
Perugia, n (%)	497 (14.8)	14 (10.9)	<0.001
Anthropometric Variables			
Men, n (%)	1591 (47.5)	72 (55.8)	0.06
Age (years)	64±5	65±6	0.02
BMI (Kg/m^2)	27.2±4.2	27.7±4.7	0.20
Waist/Hip ratio	0.9±0.1	0.9±0.1	0.22
DBP (mmHg)	82±10	82±10	0.91
SBP(mmHg)	142±18	145±21	0.05
Smoking habits			
Current smokers, n (%)	479 (14.3)	29 (22.5)	
Former smokers, n (%)	1229 (36.7)	53 (41.1)	
Never smokers, n (%)	1642 (49.0)	47 (36.4)	<0.01
Pack-years	10.7±16.9	14.4±16.2	0.01
Biochemical risk factors			
Total Cholesterol (mmol/L)	5.49±1.13	5.43±1.03	0.53
HDL Cholesterol (mmol/L)	1.27±0.36	1.17±0.29	<0.001
Triglycerides (mmol/L)	1.29 (0.92,1.87)	1.44 (1.08,2.11)	0.06
LDL Cholesterol (mmol/L)	3.55±1.01	3.52±0.94	0.72
Uric Acid (µmol/L)	313.8±71.6	324.4±71.4	0.10
hs-CRP (mg/L)	1.80 (0.75, 3.50)	2.16 (0.73, 4.42)	0.10
Blood Glucose (mmol/L)	5.5 (4.95, 6.3)	5.6 (5.1, 6.5)	0.11
Creatinine (µmol/L)	78.9 (68.1, 90.4)	81.4 (72.5, 94.4)	0.47
Framingham risk score	22.2 (14.6, 33.8)	29 (17, 44.6)	<0.001
Framingham risk score classes:			
≤5%, n (%)	40 (1.24)	1 (0.83)	
>5, ≤10%, n (%)	339 (10.5)	7 (5.8)	
>10, ≤15%, n (%)	520 (16.1)	18 (14.9)	
>15, ≤20%, n (%)	520 (16.1)	10 (8.3)	
>20%, n (%)	1806 (56.0)	85 (70.3)	0.01
Pharmacological treatments			
Lipid lowering drugs, n (%)	1384 (41)	45 (35)	0.15
Statins, n (%)	1349 (40)	43 (33)	0.12
Fibrates, n (%)	261 (8)	7 (5)	0.32
Fish oil, n (%)	115 (3)	4 (3)	0.84
Antihypertensives, n (%)	1889 (56)	74 (57)	0.82
Beta blockers, n (%)	774 (23)	39 (30)	0.06
Calcium antagonists, n (%)	535 (16)	22 (17)	0.74
ACE inhibitors, n (%)	655 (20)	18 (14)	0.12
Sartans, n (%)	505 (15)	20 (16)	0.89
Diuretics, n (%)	769 (23)	30 (23)	0.93
Insulin, n (%)	123 (4)	8 (6)	0.14
Estrogens supplementation, n (%)	213 (6)	2 (2)	0.03

Data are mean±SD and median (interquartile ranges) for continuous variables or number (percent) of subjects in group for categorical variables. Duration of smoking, Cigarettes/day, Pack-years have been calculated excluding never smokers; P values were calculated by Wilcoxon Rank-Sum Test or by Chi-square when appropriate.

Adherence to MLDP

Patient's characteristics according to classes of MLDP score are detailed in **Table 2**. MLDP score was negatively and significantly associated with almost all traditional VRFs. As expected the proportion of participants with a MLDP score >3 was the highest in Italy, intermediate in France and the lowest in northern Europe (**Figure 3**)

Table 2. Patient's characteristics according to classes of MLDP score

Characteristics	MLDP SCORE 0-1 (N=1098)	MLDP SCORE 2-3 (N=1868)	MLDP SCORE 4-7 (N=737)	P-trend
Latitude				
Kuopio	472 (43)	520 (27.8)	56 (7.6)	
Stockholm	177 (16.1)	291 (15.6)	64 (8.7)	
Groningen	319 (29.1)	191 (10.2)	17 (2.3)	
Paris	68 (6.2)	275 (14.7)	158 (21.4)	<0.0001
Milan	37 (3.4)	282 (15.1)	234 (31.8)	
Perugia	25 (2.3)	309 (16.5)	208 (28.2)	
Vascular Risk Factors				
Framingham Risk Score	27.4 (18.7, 40.7)	22.1 (14.3, 33.0)	17.5 (11.2, 27.6)	<0.0001
European Score	5.4 (3.2, 9.0)	3.8 (2.3, 6.7)	2.9 (1.8, 5.1)	<0.0001
Male	580 (52.8)	919 (49.2)	275(37.3)	<0.0001
Age	64.8 ± 5.4	63.9 ± 5.31	63.9 ± 5.7	0.0001
BMI (kg/m^2)	28.4 ± 4.43	27.18 ± 4.22	25.83 ± 3.64	<0.0001
Waist/hip ratio	0.93 ± 0.09	0.92 ± 0.09	0.9 ± 0.09	<0.0001
DBP (mm Hg)	84 ± 10	82 ± 10	79 ± 9	<0.0001
SBP(mm Hg)	147 ± 18	142 ± 18	135 ± 18	<0.0001
Hypertension	844 (76.9)	1288 (69.0)	420 (57.0)	<0.0001
Diabetes mellitus	375 (34.2)	425 (22.8)	113 (15.3)	<0.0001
Smoking habits				
Never smokers	498 (45.4)	878 (47)	407 (55.2)	
Former smokers	398 (36.2)	727 (38.9)	246 (33.4)	<0.0001
Current smokers	202 (18.4)	263 (14.1)	84 (11.4)	
Pack-years	20 (9, 33.2)	17 (7.8, 28.1)	16.3 (7.5, 30.0)	0.03
Family history of:				
Coronary artery Disease	738 (70.7)	1170 (64.7)	407 (57.8)	<0.0001
Cerebrovascular Disease	374 (34.1)	689 (36.9)	259 (35.1)	0.49
Peripheral vascular disease	118 (10.7)	236 (12.6)	89 (12.1)	0.3
Social Class				
Office work	322 (32.0)	743 (42.9)	314 (47.7)	
Service work	374 (37.2)	562 (32.4)	202 (30.7)	<0.0001
Manual work	309 (30.7)	428 (24.7)	142 (21.6)	
Study years	10.0 ± 3.4	10.6 ± 4.1	10.5 ± 4.2	0.002
Biochemical variables				
Total Cholesterol, mmol/L	5.32 ± 1.11	5.47 ± 1.12	5.8 ± 1.12	<0.0001
HDL Cholesterol, mmol/L	1.23 ± 0.36	1.26 ± 0.36	1.32 ± 0.37	<0.0001
Triglycerides, mmol/L	1.31 (0.94, 1.91)	1.31 (0.94, 1.91)	1.28 (0.9, 1.8)	0.09
LDL Cholesterol, mmol/L	3.39 ± 0.96	3.52 ± 1.02	3.83 ± 0.97	<0.0001
Uric Acid, µmol/L	320 (273, 369)	310 (263, 357)	298 (253, 352)	<0.0001
hs-CRP, mg/L	2.14 (0.89, 3.90)	1.77 (0.76, 3.53)	1.55 (0.62, 3.12)	<0.0001
Blood glucose, mmol/L	6.26 ± 1.82	5.9 ± 1.64	5.43 ± 1.15	<0.0001

Values are mean ± SD, or median (interquartile range) or n (%). P values were calculated by linear regression or by chi square for trend.

Figure 3. MLDP score adherence and latitude

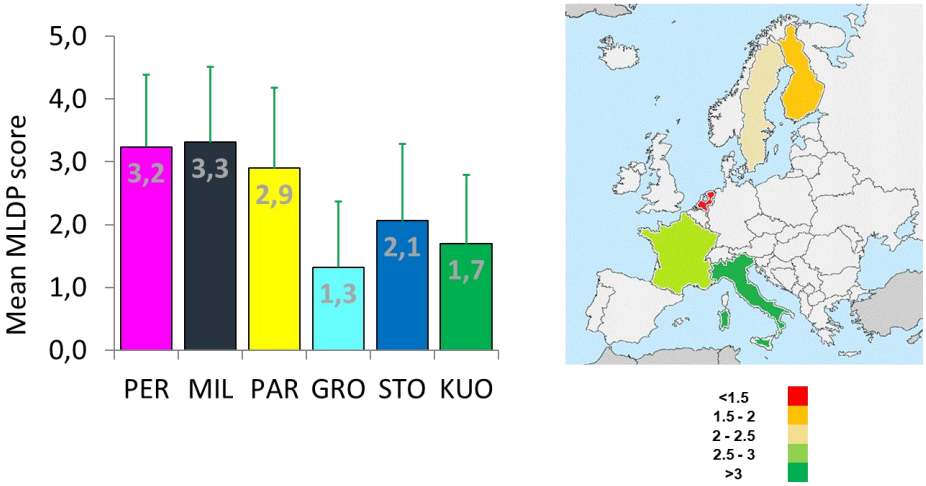
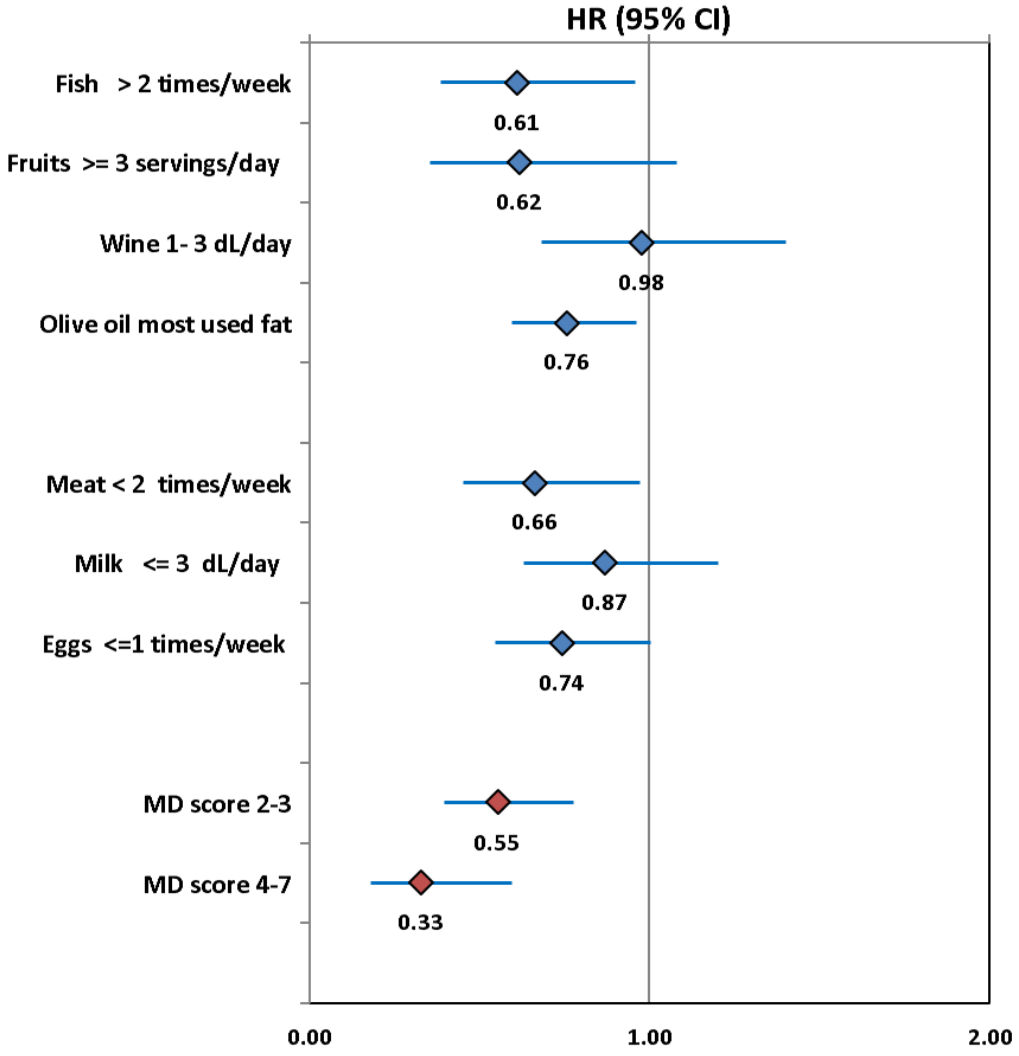


Figure 4 shows that the risk of event in subject consuming items present in a MLDP was lower than the risk of those in whom the consumption threshold was not reached. This is especially clear for those consuming fish >2 times/week whose risk of event was 39% lower than that of the ones that consumed fish < 2 times/week; a similar risk reduction was found in those consuming meat <2 times/week (that have a 34% risk reduction) compared to than those that consume meat > 2 times/week.

Figure 4 Single items of an MLDP t and the risk of events



At multivariable analysis (**Table 3**), latitude was the strongest (r^2=0.1768) independent determinant of MLDP score. Social and behavior variables such as BMI; smoke; pulse pressure; Hs-CRP; creatinine; education (expressed as study years); occupation; physical activity; HDL-cholesterol; use of antiplatelet agents; insulin treatment and statin treatment were also significantly associated. By contrast, sex, age, blood glucose, triglycerides, uric acid, white blood cell (WBC) count, family history of PAD, CVD and CAD and pharmacological treatments (β-blockers, calcium antagonists, ACE inhibitors, ARB, diuretics, insulin, oestrogens, statin, fibrates and fish-oil) were not significantly associated to MLDP score.

Table 3. Variables independently associated with Mediterranean-like dietary pattern (MLDP) score by multivariable linear regression with stepwise selection.

Variables	Beta*	SE	P value	Partial r^2
Latitude (degrees)	-0.09	0.003	<0.0001	0.1768
Education (study years)	0.03	0.01	<0.0001	0.0074
Use of antiplatelet agents	0.19	0.06	0.0007	0.0050
Physical activity (1 step)	0.11	0.03	0.0003	0.0038
Log Hs-CRP (mg/L)	-0.14	0.04	0.0009	0.0036
Pack-years of cigarettes	-0.003	0.001	0.009	0.0034
HDL-cholesterol (mmol/L)	0.20	0.06	0.002	0.0033
Creatinine (µmol/L)	-0.003	0.001	0.02	0.0024
Body mass index (Kg/m^2)	-0.02	0.01	0.002	0.0022
Insulin treatment	-0.25	0.11	0.03	0.0020
Pulse pressure (mmHg)	-0.004	0.002	0.02	0.0020
Statin treatment	0.12	0.04	0.006	0.0019
Occupation (office work)	0.10	0.05	0.03	0.0015

*β values indicate changes in MLDP score associated with a unit increment in the predictor. Variables not significantly associated were: sex, age, blood glucose, triglycerides, uric acid, WBC count, family history of PAD, CAD and CAD and other pharmacological treatments (β-blockers, calcium antagonists, ACE inhibitors, ARB, diuretics, insulin, estrogens, fibrates and fish-oil).

When the variables independently associated with MLDP were evaluated as related to each center, a significant positive correlation between MLDP and education, as indexed by study years, was observed in Paris p=0.04; Groningen p=0.03; Stockholm p<0.0001; Kuopio p<0.0001 Centers,) but not in Italian Centers.

Validation analysis (MLDP score vs VEs)

Before testing the MLDP adherence score versus the primary endpoint (i.e. the association with c-IMT-progression), whether MLDP score was associated with VEs was analyzed. Among the 3,703 subjects enrolled in the IMPROVE Study, 215 (7.96%) developed a first VE within the 36-month follow-up. Of the VEs, 32 were hard ischemic strokes and 37 were hard coronary events. Number of events according to MLDP score classes are in **Table 4**.

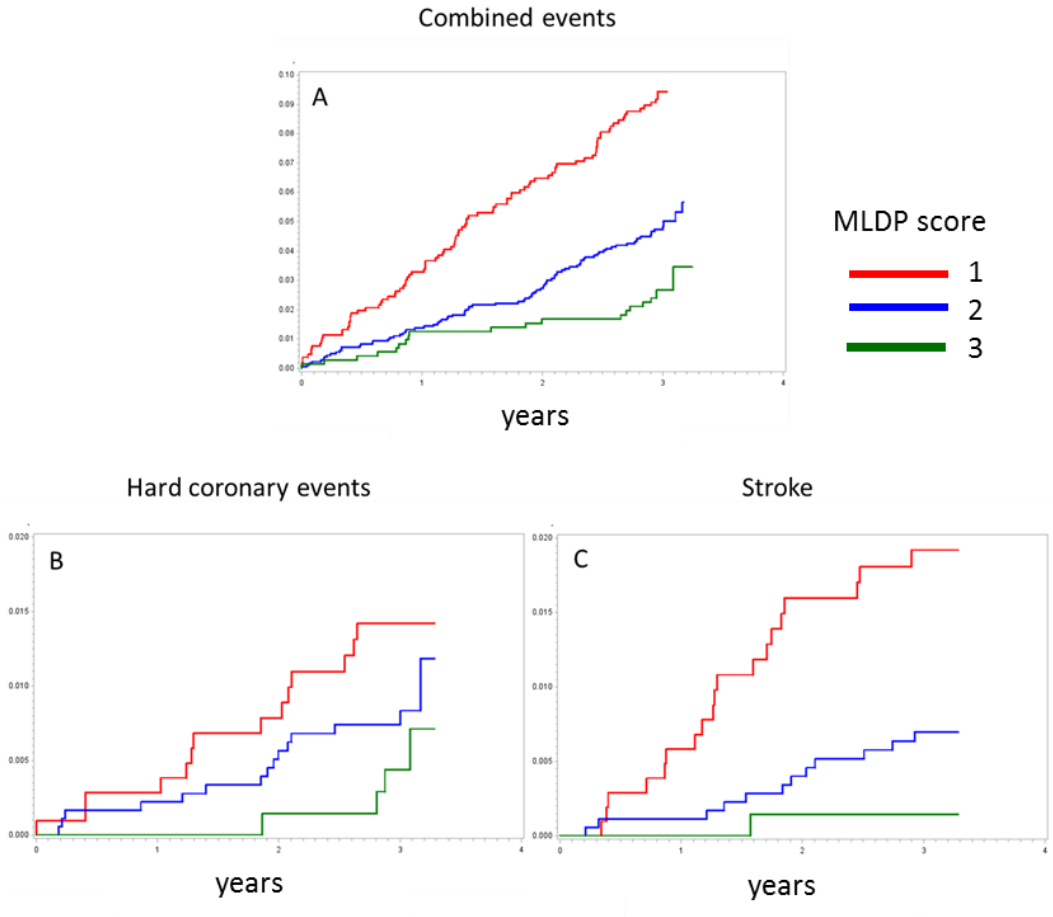
Table 4. Vascular events stratified according to the Mediterranean-like dietary pattern (MLDP) score.

Type of events	**MLDP Score**		
	0-1 n (%)	**2-3 n (%)**	**4-7 n (%)**
Total Events (cardio+ cerebro + peripheral vascular events)	101 (9.2)	94 (5)	20 (2.7)
Hard cerebrovascular events (ischemic stroke)	19 (1.7)	12 (0.6)	1 (0.1)
Hard coronary events (AMI + sudden cardiac death)	15 (1.4)	17 (0.9)	5 (0.7)

Table 3 shows that latitude was the strongest independent determinant of MLDP score, accounting for approximately 83% of the variation explained by the regression model (p<0.0001).

Figure 5 panel A shows the Kaplan-Meier incidence curves of the combined end-point stratified by MLDP score classes. The rate of events was highest in subjects with score 0-1; lower in those with score 2-3 and lowest in those with score 4-7.

Figure 5. Association of the IMPROVE MLDP score with CV events (composite end-point). Kaplan Meier event-free survival curves.



When hard coronary and hard cerebrovascular events were analyzed separately, the protective effect of MLDP was stronger with respect to cerebrovascular than to coronary endpoints (**Figure 5, Panel B and C**). Being the association with VEs highly significant, MLDP adherence score was considered as being validated and, as such, suitable for the assessment of its association with c-IMT change over time.

MLDP score and c-IMT

In an unadjusted model, MLDP was significantly associated with baseline c-IMT variables. The associations, however, disappeared when smoking, physical activity, education, occupation, and CV risk factors (BMI, heart rate, lipids, glucose, creatinine, uric acid, CRP) were included into the model (**Table 5**).
Table 6 shows the mean (±SD) values of c-IMT progression variables stratified by MLDP score categories and association between MLDP score and c-IMT progressions by unadjusted (Model 1) or adjusted (Models 2-3) linear regression models. A significant negative trend was found between MLDP score and most of c-IMT-progression variables, regardless of adjustment for all potential confounders (**Table 6, Models 1-3**).

Table 5 Association between MLDP score and baseline carotid C- IMT by multivariable unadjusted or adjusted linear regression models§*.

	Model 1		Model 2		Model 3	
MLDP score	**Beta (95% CI)**	**P value**	**Beta (95% CI)**	**P value**	**Beta (95% CI)**	**P value**
IMT_{mean}						
2-3 vs 0-1	-0.05 (-0.06, -0.03)	**<0001**	-0.02 (-0.03, 0.00)	**0.03**	0.00 (-0.02, 0.01)	0.69
4-7 vs 0-1	-0.08 (-0.10, -0.07)	**<0001**	-0.02 (-0.03, 0.00)	0.11	0.01 (-0.01, 0.03)	0.46
Trend	-0.02 (-0.03, -0.02)	**<0001**	-0.01 (-0.01, 0.00)	**0.03**	0.00 (0.00, 0.01)	0.64
IMT_{max}						
2-3 vs 0-1	-0.17 (-0.23, -0.11)	**<0001**	-0.06 (-0.12, 0.00)	0.06	-0.01 (-0.08, 0.05)	0.66
4-7 vs 0-1	-0.27 (-0.35, -0.20)	**<0001**	-0.03 (-0.11, 0.05)	0.48	0.04 (-0.05, 0.12)	0.40
Trend	-0.08 (-0.10, -0.06)	**<0001**	-0.02 (-0.04, 0.00)	0.14	0.01 (-0.02, 0.03)	0.65
$IMT_{mean-max}$						
2-3 vs 0-1	-0.10 (-0.13, -0.07)	**<0001**	-0.03 (-0.06, 0.00)	**0.04**	-0.01 (-0.04, 0.03)	0.74
4-7 vs 0-1	-0.16 (-0.20, -0.12)	**<0001**	-0.02 (-0.06, 0.02)	0.30	0.02 (-0.02, 0.06)	0.32
Trend	-0.04 (-0.05, -0.04)	**<0001**	-0.01 (-0.02, 0.00)	0.15	0.00 (-0.01, 0.02)	0.36
1° CC mean						
2-3 vs 0-1	-0.02 (-0.04, -0.01)	**<0001**	-0.01 (-0.02, 0.01)	0.35	0.00 (-0.01, 0.02)	0.63
4-7 vs 0-1	-0.05 (-0.06, -0.03)	**<0001**	-0.01 (-0.03, 0.01)	0.24	0.01 (-0.01, 0.02)	0.47
Trend	-0.01 (-0.02, -0.01)	**<0001**	0.00 (-0.01, 0.00)	0.16	0.00 (0.00, 0.01)	0.49

§. Definitions of such variables and their abbreviations are reported in the Additional material of this study.
Model 1: unadjusted; **Model 2**: adjusted for age, sex, latitude; **Model 3**: as model 2 plus smoking, body mass index, education, physical activity, occupation, lipids (LDL and HDL cholesterol and triglycerides), Hs-CRP, creatinine, plasma glucose, pulse pressure.

Table 6. Progression of ultrasonographic variables according to classes of Mediterranean-like dietary pattern (MLDP) score (column 3) and association between MLDP Score and carotid artery intima media thickness (c-IMT) progression by multivariable unadjusted or adjusted linear regression models (Models 1-3).

				Model 1		Model 2		Model 3	
Progression variables	**Classes of MLDP score**	**Mean ±SD**	**Contrasts**	**Beta (95% C.I.)**	**P value**	**Beta (95% C.I.)**	**P value**	**Beta (95% C.I.)**	**P value**
PF CC-IMT$_{mean-progr}$	0-1	5.9±37	—	—	—	—	—	—	—
	2-3	1.6±35.5	2-3 vs 0-1	-4.3 (-7, -1.5)	**0.002**	-2.2 (-5, 0.6)	0.1	-1.9 (-4.9, 1.2)	0.2
	4-7	-0.04±31.6	4-7 vs 0-1	-5.9 (-9.3, -2.5)	**0.0007**	-1.5 (-5.3, 2.2)	0.4	-1 (-5.1, 3.2)	0.6
			trend	-1.4 (-2.3, -0.6)	**0.001**	-0.2 (-1.2, 0.7)	0.6	-0.1 (-1.2, 1)	0.9
IMT$_{mean-progr}$	0-1	16.9±66.8	—	—	—	—	—	—	—
	2-3	11.5±51.9	2-3 vs 0-1	-5.4 (-9.7, -1.2)	**0.01**	-3.9 (-8.3, 0.5)	0.08	-4.1 (-8.8, 0.5)	0.08
	4-7	5.9±45.3	4-7 vs 0-1	-11 (-16.3, -5.7)	**<0.0001**	-7.6 (-13.4, -1.7)	**0.01**	-8.2 (-14.4, -1.9)	**0.01**
			trend	-2.8 (-4.2, -1.5)	**<0.0001**	-2 (-3.5, -0.5)	**0.01**	-2.1 (-3.8, -0.5)	**0.01**
IMT$_{max-progr}$	0-1	43.8±322.3	—	—	—	—	—	—	—
	2-3	45.2±289.9	2-3 vs 0-1	1.3 (-21, 23.6)	0.91	-2.0 (-25, 21.1)	0.9	-4.4 (-29.2, 20.5)	0.73
	4-7	20.5±245.9	4-7 vs 0-1	-23.3 (-51.1, 4.4)	0.10	-30.6 (-61.4, 0.2)	0.05	-35.3 (-69, -1.7)	**0.04**
			trend	-6.8 (-13.7, 0.2)	0.06	-9.2 (-17, -1.3)	**0.02**	-11.4 (-20, -2.7)	**0.01**
IMT$_{mean-max-progr}$	0-1	33.9±148.8	—	—	—	—	—	—	—
	2-3	32.6±123.8	2-3 vs 0-1	-1.3 (-11.2, 8.6)	0.80	-1.6 (-11.8, 8.7)	0.8	-2.6 (-13.7, 8.5)	0.65
	4-7	19.4±111.6	4-7 vs 0-1	-14.5 (-26.8, -2.2)	**0.02**	-15.2 (-28.9, -1.6)	**0.03**	-16.1 (-31.1, -1.1)	**0.04**
			trend	-4.1 (-7.1, -1)	**0.01**	-4.5 (-7.9, -1)	**0.01**	-5 (-8.9, -1.2)	**0.01**
Fastest IMT$_{max-progr}$	0-1	292±294.8	—	—	—	—	—	—	—
	2-3	257±271.1	2-3 vs 0-1	-35 (-55.6, -14.4)	**0.0009**	-23 (-44.2, -1.8)	**0.03**	-22.6 (-45.6, 0.5)	0.06
	4-7	215.2±225.1	4-7 vs 0-1	-76.8 (-102.5, -51.2)	**<0.0001**	-50.8 (-79.2, -22.5)	**0.0004**	-44.1 (-75.4, -12.9)	**0.01**
			trend	-20.8 (-27.2, -14.4)	**<0.0001**	-14.4 (-21.6, -7.3)	**<0.0001**	-13.4 (-21.4, -5.4)	**0.001**

Model 1: unadjusted; **Model 2**: adjusted for age, sex, latitude; **Model 3**: as model 2 plus pack-years, body mass index, education, physical activity, occupation, lipids (LDL and HDL cholesterol and triglycerides), plasma glucose, high sensitive C-Reactive Protein, creatinine, pulse pressure, antiplatelet,

A significant association was also found between MLDP score and c-IMT progression variables (**Figure 5; Figure 6, and Table 7**) Accordingly, when plotted vs the MLDP score, progression of $IMT_{mean-max}$ was nearly 2-fold lower in those with MLDP scores 4-5 than in those with MD scores 0-1 (0.019 vs. 0.035 mm/yr, P_{trend}=0.01). No association was found between MLDP score and baseline c-IMT (**Figure 6**). The significant association between MLDP score and Fastest-$IMT_{max-progr}$ is shown in **Figure 7**.

Figure 5. Carotid IMT and IMT progression according to MLDP score.

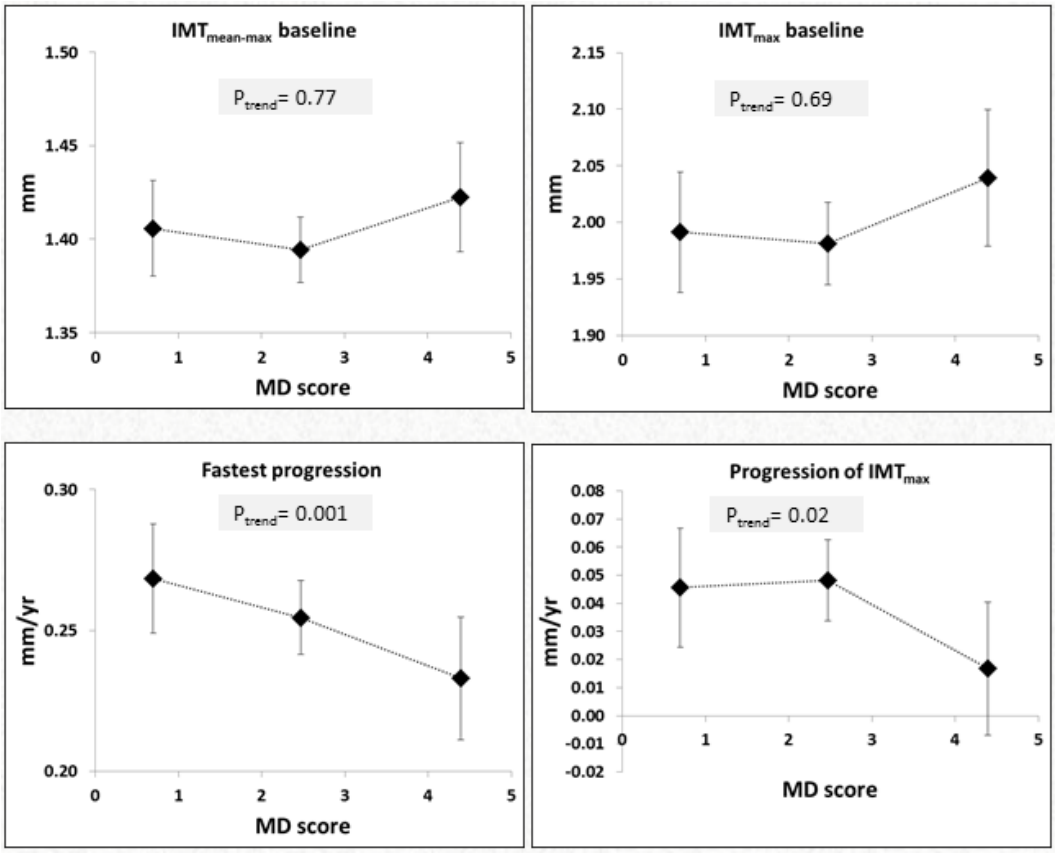


Figure 6. MLDP and IMT in the IMPROVE Study

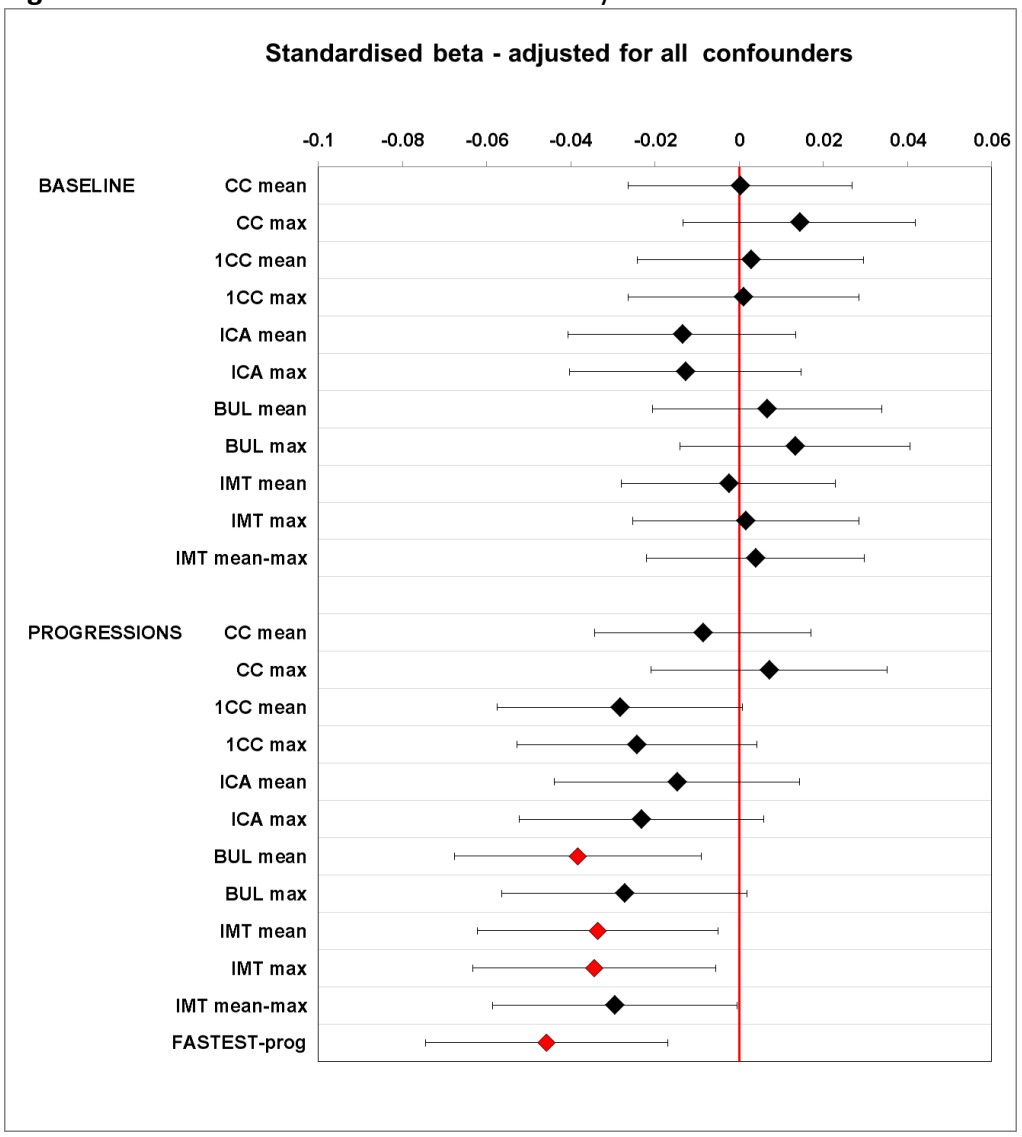


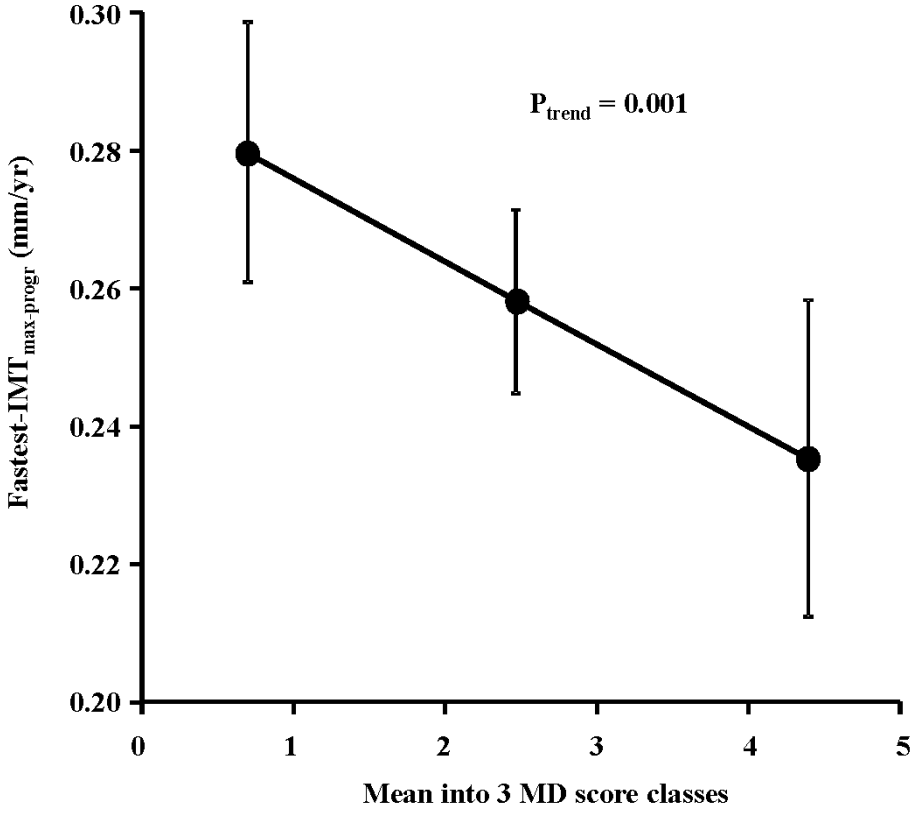
Table 7. Association between MLDP and C-IMT progressions by multivariable unadjusted or adjusted linear regression models.

	Beta (95% C.I.)	P value	Beta (95% C.I.)	P value	Beta (95% C.I.)	P value
$IMT_{mean-max-progr}$						
MD score 2-3 vs 0-1	-1.3 (-11.2, 8.6)	0.80	-1.6 (-11.8, 8.7)	0.76	-1.5 (-12.6, 9.5)	0.79
MD score 4-7 vs 0-1	-14.5 (-26.9, -2.2)	0.02	-15.2 (-28.9, -1.6)	0.03	-14.8 (-29.8, 0.19)	0.05
Trend	-4.1 (-7.2, -1.0)	0.01	-4.5 (-7.9, -1.0)	0.01	-4.6 (-8.5, -0.80)	0.02
$IMT_{mean-progr}$						
MD score 2-3 vs 0-1	-5.4 (-9.7, -1.2)	0.01	-3.9 (-8.3, 0.5)	0.08	-4.0 (-8.6, 0.6)	0.09
MD score 4-7 vs 0-1	-11.0 (-16.3, -5.7)	<0.0001	-7.6 (-13.5, -1.7)	0.01	-8.0 (-14.2, -1.7)	0.01
Trend	-2.9 (-4.2, -1.5)	<0.0001	-2.0 (-3.5, -0.5)	0.01	-2.1 (-3.7, -0.5)	0.01
$IMT_{max-progr}$						
MD score 2-3 vs 0-1	1.3 (-21.0, 23.6)	0.91	-2.0 (-25.0, 21.1)	0.87	-3.0 (-27.7, 21.8)	0.81
MD score 4-7 vs 0-1	-23.3 (-51.1, 4.4)	0.10	-30.6 (-61.4, 0.23)	0.05	-33.4 (-67.0, 0.14)	0.05
Trend	-6.8 (-13.7, 0.17)	0.06	-9.2 (-17.0, -1.4)	0.02	-10.8 (-19.4, -2.2)	0.01
Fastest IMTmax-prog						
MS score 2-3 vs 0-1	-0.03 (-0.06-0.01)	**0.0009**	-0.02 (-0.04-0.00)	**0.03**	-0.02 (-0.04-0.00)	0.12
MS score 4-7 vs 0-1	-0.08 (-0.10-0.05)	**<0001**	-0.05 (-0.08-0.02)	**0.0004**	-0.04 (-0.07-0.01)	**0.01**
Trend	-0.021(-0.03-0.01)	**<0001**	-0.014 (-0.02-0.01)	**<0001**	-0.012 (-0.02-0.04)	**0.003**

Model 1: unadjusted; **Model 2**: adjusted for age, sex, latitude; **Model 3**: as model 2 plus smoking, body mass index, education, physical activity, occupation, lipids (LDL and HDL cholesterol and triglycerides), Hs-CRP, creatinine, plasma glucose, pulse pressure.

Model 4: model 4+baseline fastest+ fastest.

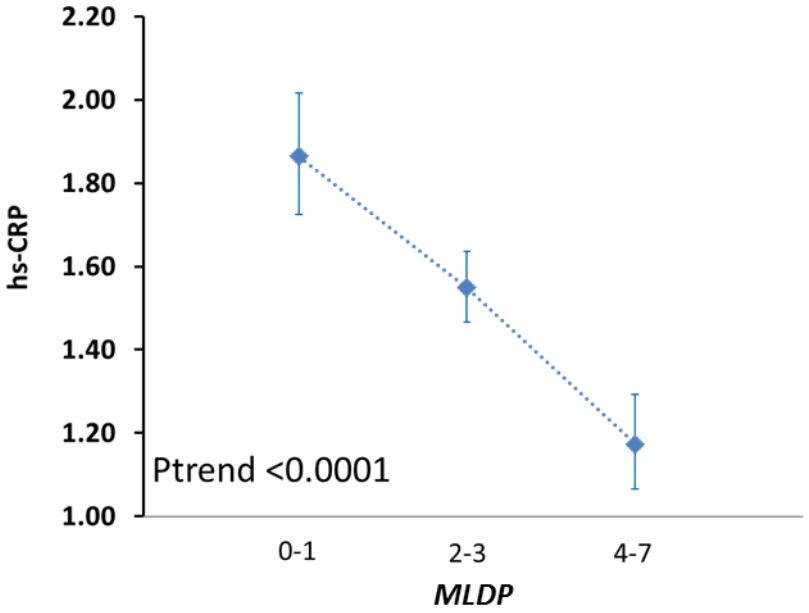
Figure 7. Association between MLDP and Fastest-IMT$_{max\text{-}progr}$



MLDP score and indices of inflammation.

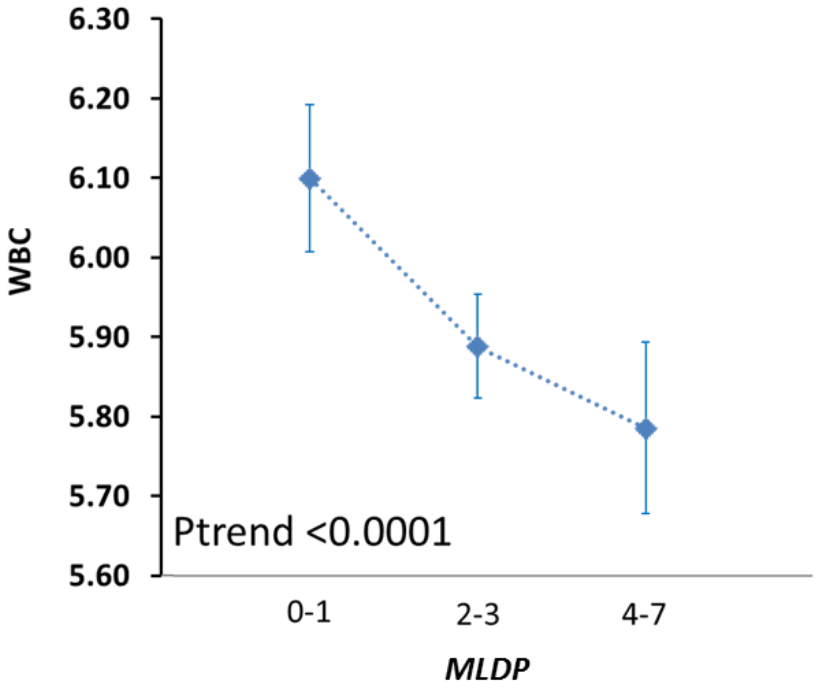
There was a significant negative correlation between hs-CRP and MLDP adherence when correcting these results for confounders (age, gender, latitude) the results were further confirmed (r= -0.034; p<0.0001). (**Figure 8**).

Figure 8. Effect of MLDP on hs-CRP



A significant negative correlation was observed when analyzing WBC and MLDP adherence and correcting these results for confounders (age, gender, latitude) (**Figure 9**).

Figure 9. Effect of MLDP on WBC



The RISMED Study

Characteristics according to diet of 52 patients (ongoing analysis) (28 LFD and 24 MD) so far analyzed are detailed in **Table 8**. Mean age was 61.3 years for MD group and 62.8 for LFD. Moreover, 83.3% of subjects in the MD group were males whereas males 89.5% in the LFD.

No difference was found by comparing the two groups as stratified according to the parameters listed above.

Table 8. Baseline characteristics of participants

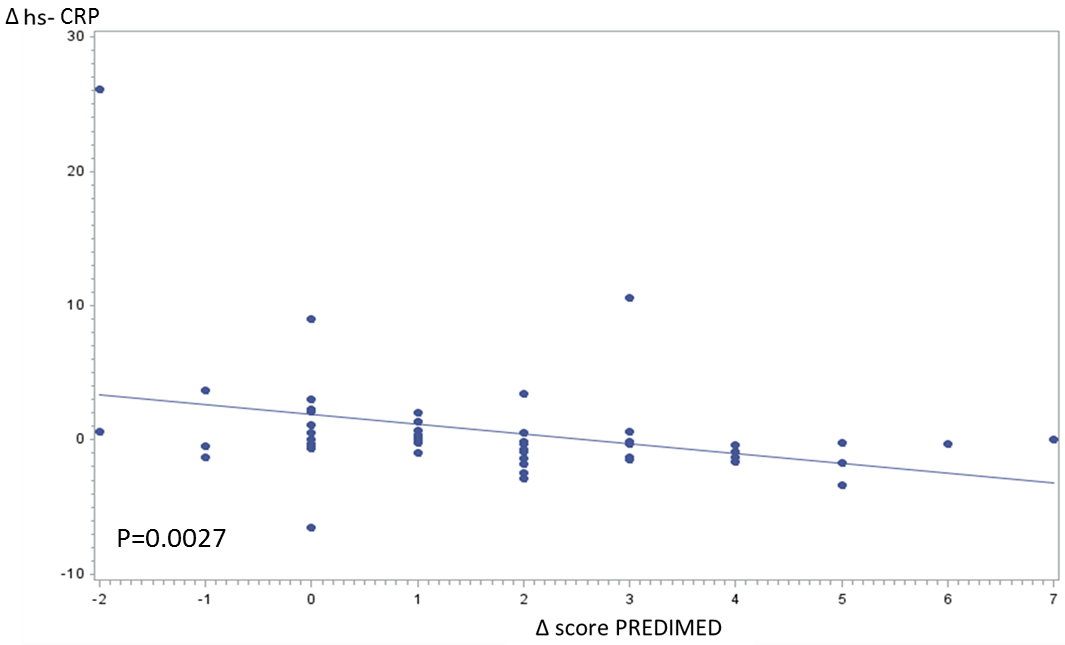
Variable	MD group (n=24)	LFD group (n=28)	*p value* (MD *vs* LFD)
Demographic characteristics			
Age - years	61.3±7.4	62.8±7.2	0,47
Male gender - no. (%)	20 (83.3)	25 (89.5)	0,53
Clinical characteristics			
BMI - Kg/m^2	27.3±3.4	27.9±4.2	0,54
Total cholesterol (mg/dL)	173.6±26.4	162.3±36.1	0,21
LDL cholesterol (mg/dL)	100.7±24.2	88.2±28.5	0,73
HDL cholesterol (mg/dL)	50±14.4	51.5±16.6	0,93
Triglycerides (mg/dL)	114.6±66.4	113.1±58.8	0,10
Fasting glycemia (mg/dL)	101.1±10.2	103.8±10.4	0,35
Pharmacological treatments			
Fibrates - no. (%)	0 (0.0)	1 (3.6)	0,35
Statins - no. (%)	19 (79.2)	26 (92.9)	0,15
β-blockers - no. (%)	13 (54.2)	18 (64.3)	0,46
ACE inhibitors - no. (%)	8 (33.3)	7 (25.0)	1,00
ARB - no. (%)	11 (45.8)	10 (35.7)	0,46
Sartans - no. (%)	1 (4.2)	0 (0.0)	0,27
Diuretics - no. (%)	8 (33.3)	8 (28.6)	0,71
Antiplatelet- no. (%)	24 (100.0)	28 (100.0)	1,00
Anticoagulants - no. (%)	0 (0)	1 (3.6)	0,35
Antinflammatory- no. (%)	5 (20.8)	3 (10.7)	0,31
Antiarrhythmics Drugs - no. (%)	3 (10.7)	3 (10.7)	0,84
Antidiabetic drugs - no. (%)	2 (8.3)	0 (0.0)	0,12
Gastroprotective drugs - no. (%)	20 (83.3)	19 (67.9)	0,20
Vitamins - no. (%)	1 (4.2)	1 (3.6)	0,91

Quantitative variables were expressed as mean±SD and categorical variables as n(%). Body mass index (BMI), myocardial infarction(MI), low density lipoprotein (LDL), high density lipoprotein (HDL), dose area product (DAP), fluoroscopy time (FT), effective dose (ED).

Correlation between inflammatory markers and MD adherence

A significant negative correlation between MD delta score adherence and hs-CRP was found (r= -0.40778; p=0.0027) following 3-mo of diet **(Figure 10)**. An inverse no significant correlation of WBC with MD delta score adherence (r= -0.03; p=0.84) was also documented.

Figure 10. Correlation between hs-PCR and MD score adherence

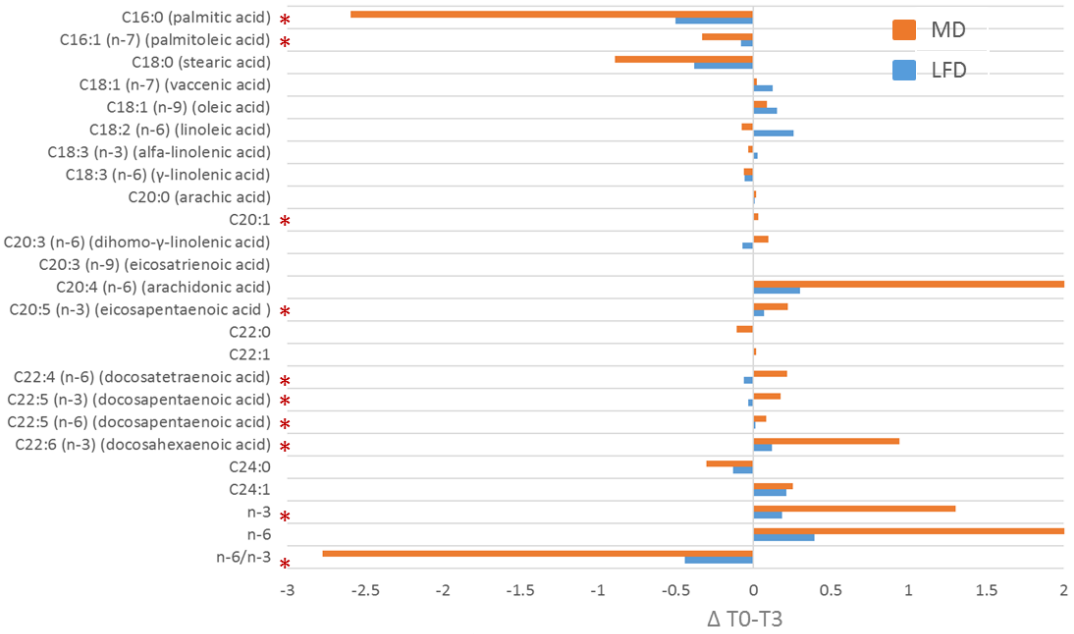


MD and FAs profile

Several components of the blood fatty acid profile differed between cases and controls (**Figure 11**) following a 3-mo of dietary intervention. MD patients had significantly lower C16:0 (Palmitic acid) content as compared to LFD patients. Total monounsaturated fatty acid (MUFA) and C18:1 (oleic acid) were similar in both groups, whereas C16:1 (palmitoleic acid) and C20:1 were higher in MD group than in LFD group. MD also had higher total PUFA-n-6, C20:3n-6 (dihomo-γ-linolenic acid DHLA), C20:4n-6 (arachidonic acid), C22:4n-6, C22:5n-6 (docosapentaenoic acid) than controls. In addition, there were also higher total PUFA-n-3, C22:5n-3 (DPA) and C22:6n-3 (DHA) in patients that followed an MD as compared to LFD

controls. **Figure 11** also shows differences between groups in FA ratios, MD diet showing higher n-3 fatty acids compared to LFD. Moreover, there was a lower n-6/n-3 ratio in patients that received an MD than in controls. Likewise, positive significant correlations between MD adherence score and C20:1 (r=0.36; p=0.03); C20:3n-6 (dihomo-γ-linolenic acid DHGLA) (r=0.33; p=0.05); C20:4n-6 (arachidonic acid) (r=0.46; p=0.005); C22:6n-3 (DHA) (r=0.47; p=0.004), and n-3 fatty acids (r=0.36; p=0.03), were observed. Finally, a significant negative correlation was documented between MD adherence score and C16:0 (Palmitic acid) and n-6/n-3 ratio (r=-0.36; p=0.03).

Figure 11. Fatty acid profile in cases and controls after 3-mo diet



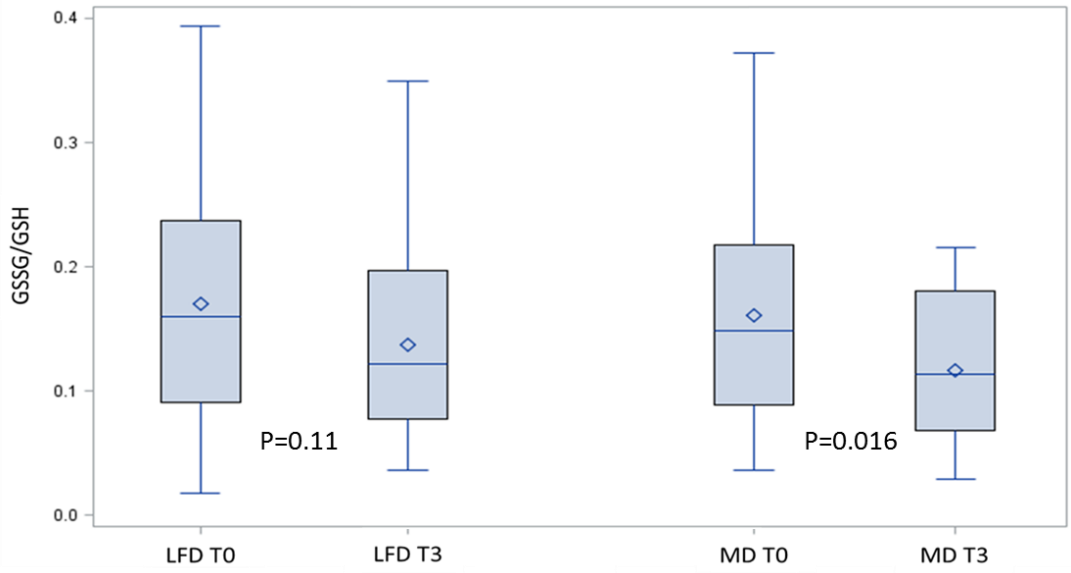
*p value ≤ 0.05

MD and Oxidative stress status

When analyzed together, the MD group and the LFD group had a significant reduction of GSSG/GSH ratio (p=0.005) 3 months after a healthier diet pattern. In parallel, the MD group and the LFD group had a similar weight loss (MD -3.20; C.I. 95% -4.49, -1.92 p<0.001; LFD -3.34; C.I. 95% -4.53, -2.16 p<0.001) and a similar waist circumference reduction (MD -3.96; C.I. 95% -5.17, -2.74 p<0.001; LFD -3.61; C.I. 95% -4.87, -2.34 p<0.001) at completion of the 3-mo healthier dietary patterns. However, when splitting the results based on the different diet followed by the patients, only those that followed a MD dietary pattern had a reduction in the oxidative stress status (p=0.016) **(Figure 12)**.

also

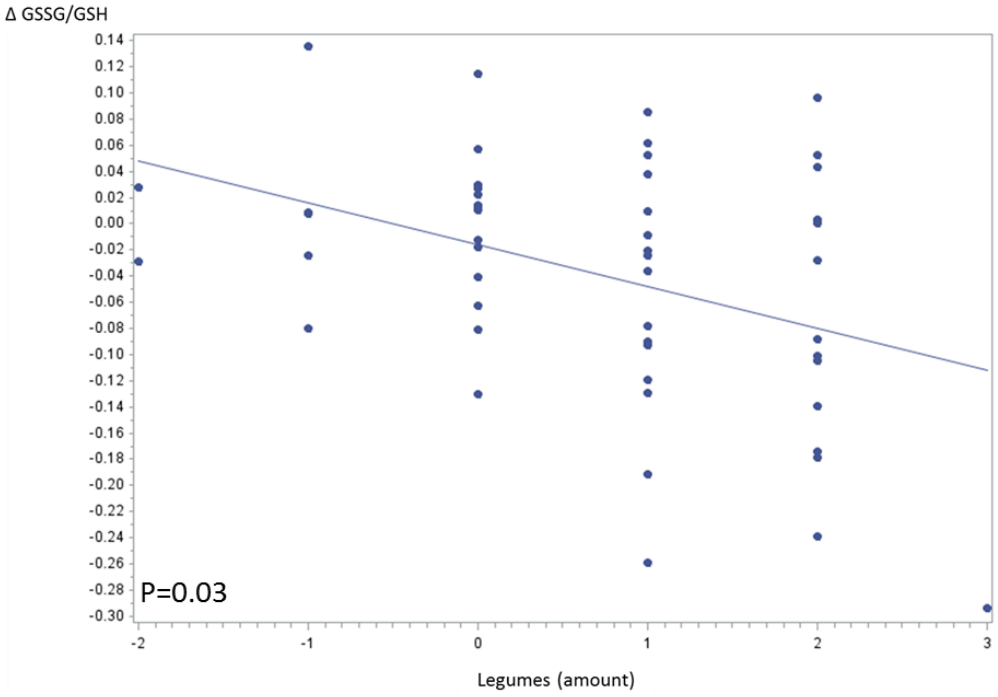
Figure 12. GSSG/GSH ratio in cases and controls after 3-mo diet



Correlation between oxidative stress and single items of the MD

A significant negative correlation between GSSG/GSH ratio (**Figure 13)** and the quantity of legumes consumed in the MD diet (r=-0.33; p=0.03) was found.

Figure 13. Correlation between GSSG/GSH ratio and legumes consumption



Correlation between oxidative stress and FAs profile

A significant negative correlation between GSSG/GSH ratio (**Figure 14)** and the alfa-linolenic acid ALA (C18:3n-3) (r=-0.43; p=0.01) content was found. Consistent with the antioxidant activity of PUFA, a significant positive correlation between GSSG/GSH ratio (**Figure 15)** and n-6/n-3 ratio (r=0.38; p=0.02) was found.

Figure 14. Correlation between GSSG/GSH ratio and alfa-linolenic acid

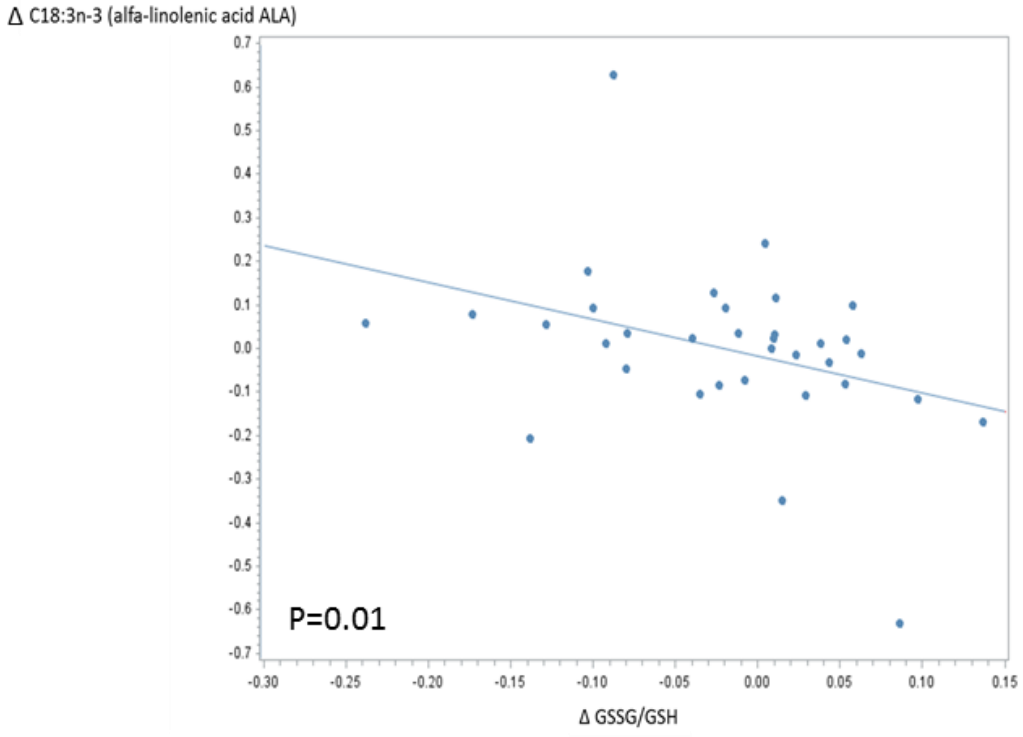
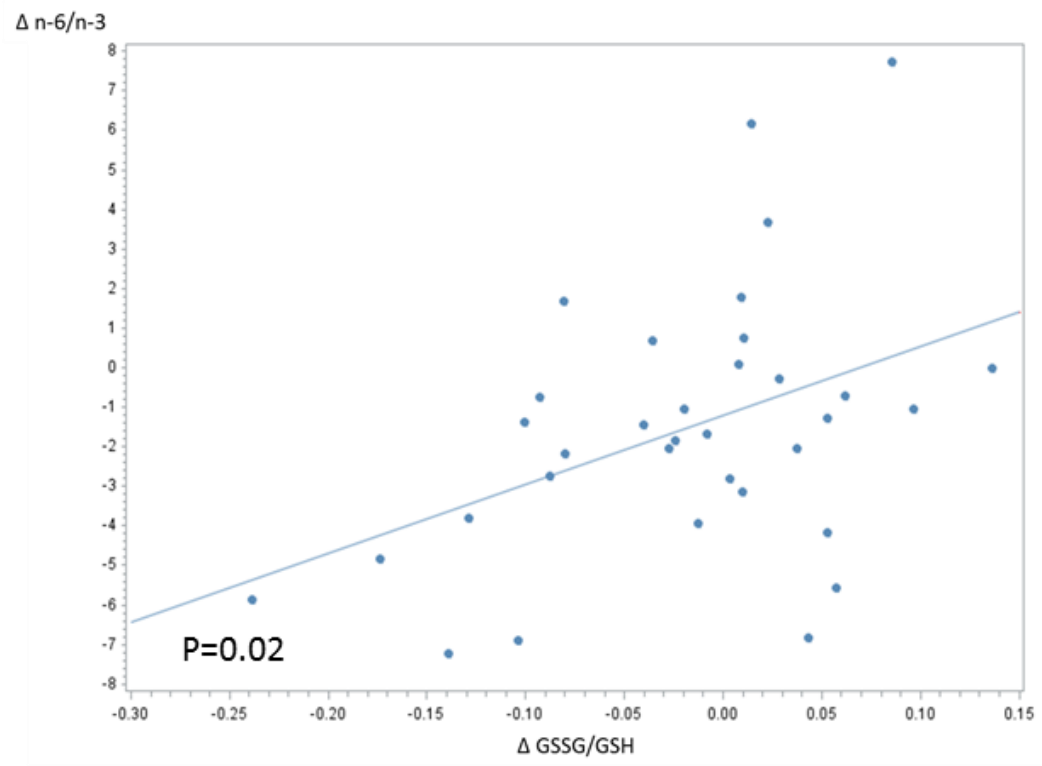


Figure 15. Correlation between GSSG/GSH ratio and n-6/n-3



Discussion

In this new analysis of the IMPROVE Study we have shown that, by using a non-invasive ultra-sonographic diagnostic technique, that allows the assessment of c-IMT changes over time, it is possible to detect the effect of a specific diet in a relatively short time (15 months). The dietary pattern considered was the Mediterranean diet, which was measured by using a relatively simple food frequency questionnaire (Mediterranean-like dietary pattern adherence score - MLDP-) validated a priori vs VEs. In addition, in this new analysis of the IMPROVE Study we have also shown the involvement of the inflammatory pattern, documenting that MLDP is associated to hs-CRP and, in North Europe only, with white blood cells. Such relationship is in keeping with data stemming from the second part of this thesis (the RISMED Study) which show: 1) that by consuming a MD it is possible to modify favorably the plasma fatty acid (FA) profile known to be altered in patients with CAD; 2) that blood FA profile is an index of the quality of food intake which might be used as a tool to estimate diet quality and compliance with nutritional advices; 3) that MD is able to affect some indices of inflammation and oxidative balance; and 4) that there is a relationship between these inflammatory/oxidative changes and the modification of the blood FA pattern.

MLDP adherence and c-IMT or c-IMT change over time.

The IMPROVE is the unique observational study that, besides the associations between MLDP and cross-sectional c-IMT, evaluated also the association with c-IMT progression. This association was best detected by using the Fastest $IMT_{max\text{-}progr}$, the unique carotid IMT progression variable that previous data of the IMPROVE study have shown to be strongly associated to VEs. [249] In addition, we have shown that these associations were stronger in carotid segments with the highest atherosclerotic involvement, i.e. carotid bifurcations, suggesting a stronger impact of MLDP on focal atherosclerosis rather than generalized thickening of the vessel wall. [250] Taken together, these data support the usefulness of carotid

atherosclerosis change over time as a surrogate endpoint to assess the effects of dietary interventions in epidemiological studies instead of baseline c-IMT measurements only. In fact, with c-IMT measured at baseline, we did not find any association with MLDP. The latter issue was previously investigated in three observational studies. [241, 250, 406]

In the first, a cross-sectional study from South of Italy carried out on 929 adults without known diabetes or atherosclerotic cardiovascular disease, no significant association was found between a dietary pattern similar to the one reported here and carotid atherosclerosis, defined as the presence of plaques and/or increased intima–media thickness.[406]

The second, included 1,374 participants of the Northern Manhattan Study. Although no cross-sectional association between MLDP and c-IMT (or presence/absence of plaque) was found, a significant association with plaque size obtained comparing the top vs 1-3 quartiles of plaque thickness and comparing the plaque area above or below the median was found in regression analyses after controlling for confounders.[250]

In the third one, a cross-sectional study carried out in 110 HIV-infected patients and 131 non-HIV-infected participants, a poor adherence to a MLDP score was associated in multivariable analysis with higher risk of subclinical atherosclerosis, indexed by both c-IMT≥0.9 mm and/or presence of at least one carotid plaque.[241] So, our results are in line with the results from Buscemi et al., [406] at least partially, with those from Gardener et al,[250] but not with those of Viskovic et al. [241]

The lack of associations between MLDP and cross-sectional c-IMT after adjustment for confounders can be explained, considering that the one-time measured c-IMT reflects the complete burden of genetic and environmental factors acting throughout the entire life of the subjects, whereas carotid IMT change over time is mainly the response to VRFs and behaviors acting in relatively recent times.

Indeed, the food questionnaire we administered to our middle age population, likely reflected the diet followed in the last years, whereas no information was available about the life-long food behavior.

Another issue that deserves to be highlighted concerns the relationship between MLDP and VEs in different European Countries. With one exception, [402] only single-nation studies have so far addressed the relation between MD scores and VEs (Greece, [24] USA,[407] north Sweden[408]). However, rather than the association itself, the important information stemming from these new analyses from the IMPROVE Study lies on the fact that such association is independent of baseline c-IMT and c-IMT progression. Such issue, never reported before, suggests that the protective effects of MD on VEs acts through mechanisms that are not directly related to the size and growth of atherosclerotic plaques, but to other mechanisms e.g. low-grade inflammation. In line with these considerations, several authors outlined the association between immune and inflammatory processes and the composition of gut microbiota [409] which, in turn, has been related to dietary habits, especially to MD.[410, 411] Notably, Hs-CRP, a widely accepted marker of low grade inflammation, is an important (although not the only) mediator of the effect of MD on VEs [412].

MLDP score adherence, vascular events and latitude.

The simplified IMPROVE-Study MLDP score, included: fruits, fish, wine, olive oil, meat, milk and eggs. Despite such simplification, we have shown that the rate of events was highest in subjects with score 0-1; intermediate in those with score 2-3 and low in those with score 4-7. This suggests that it is possible to gather information of the effect of a Mediterranean-type of diet on CV disease even considering few dietary items. The present results are in keeping with other reports in the area.

In the study performed by Estruch et al., the Authors observed that in subjects at high risk of cardiovascular disease, MD plus olive oil and MD plus nuts are able to reduce the incidence of major cardiovascular events.[413]

With the exception of the EPIC-Netherlands cohort,[414] the concept of high consumption of eggs, meat, and fried foods and high risk of acute VEs is true in 52 countries in the INTERHEART study;[415] in the US population,[416-419] and in China.[420] On the other hand, at variance with the results of some,[415, 417-423] but not all studies, [424, 425] a low risk of developing CAD is seen with the use of plant-based nutritional patterns (as in the REGARDS Study).[426-428]

Despite European countries have increasingly similar patterns of food availability, likely due to the invading fast-food culture that has spread dramatically in the Mediterranean area,[429] the new information raised from the IMPROVE study results documents that the rate of VEs across the 5 European countries involved in the study vary along a north to south geographical gradient and correlate significantly with the intake of major components of the MD food, with a greater adherence to the MLDP score being associated with a lower likelihood of having VEs.

We have also shown that the association between VEs and the MLDP score is stronger in North than in South European Countries, independently of anthropometric, lifestyle and other clinical characteristics.

Of note, also the association between MLDP score and the level of education is stronger in northern than in southern countries, thus confirming the results of Regmi et al.,[430] which showed that the income and the economic environment may have a crucial role in the context of the dietary pattern. One of the possible explanations is that while the items of MD diet are easily available to everybody and relatively cheap in the south of Europe, they are more expensive in northern countries and, as such, they are available only to the highest socio-economic classes. [247, 431-433] An alternative explanation might be the differences between

Mediterranean and non-Mediterranean countries in terms of socio-demographic, anthropometric and lifestyle factors. For example, a low socioeconomic environment has been associated with high stroke mortality in cohort studies[434, 435] and in studies investigating the geographic distribution of stroke.[436, 437]

The issue of different nutritional patterns in Europe has been previously analyzed. A large variability in nutrient intake across European populations with a geographical gradient within and between European countries has been described.[438] Three main region-specific patterns have been identified. In Mediterranean regions, including Greece, Italy, and the Southern of Spain, the nutrient patterns are mainly governed by a relatively high intakes of vitamin E and MUFA, whereas retinol and vitamin D intakes are relatively low. The general population in Germany, in the Netherlands and in the UK share relatively high intakes of PUFA and saturated fatty acids, and relatively low intakes of MUFA, in combination with a relatively high intake of sugar. These differences are justified by a diet rich in plant foods and olive oil in Greece, Italy and, to some extent, also in Spain.[439, 440] By contrast, the diet in the general population of northern countries (Germany, the Netherlands, and UK) is characterized by a higher consumption of animal, processed, and sweetened foods -including nonalcoholic beverages and soft drinks-, and, more specifically, added fats and dairy products, which are often fortified with retinol and vitamin D.[439-442] Margarine and processed meat are the main food sources of PUFA in Germany and the Netherlands.[443] Recently, a general north-south gradient for the contribution of highly industrially processed foods to overall food consumption in Europe has been shown.[444]

MD and inflammation

As far as the relationship between MD and inflammation is concerned, the absence of correlation between MLDP score adherence and WBC and the

significant negative correlation with hs-CRP (Figure 8 and Figure 9) were documented in the IMPROVE study and confirmed in the RISMED Study.

These results, which suggest an involvement of the anti-inflammatory effect of MD, corroborate several previously reported data. For example, in the study of Jiang et al it was shown that a frequent intake of nuts is associated with decreased levels of IL-6, C-reactive protein, and fibrinogen in a population free of CAD.[445] Similarly, the study of Dell'Agli et al., showed that olive oil down-regulates vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and E-selectin expression in the endothelium,[446] and decreases plasma levels of soluble intercellular adhesion molecule-1; soluble E-selectin; IL-6, and hs-CRP, in high-risk patients;[447-449] all widely accepted markers of inflammation.
MD supplemented with walnuts also decreases the level of soluble vascular cell adhesion molecule-1 in healthy subjects and in patients with hypercholesterolemia.[450] Moreover, two cross-sectional studies[451, 452] and a feeding trial in Mediterranean populations [357] further support the concept that MD exerts anti-inflammatory effects.[453]

The involvement of inflammation in such contest is also by an interventional study (the pilot study of the PREDIMED trial) in which the effects of the three dietary interventions (MD plus olive oil, MD plus nuts and low-fat diet) modify the expression of soluble adhesion molecules and cytokines related to atherosclerosis.[308] Such concept was previously suggested by studies showing that MD -or its components- are associated with a lower inflammatory status and/or an improved endothelial function.[24, 358, 402, 450, 454] Similar findings have been obtained by considering other healthy dietary patterns.[358]

In a sub-analysis carried out in a high-CAD-risk group of the PREDIMED population, it has been addressed also the role of immune cell activation and of soluble inflammatory biomarkers.[455] The authors observed, after 3 months of the

aforementioned three-dietary interventions, that the expression of CD49d (an adhesion molecule crucial for leucocyte homing), and of CD40 (a pro-inflammatory ligand) decreased in the MD plus olive oil group, in the MD plus nuts group but not in the low-fat diet group. In the two MD groups, a reduction in serum IL-6 and in the soluble intercellular adhesion molecule-1 was also observed while both these soluble markers resulted to be increased in the low-fat diet group. On the other hand, CRP decreased only in the MD plus olive oil group. This inhibition 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 both the IMPROVE and the RISMED Study, the beneficial effects of MD on inflammatory markers were comparable both considering the subjects treated with risk-reducing agents known to have anti-inflammatory properties (e.g. statins and angiotensin-converting enzyme inhibitors), and those not treated with such drugs. This suggests that the anti-inflammatory effect of MD is complementary to that provided by potentially anti-inflammatory pharmacological treatments.

The benefit of dietary change in the IMPROVE and in the RISMED Study occurred regardless of patients' age. This suggests that the anti-atherosclerotic effects of healthy foods are not limited to a specific phase of the disease (earlier or overt) and supports the recommendation that the MD is a tool potentially useful against CAD in all its stages. [456] Similar findings have been described by Estruch in a commentary of the PREDIMED study.[456] In such commentary, the author concluded that the adherence to MD might be considered as a health tool even in elderly patients at high risk of cardiovascular diseases.

MD and Oxidative stress

As to the inverse association between adherence to MD and oxidative stress here reported, we believe that this result is relevant in view of the lack of randomized controlled trials assessing the effects of MD on glutathione redox pathways in the

general population. Our result is in keeping with data from animal experiments showing that polyphenols naturally present in Mediterranean foods decrease the GSSG/GSH ratio and GSSG concentrations without changing GSH concentrations.[457, 458]
One possible explanation of the GSSG/GSH ratio reduction can be found in the diverse nutrients present in a typical MD that can provide NADPH[459] (oxidized by gluthathione reductase to revert GSSG to GSH). [460]

A "sparing effect" on the GSH/GSSG redox cycle may indeed contribute to an increased GSH/GSSG. By providing additional antioxidants (e.g. vitamin C, vitamin E, carotenoids, polyphenols, zinc, selenium) and ensuring adequate activity and efficiency of antioxidant enzymes, [373] MD may decrease the need for the GSH/GSSG antioxidant pathway, which may "spare" the GSH/GSSG recycle.[461]

As in the RISMED Study, several reports have addressed the possibility of an (inverse) association between MD adherence and oxidative stress. However, the results were not entirely consistent.

A few small trials[462-465] and one large trial[385], examined short- or intermediate-term effects of MD on other circulating markers of oxidative stress, including urinary F2-isoprostanes[463], plasma malondialdehyde[464], and oxidized LDL.[385, 458, 462, 465] In the largest of these trials, subjects assigned to MD had lower oxidized LDL than did those following the control diet.[385] Other trials, however, yielded different results.

This inconsistency might be due to the different biomarkers measured. Individual markers may signal different metabolic pathways, [369] and it is conceivable that only some pathways are influenced by diet. The possibility of unmeasured confounding related to different genetic backgrounds and familial factors should also be considered.

MD and FAs profile

Concerning variations of blood fatty acids, our results are in line with those from a study on 1,050 French elderly community dweller from Bordeaux (mean age 75·9 years), in whom MD adherence (scored as 0–9) was computed from a FFQ and 24 hours recall.[395] As in our study, after adjusting for several personal and environmental variables, plasma DHA, the EPA+DHA index and total n-3 PUFA were positively associated with MD adherence. At variance from our study, however, plasma palmitoleic acid was significantly inversely associated with MD adherence, and the n-6:n-3 PUFA, AA:EPA, AA:DHA and AA:(EPA+DHA) ratios were inversely associated with MD adherence. Similarities and differences between these two studies are now under intensive investigation in our laboratory. The possibility that higher than expected palmitoleic acid levels might be due to an enhanced conversion of palmitic acid in subjects receiving a MD has been recently proposed by our group.[396]

Moreover, the lower palmitic acid content observed in our study, is in line with that observed in the study by Shearer et al.,[356] that pbserved that blood levels of palmitic acid are strongly associated with CAD.

As to the negative correlation between GSSG/GSH and α-linolenic acid (C18:3n-3) levels, and the simultaneous positive correlation between GSSG/GSH ratio and n-6/n-3 ratio, our data are in keeping with animal studies. A reduced hepatic lipid content, and a concomitant reduction in antioxidant response with higher GSSG/GSH ratio and reduction of indices of inflammation (i.e. serum tumor necrosis factor-α, IL-1β, and IL-6 levels) occurs following dietary supplementation of PUFA in obese male C57BL/6J mice fed a high fat diet.[466]

All in all, the relationship between oxidative changes and blood FA pattern modifications that emerges from the ongoing results of the RISMED argues for a direction to be pursued to identify mechanisms of the protective effect of MD.

Limitations

The two studies analyzed here (and the combined discussion of their results) have some potential limitations: 1) being both observational studies, no causal relation can be demonstrated between MD and clinical or subclinical disease; 2) although a variety of potential confounders have been taken into account, a residual confounding effect of unmeasured factors cannot be ruled out; 3) the IMPROVE study has been carried out in different Countries whereas the RISMED Study has only taken into account Italian individuals; 4) at variance with the RISMED Study, in the IMPROVE Study diet was assessed only at the beginning of the study and dietary changes occurring later may have led to non-differential misclassification and underestimation of the true associations; 4) in both cases the dietary assessment was based on a food frequency questionnaire much simpler than that employed in other studies (e.g. the EPIC questionnaire[24]), and 5) pre-vs post analyses of the only RISMED Study have been carried out after only a 3-mo intensively advised MD in Mediterranean males and females with a recent history of coronary revascularization. However, the strength and the consistency of the data presented here suggest that the beneficial effect of an MD nutritional pattern can be easily extrapolated using a limited number of food items, and that its association with VEs is so strong to be detectable even in the presence of a certain degree of misclassification, including time of exposure to MD. In addition, the Italian population examined in the RISMED Study is superimposable to the Italian population evaluated in the IMPROVE Study. Furthermore, as in previous reports in the area, [450] in both studies, the beneficial effect of MD on inflammatory markers was comparable in subjects under stable treatment with agents that have demonstrable anti-inflammatory properties, -e.g. angiotensin- converting enzyme inhibitors and statins-, and in those not treated with these drugs, suggesting that the anti-inflammatory effect of the MD is in both cases complementary to that of the pharmacological treatment.

Conclusions

Using a non-invasive ultrasonographic diagnostic technique to assess c-IMT changes over time, it is possible to detect the effect of a specific diet in a relatively short time (15 months), in this new analysis of the IMPROVE Study. In addition, by using a relatively simple food frequency questionnaire (MLPD score), validated *a priori* vs VEs in different European Countries, we have found that: 1) the significant association between MLDP adherence and VEs is independent of baseline c-IMT and c-IMT progression; 2) regardless of adjustment for potential confounders, a significant negative trend exists between MLDP score and most of c-IMT-progression variables (but not baseline c-IMT variables); 3) MLDP adherence is associated to both hs-CRP and white blood cells, the latter having a north to south gradient with stronger association in northern countries; 4) the latter relationship is in keeping with data stemming from the RISMED Study (showing that MD adherence may affect some indices of inflammation and the oxidative stress balance); 5) blood FA profile in the RISMED Study is an index of the quality of food intake which might be used as a tool to estimate diet quality and compliance with nutritional advices; 6) by consuming a MD it is possible to modify favorably the plasma FA profile known to be altered in patients with CAD, and 7) there is a relationship between inflammatory/oxidative changes and the modification of the blood FA pattern that occur adhering to a MD.

To test the diet heart concept, Ancel Keys organized a pilot study in 1957 in Nicotera, Calabria, where CAD was almost non-existent. Keys and his wife Margaret were highly impressed by the eating patterns of that and other Mediterranean cohorts, and decided to write, in the late '50s of the last Century, the book that became a best seller, "Eat Well and Stay Well" where great merit is given to MD for longevity.[467, 468] Common approaches that target multiple risk factors, are expected to improve the probability of a better health at older ages.[469] Reduced oxidative damage has been documented in calories-restricted, longer-surviving rodents. [470] Reduced oxidative stress and inflammation, and control of

body weight are important effects of MD. Thus, MD has the potential to be considered to prolong a healthy life in man.[24 , 339, 402, 471]

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