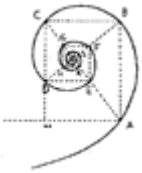
Università degli Studi di Milano

Dottorato in Medicina Molecolare e Traslazionale



TESI DI DOTTORATO

Ciclo XXIX

Anno Accademico 2015/2016

Dottoranda: Costanza CONTI

EFFECTS OF DIETARY COMPONENTS ON CARDIOVASCULAR DISEASE RISK FACTORS AND THEIR INTERACTION WITH CANDIDATE GENES

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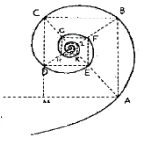

DOTTORATO IN MEDICINA MOLECOLARE E TRASLAZIONALE

CICLO XXIX

Anno Accademico 2015/2016

TESI DI DOTTORATO DI RICERCA

EFFECTS OF DIETARY COMPONENTS ON CARDIOVASCULAR DISEASE RISK FACTORS AND THEIR INTERACTION WITH CANDIDATE GENES

Dottorando:
Costanza CONTI

Matricola N° R10711

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SOMMARIO

Introduzione: Diete ricche di vegetali sono state ripetutamente associate ad un effetto protettivo sul rischio di malattie cardiovascolari. In particolare diverse evidenze sottolineano gli effetti della dieta vegetariana e della dieta Mediterranea nel ridurre il colesterolo LDL e il colesterolo totale. E' stato ipotizzato che tali effetti siano il risultato di una diminuzione dell'assorbimento del colesterolo alimentare e/o di una diminuzione della sua sintesi. Inoltre l'ampia variabilità inter individuale nell'effetto della dieta sui livelli di colesterolo potrebbe essere collegata ad interazioni tra i geni coinvolti nel metabolismo del colesterolo e fattori nutrizionali.

Scopo ed obiettivi: Nell'ambito del progetto ATHENA (7 PQ) abbiamo studiato gli effetti di componenti dietetici, in particolare antocianine e polifenoli, su fattori di rischio cardiovascolare e i possibili meccanismi molecolari alla base della variabilità interindividuale nella risposta alla dieta.

Metodi: Abbiamo reclutato 450 volontari apparentemente sani. I dati raccolti includono informazioni demografiche, cliniche, nutrizionali, biochimiche e genetiche. Il consumo di macro e micronutrienti è stato calcolato tramite interviste *24h dietary recall*, ripetute almeno due volte in un anno per apprezzare variazioni stagionali. I pattern dietetici (onnivoro/vegetariano) sono stati riportati dai soggetti reclutati, mentre l'aderenza alla dieta mediterranea è stata valutata tramite un questionario. Abbiamo analizzato le associazioni tra steroli plasmatici, implicati nell'omeostasi del colesterolo, e fattori nutrizionali tramite regressione multiparametrica. Abbiamo esplorato la presenza di interazioni Gene*Environment (G*E) in cinque geni candidati precedentemente riportati in studi di nutrigenomica o che codificavamo per proteine modulate dalla dieta: per LDL (CYP7A1, INSIG2, LPA, PCSK9) e per HDL (PON1). Inoltre abbiamo sviluppato uno strumento per la gestione

degli studi di nutrigenomica che include una piattaforma web e una mHealth app.

Resultati: Gli individui che assumevano diete ricche di vegetali (vegetariani, soggetti con alta aderenza alla dieta Mediterranea e soggetti con alto consumo di polifenoli) avevano BMI e pressione sanguinea più bassi degli individui con diete povere di vegetali. Nei vegetariani i livelli di colesterolo totale ed LDL erano più bassi.

I fitosteroli erano significativamente aumentati nei soggetti con diete ricche di vegetali. Il latosterolo era significativamente diminuito negli individui con alta aderenza alla dieta Mediterranea. Il 27-idrossicolesterolo (27-OHC) è risultato aumentato in tutti i gruppi con diete ricche di vegetali. Quattro SNP indipendenti del gene PON1 modulavano in modo significativo l'effetto delle antocianine/polifenoli su fattori di rischio cardiovascolare. Uno SNP di PCSK9, già noto per determinare una mutazione funzionale, ha dimostrato di essere in significativa interazione con la dieta nel determinare i livelli plasmatici di LDL.

Discussione: Il nostro studio mostra che diete ricche di vegetali aumentano il turnover del colesterolo nei tessuti periferici. Questo effetto si potrebbe sommare a quelli osservati sull'assunzione e sulla sintesi endogena, contribuendo a determinare gli effetti positivi delle diete ricche di vegetali sul colesterolo plasmatico. I risultati delle interazioni dei geni PON1 e PCSK9 con la dieta nel modificare il profilo lipidico necessitano validazione. La soluzione mHealth implementata fornisce indicazioni in maniera semi automatica, utilizzando output di un sistema di decisione assistita che devono essere validati da un professionista della salute prima di essere visualizzati dal paziente. Inoltre offre la possibilità di monitorare il paziente e di raccogliere in modo semplice i dati sugli stili di vita nell'ambito di studi nutrizionali o nutrigenetici.

ABSTRACT

Introduction: Plants-rich diets have been consistently associated with protective effects on Cardiovascular Disease (CVD), in particular a significant body of evidence underlines the effects of vegetarian diet and Mediterranean diets on LDL and total cholesterol levels. It has been postulated that modifications are the effect of decreased dietary absorption of cholesterol and/or decreased synthesis. In addition the large individual variability in the response to diet-induced changes in cholesterol levels could be related to interaction between genes involved in cholesterol metabolism and dietary factors.

Aim and objectives: In the context of the ATHENA study (7FP), we explored the effects of dietary components, particularly anthocyanins and polyphenols, on CVD risk factors and the possible molecular mechanisms underlying inter-individuals variability in the response to diet.

Methods: We recruited 450 apparently healthy volunteers. We collected demographic, clinical, dietary, biochemical and genetic data. Macro and micro nutrients intake was estimated through 24 hour dietary interview repeated at least twice to get seasonal variations. Overall dietary patterns (vegetarian/omnivore) were self-reported while adherence to Mediterranean diet was evaluated through a questionnaire. We analyzed the association between non-cholesterol sterols which are implicated in cholesterol homeostasis and dietary components using multiparametric regression models. We explored Gene*Environment (G*E) interactions, on five candidate genes previously reported in nutrigenomic studies or encoding proteins modulated by diet: for LDL (CYP7A1, INSIG2, LPA, PCSK9) and for HDL (PON1). In addition we developed a tool to manage nutrigenomic studies that includes a web-platform and a mHealth app.

Results: Individuals with plants-rich diets (i.e. vegetarians, those with high adherence to Mediterranean diet and those with high polyphenols intake) had lower BMI and lower blood pressure compared to individuals with low-plants diets. Vegetarians showed decreased total cholesterol and LDL levels. Phytosterols were significantly increased in plants-rich diets. Lathosterol was significantly decreased in relation to adherence to Mediterranean diet. 27-hydroxycholesterol (27OHC) levels resulted increased in all plants-rich diets. Four independent SNPs of PON1 were shown to modulate the activity of polyphenols/anthocyanins on CVD risk factors. A SNP of PCSK9, already reported in relation to a “gain of function” mutation showed interaction with overall diet in determining LDL plasma levels.

Discussion: Our study showed that plant-rich diets increase the cholesterol turnover in the peripheral tissues. This mechanism, combined with already reported modifications in the absorption of cholesterol and decrease *de novo* synthesis, can contribute to the widely recognized beneficial effects of plant-rich diets on cholesterol levels. The results reported for G*E interactions of PON1 and PCSK9 genes with dietary factors in modifying the lipid profile warrant replication. The mHealth solution implemented, delivering semi-automatic suggestions, based on the outputs of a Decision Support System than needs to be validated by healthcare professionals before being displayed at patient side, offers the possibility to track the patient lifestyles and to collect valuable information for nutritional and nutrigenetic studies.

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1 INTRODUCTION

1.1 Concept

Cardiovascular diseases (CVD) are the first cause of death worldwide, determining 17.9 millions of deaths per year. Coronary heart disease and stroke account for more than 85% of these deaths [1].

Two large case control studies INTERHEART [2] and INTERSTROKE [3], that collected data from 52 different countries all over the world, indicate that up to 90% of the risk of developing acute myocardial infarction and 90.7% of the risk of developing acute stroke can be accounted for by modifiable risk factors (such as smoking, hypertension, diabetes, waist/hip ratio, psycho-social factors, physical activity, alcohol consumption and fruit and vegetable consumption).

Such evidences guided the definition of recent guidelines for cardiovascular disease management.

According to the 2016 European Guidelines on Cardiovascular Disease Prevention: *"Prevention should be delivered (i) at the general population level by promoting healthy lifestyle behavior and (ii) at the individual level, i.e. in those subjects at moderate to high risk of CVD or patients with established CVD, by tackling unhealthy lifestyles (e.g. poor-quality diet, physical inactivity, smoking) and by optimizing risk"* [4].

In addition, International clinical guidelines recommend that patients without prior CVD have their CVD risk estimated by risk scores, to inform the intensity of risk factor management. The risk of experiencing a CVD over a certain time period (typically 5-10 years) is usually calculated using risk scores derived from the Framingham risk score (from the first publication by Anderson et al, [5] to more recent updates). Existing risk score does not include any lifestyle risk factor apart from smoke. The SCORE - Systematic Coronary Risk Evaluation- is currently the most widely accepted risk score

tool in Europe. It is based on national data sets from European countries, totaling around 250.000 people [6].

A substantial body of evidence implicated several aspects of diet with the occurrence of CVD. Studied dietary factors include nutrients (macronutrients, micronutrients, etc.) foods and overall dietary patterns (e.g. Mediterranean Diet [7]). Several evidences from high risk groups (e.g. diabetics) support the use of dietary and more in general lifestyle interventions. Fewer studies are conducted on “apparently healthy” individuals, as in this population longer follow up periods are needed to observe changes in clinical outcomes.

There is wide consensus in the scientific community and among policy makers on the need to develop targeted lifestyle interventions, stratified according to the patient risk. Among those, personalized nutrition interventions were proposed, taking advantage of the increasing interest of citizens in participating actively to their prevention. Current barriers to the use of personalized nutrition include: lack of validation (often the biological meaning is unclear) and lack of acceptability by medical community.

1.2 State of the art on CVD prevention through dietary interventions

1.2.1 Diet as risk factor for CVD

The risk of CVD is strongly associated with socio-behavioral risk factors: smoking, insufficient physical activity and unhealthy diet. The role of diet in health promotion was already recognized in the 5th Century BC, as witnessed by the Plato, which introduces the concept of "healthy diet" in his dialogues. However we became aware of the actual relevance of diet in disease prevention and health promotion only in the last decades.

A number of epidemiological studies, systematic reviews and meta-analyses indicated that healthy diets (such as vegetarian or vegan diets [8]) are associated to a reduced risk of CVD (but also of cancer and neurodegenerative disorders). A recent review considering 86 cross-sectional studies, reported that vegetarian diet is consistently associated with an improved lipid profile in particular with a reduction of Body Mass Index (BMI), Low Density Lipoprotein (LDL) cholesterol and total cholesterol [8].

A well-studied example of healthy dietary pattern is the Mediterranean diet, typical of southern European countries, which includes several daily servings of fruit/vegetables, whole-grain cereals, legumes, accompanied by wine in moderation and less frequently meat and dairy products. According with the meta-analysis conducted by Sofi *et al*, including12 studies, for a total of 1.574.299 subjects, high adherence to Mediterranean Diet resulted in 9% decrease in CVD mortality [9]. In Spain a large interventional trial (N=7,447) demonstrated that Mediterranean diet, supplemented with olive oil or nuts, reduced the incidence of major cardiovascular events compared to control diet in persons with high cardiovascular risk [10].

Few studies analyzed the effects of dietary athocyanins on CVD risk factors in an ecological context, despite the increasing number of interventional studies supporting their role in influencing lipid profile and the strong evidences obtained in animal and in vitro studies [11].

Anthocyanins are red-blue-violet pigments present in many fruits, vegetables and flowers that belong to the large class of polyphenols. Polyphenols include diverse molecules: while they all serve as antioxidants, they are not equally effective, they differ widely in their concentrations in different foods, they differ in their bioavailability and their metabolism by the microflora of the gastrointestinal tract [12]. The most “famous” polyphenol is resveratrol, responsible of the “French Paradox”, but other well-known classes of polyphenols include catechins and flavonoids (mainly isoflavones from soy), which have been widely studied. Anthocyanins are generally widely available in traditional diets such as the Mediterranean diet. Despite the long history of consumption of anthocyanins, particularly in traditional diets, there was relatively little attention focused on this class of polyphenols until recently. Preclinical studies within the FLORA project, co-funded by the EC under the 6FP, demonstrated that dietary anthocyanins can offer cardio-protection, reduce the progression of cancer and limit weight gain in animals fed a high fat diet. The bioavailability and the beneficial effects of dietary anthocyanins depend largely from the chemical structure of each anthocyanin and from the food matrix [13].

ATHENA “AnThocyanin and polyphenol bioactives for Health Enhancement through Nutritional Advancement”, an EU funded collaborative project (Grant Agreement 245121), aimed to explore the role of anthocyanins and polyphenols in health enhancement. The project has taken the advantage of the multidisciplinary interactions among and the complementary expertise of biotechnologists, human and plant geneticists, nutritionists and clinical epidemiologists. In particular, one of the aims of the project was to determine whether anthocyanin/polyphenols rich diets can offer protection against CVD in humans. This goal has been addressed through an observational study on 500 healthy Italian subjects, divided according to their dietary habits in omnivores and vegetarians.

Postulated disease-preventive mechanisms of rich-plants diets are related to biologically active compounds (i.e. vitamins, minerals, fiber etc.) that are highly represented in fruits and vegetables and that impact on the following processes.

Antioxidant activity: vegetables and fruits contain several micronutrients which have antioxidant properties. For long times studies have focused on vitamins with antioxidant properties (vitamins C and E and carotenoids), but more recently polyphenols are gaining attention, since they show stronger antioxidant properties and since they are (collectively) the most represented antioxidants in the diet. Some studies indicate that polyphenols exert a direct activity, as scavengers of reactive oxygen species and indirect activity, stimulating endogenous antioxidant systems [14].

Decrease of platelet aggregation: Data from studies on garlic and on some flavonoids suggest that they may act through inhibition of lypoxygenase and cyclooxygenase activity [15].

Decrease of blood pressure: High blood pressure is an important risk factor for coronary heart disease and stroke. Sodium intake is a well-known dietary determinant of hypertension while the intake of fiber, magnesium and potassium with diet is inversely associated with systolic and diastolic blood pressure [16]. Vegetarian-diets and plants-rich diets such as the DASH ("Dietary Approaches to Stop Hypertension") diet, which are rich in these nutrients, were associated to a significant reduction in blood pressure [17-18].

Modification of cholesterol metabolism: Animal studies suggest that dietary fiber is determinant in reducing cholesterol, through different mechanisms, which are partially source-specific.

On the basis of the effects observed in animals and humans, possible mechanisms implicated in cholesterol reduction include: *"1) increased excretion of fecal bile acids and neutral steroids, 2) altered ratios of primary to secondary bile acids",* 3) decreased cholesterol absorption, due to competition with other components and to decrease in high-cholesterol-containing foods in the diet [15].
In addition, a vegetable-rich diet could affect lipid profile since it reduces BMI. Despite the fact that the possible mechanism of action are not fully elucidated, the evidences on the beneficial effects of plants-rich diets on cholesterol levels are so consistent that most Scientific Societies recommend dietary changes as first-line therapy for dyslipidemia, because they are safe and inexpensive.

The relation between a healthy diet and endothelial function has been investigated by van Bussell et al, in a longitudinal study on 557 subjects of the CODAM ("Cohort on Diabetes and Atherosclerosis Maastricht") Study, at increased risk of CVD. This study demonstrated that a higher consumption of some foods, i.e. fish, but not vegetables, dairy products and meat were associated with endothelial dysfunction scores [19].
Intima Media Thickness (IMT) of the carotid artery in adults, is a well-established noninvasive measure of the extent and severity of subclinical atherosclerosis, associated to endothelial dysfunction. A recent systematic review indicates that the current evidence on the effects of specific micronutrient consumption and carotid IMT is largely inconclusive [20].

These evidences reinforce the concept of a "common soil" for most cardiovascular disease, which is supported by the observation that all these diseases share common risk factors, such as smoking, unhealthy eating, physical inactivity, and, even more importantly, predisposing conditions, such as obesity, diabetes, metabolic syndrome, dyslipidemia.

1.2.2 Prevention programmes targeting dietary habits

In 1972 the North Karelia Project was launched in Finland, the Country with the highest CVD mortality in the world at the time. North Karelia was chosen since it was a County with record CVD events and particularly Coronary Heath Disease (CHD) mortality as a result of a diet high in salt and saturated fat, and low in vegetables, in addition to high rates of smoking and physical inactivity.

It was the first major community-based project for CVD prevention and combined integrated interventions to promote healthier dietary habits, exercise patterns and smoking reduction. The three main risk factors that were targeted in the prevention programme were: total cholesterol; hypertension and smoking. Within 5 years, the project resulted in an amazingly rapid decline in CHD mortality: of 17.4% in men and of 11.5% in women. Smoking habits did not significantly change in the study period while the reduction of hypertension and total cholesterol were significant. In particular a 4.1 and 1.2% reduction in serum cholesterol was exhibited in men and women, respectively [21].

Preventable risk factors for CVD are now well studied and understood but despite the number of supporting evidences, prevention programmes targeting dietary habits are still uncommon ("spot") and their efficacy is very variable and very much dependent of the programme characteristics.

According with WHO Action Plan 2013-2020 the policy measures that should be promoted at global level include:

- *"Reduce the level of salt/sodium added to food (prepared or processed).*
- *Increase availability, affordability and consumption of fruit and vegetables.*

- *Reduce saturated fatty acids in food and replace them with unsaturated fatty acids.*
- *Replace trans-fats with unsaturated fats.*
- *Reduce the content of free and added sugars in food and non-alcoholic beverages.*
- *Limit excess calorie intake, reduce portion size and energy density of foods."*

On other hand, different strong socio-economic factors steer lifestyle habits in an opposite direction. For example, the Mediterranean diet is progressively losing its diffusion in those countries of the Mediterranean area where it was first described. This is due to marked changes in life styles and working conditions, resulting in a drastic reduction in calories consumed in job-related activities and in time dedicated to cooking.

Research on how food environment influences food choices has grown and emphasized that our food environment makes it difficult for consumers to choose healthy foods, especially when food marketing drives unhealthy choices and low nutrient and high calories foods. In addition the last economic crisis emphasized the fact that the major ingredients of the Mediterranean diet (e.g. fruits, vegetables and fish) may not be affordable for the lower socio-economic strata, which is the strata with higher CVD incidence. In fact, despite existing evidences and suggestions by WHO, the report "The rising cost of a healthy diets" estimates that over the last 30 years the relative cost of fresh fruit and vegetables has increased more rapidly than other types of foods including fast foods. These trends are particularly evident in emerging Countries, but they can be detected also in UK and US [22].

Fast foods, rich in fats, sugar and salt, and low in fiber and phytonutrients, have invaded European tables. The impact of such "westernization" of diet is

stronger in younger generations that are at increased risk of obesity and associated complications. In the WHO European Region 30% of 11 years old children is overweight or obese and this figures are ten times higher than in 1979 [23]. The European Commission implemented different policies to face obesity prevention, but the phenomenon does not show decreasing trends.

1.2.3 State of the art on diet and cholesterol metabolism

1.2.3.1 Overview of cholesterol metabolisms

Cholesterol is an essential component of human cell membranes and it is the precursor of steroid hormones and bile acids. Regulation of cholesterol homeostasis is controlled by three main fluxes: intestinal absorption, de novo synthesis and catabolism, mainly through bile acid synthesis. Thanks to a number of control and feedback mechanisms, cholesterol absorption, de novo synthesis and bile acid production are interactive, so that an increased absorption through diet is compensated by lower synthesis and increased excretion. If such homeostasis is impaired, due to malfunctioning of mechanisms regulating cholesterol exchange from plasm to tissues, or cholesterol absorption, or de novo synthesis or excretion, plasma cholesterol increases.

Dietary cholesterol enters the small intestine, where absorption occurs[24]. The rate of absorption varies from 29.0 to 80.1% in healthy subjects [25]. Cholesterol absorption is regulated by receptors on the apical membrane, Niemann-Pick C1-like 1 protein (NPC1L1) and ATP-binding cassette (ABC) transporters 5 and 8 (ABCG5/G8). In the enterocyte chylomicrons are formed and transferred to the liver via lymphatic system.

About half of the cholesterol in the body derives from endogenous (de novo) synthesis, which occurs mainly in the liver and the intestine.

Synthesis of cholesterol begins from the acetyl-CoA, and it is composed of the following major steps: 1. Acetyl-CoAs are converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA); 2. HMG-CoA is converted to mevalonate; 3.Mevalonate is converted to isopentenyl pyrophosphate (IPP); 4. IPP is converted to squalene; 5. Squalene undergoes a two-step cyclization to yield lanosterol; 6. Two alternative pathways leading to cholesterol formation can be activated.

Rate limiting step occurs at the HMG-CoA reductase followed by mevalonate formation.

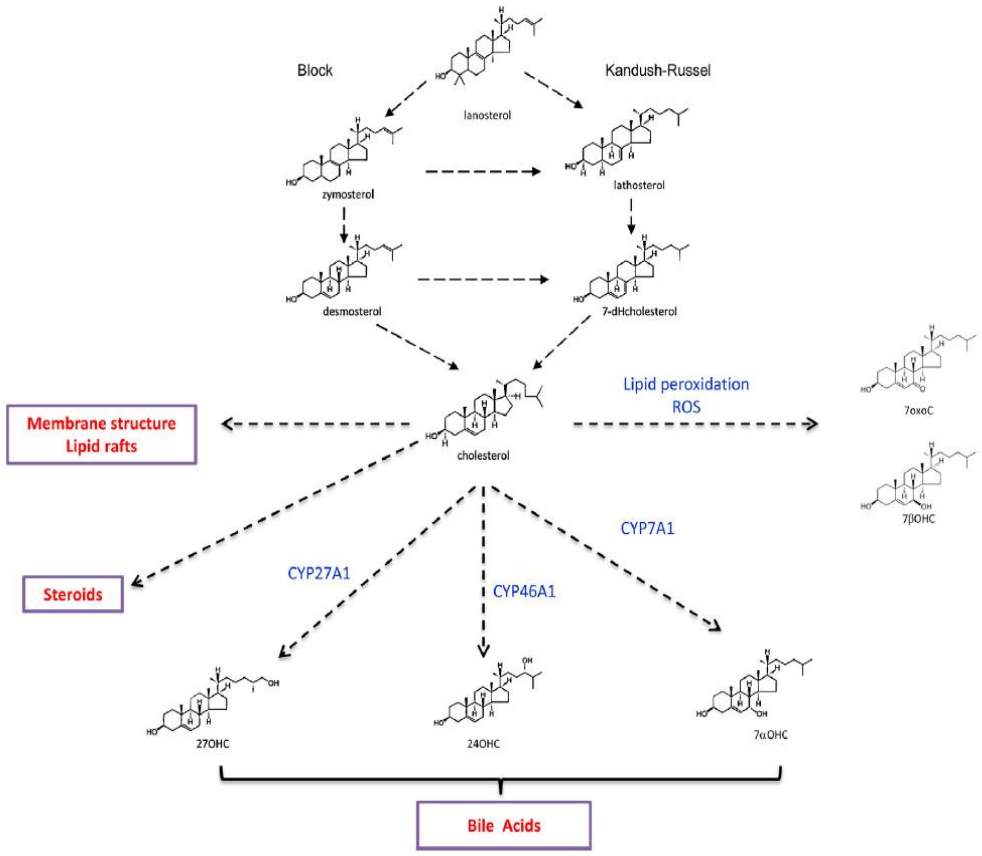


Figure 1: Simplified diagram of cholesterol and oxysterols metabolism (From Leoni et al, 2013). Liver cholesterol 7a-hydroxylase (CYP7A1) converts cholesterol into 7a – hydroxycholesterol (7aOHC), the main precursor of the neutral bile acid pathway. Cholesterol 27-hydroxylase (27OHC) expressed in different cell types converts cholesterol into 27-hydroxycholesterol (27OHC), precursor of the acidic bile acid pathway. Neuronal specific cholesterol 24-hydroxylase (CYP46A1) is responsible for 24S-hydroxycholesterol (24OHC) formation. Cholesterol autoxidation in presence of lipid peroxidation and reactive oxygen species (ROS) results into formation of several oxysterols [26].

The two possible pathways activated to obtain cholesterol are: via lathosterol (Kandutsch-Russell pathway) or via desmosterol (Bloch pathway), as summarized in the figure below.

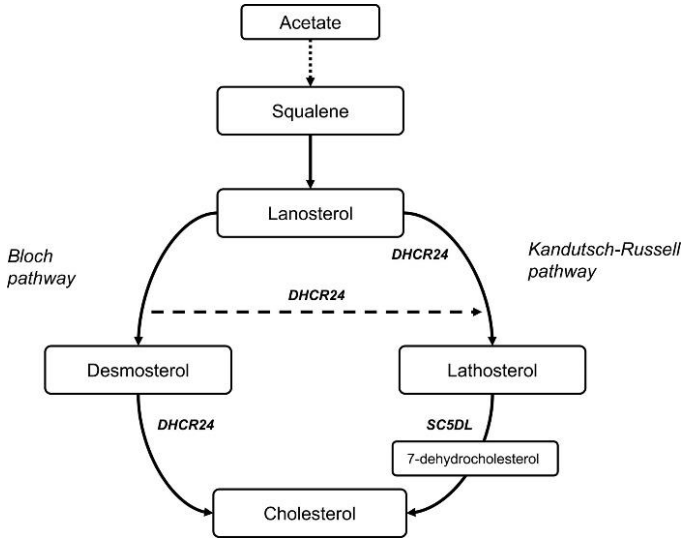


Figure 2: Overview of cholesterol metabolism.

Cholesterol is transported in the serum within Low Dense Lipoproteins (LDLs).

The liver synthesizes and secretes VLDLs (Very Low Density Lipoprotein) which are rich in triglycerides and apolipoproteins. Their degradation, through loss of triglycerides and apolipoproteins gives origin to Intermediate Density Lipoproteins and LDL, through the action of endothelial cell-associated lipoprotein lipase.

In peripheral tissues, the cellular upload of LDL is mediated by the LDL receptor (LDLR). The transcription of LDLR is regulated by SREBPs transcriptional factors and is downregulated when cellular concentration of cholesterol increases, so that LDL remains in the blood.

The removal of excess peripheral cholesterol to the liver involves a complex pathways and it is referred as **"**Reverse cholesterol transport"**.**

Cellular cholesterol efflux, in peripheral tissues, is mediated by HDLs. It can then be transferred to VLDLs and LDLs via the action of cholesteryl ester transfer protein (CETP) which is associated with HDLs.

Ultimately, cholesterol is excreted in the bile as free cholesterol or as bile salts following conversion to bile acids in the liver.

The catabolism of cholesterol into bile acids occurs by two major pathways: the classical and the alternative (acidic pathway). In the classical pathway 7α-hydroxycholesterol is formed, while in the alternative pathway, the first step involves the oxidation of cholesterol to 27-hydroxycholesterol.

1.2.3.2 Phytosterols and diet

Phytosterols cannot be synthesized by humans and are always derived from the diet. Phystosterols (or plant sterols) occur naturally in vegetable oils and in smaller amounts in vegetables. They have been used as marker of cholesterol absorption, since cholesterol absorption is highly variable, and influenced by several factors (e.g. diet, genetics etc.).

The most abundant plant sterols are sitosterol and campesterol and several studies suggest that a high intake of phytosterols is associated with lower plasma cholesterol levels, in particular with lower total cholesterol and LDL. Their mechanism of action is related to competition with cholesterol (i.e. introduced with the diet from foods of animal origin) for intestinal absorption.

Sarkinnen et al in 1997 reported a change of phystosterol levels in relation to the addition of vegetable oils in the diet in hypercholesterolemic subjects [27]. Another study reported that vegans have significantly higher campesterol (both absolute value and ratio over total cholesterol) and sitosterol (only absolute value) compared to omnivores [28].

On the contrary, a recent study showed no associations between absolute plasma campesterol and sitosterol with dietary patterns [29].The authors suggest as possible explanation of this finding the fact that in humans less than 5% of ingested plant sterols are absorbed and that the absorption of plant sterols is much less and their excretion into bile is much faster than cholesterol.

Based on the results of recent meta-analysis that associated phytosterols administration with decreased LDL plasma levels [30] several Scientific Societies suggest to administer 2g/day of phytosterols to hypercholesterolemic patients [31-32].

However the cholesterol lowering effect of functional foods and specific diets rich in plat sterols is still matter of debate.

1.2.3.3 De novo cholesterol synthesis and diet

Endogenous synthesis of cholesterol is controlled by several factors including: genetics, age and metabolic state. For example individuals with higher BMI show an increased cholesterol synthesis which is accompanied by a decreased intestinal absorption [33].

Lanosterol is an intermediate of cholesterol synthesis that derives from squalene and that can be processed into lathosterol or desmosterol. Studies analysing lipids metabolism have been using its serum concentration corrected for total cholesterol concentration as biomarker of cholesterol endogenous synthesis. It exert a regulating role in the de novo sinthesys. In facts when intracellular levels of lanosterol are high, throuhg INSIG1 protein, it promotes the degradation of HMG-CoA reductase, the rate limiting enzyme in cholesterol synthesis.

Lathosterol is produced from lanosterol in the Kandutsch-Russell pathway as intermediate of cholesterol synthesis. Its serum concentration corrected

for cholesterol concentration is the most commonly reported marker of cholesterol de no novo synthesis, and recently it was validated in a methodological study aimed at studying the correlations between non-cholesterol sterols and gold standard functional techniques [28].
Several evidences suggest a role of plant-based diets in decreasing serum levels of lathosterol. A study conducted in patients with Rheumatoid Arthritis showed that a vegan diet determined a decrease in serum cholesterol and lathosterol concentrations [34]. This observation was confirmed in a trial comparing surrogate markers of cholesterol metabolism, reporting a significant difference of 272 mg/d between vegans and omnivores [28].
Another study reports that lathosterol to cholesterol ratio significantly decreased in response to very-high-fructose diet, in health subjects [35].
Individuals with Metabolic Syndrome or Diabetes show higher desmosterol and lathosterol concentrations accompanied by lower concentrations of cholesterol absorption markers [36].

Desmosterol is produced from lanosterol in the Bloch pathway as intermediate of cholesterol synthesis. Studies analysing lipids metabolism have been using its serum concentration corrected for total cholesterol concentration as biomarker of cholesterol synthesis. Desmosterol levels has been associated to gender, being higher in women than man [36]. Desmosterol is also a regulator of cholesterol metabolism that was associated to activation of cholesterol efflux and downregulation of de novo cholesterol synthesis [37].

A small dietary trial, showed that, compared to placebo, a diet supplementation with plant sterols increased serum levels of plant sterols such as campesterol and sitosterol and increased markers of cholesterol synthesis such as desmosterol and lathosterol, without changing cholesterol serum levels [38].

1.2.3.4 Cholesterol turnover and diet

Cholesterol can be removed from the body by two mechanisms: it can be removed directly as free cholesterol or after conversion to bile acids for faecal excretion. Bile acids synthesis is the main pathway of cholesterol excretion.

"HDL-mediated reverse cholesterol transport is the most important mechanism regulating cholesterol homeostasis, allowing a flux of cholesterol from extrahepatic tissues to the liver.
Oxidative mechanisms involving formation of side-chain oxidized oxysterols can be regarded as alternatives to this mechanism" [39], as summarized in the figure below.

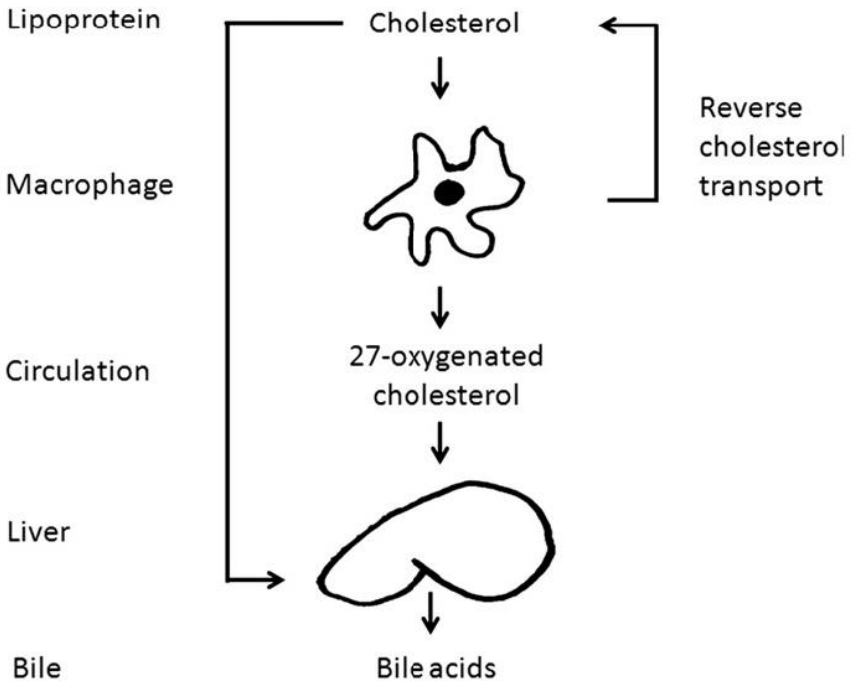


Figure 3: Elimination of cholesterol from macrophages by the sterol 27-hydroxylase mechanism an alternative to the HDL-dependent reverse cholesterol transport (From Bjorkhen 2012)[39]

Cholesterol from peripheral tissues is converted to **27-hydroxycholesterol** (27-OHC), by sterol 27-hydroxylase (CYP27A1) which is widespread, but particularly represented in the vascular endothelium and macrophages. The severity of cerebrotendinous xanthomatosis, due to CYP27A1 deficiency, indicates the critical role of 27-OHC in cholesterol homeostasis.
27-OHC is then transported from the tissues to the liver, where it is converted to bile acids (via acidic pathway), contributing to cholesterol efflux independently from HDL [40].
A study on healthy males showed that subjects with low HDL plasma levels have increased 27-OHC over cholesterol ration and supports the role of oxysterols as alternative mechanism of cholesterol excretion. "*This pathway could be a compensatory mechanism for the ineffective reverse cholesterol transport of individuals with very low levels of HDL-C"* [41].

24S-hydroxycholesterol (24-OHC) is mainly produced by cholesterol degradation in the brain and it reflects the brain turnover. The enzyme converting cholesterol in 24-OHC (24S-hydroxylase, known as CYP46) is located exclusively in neuronal cells and levels of 24-OHC in cerebrospinal fluid (but not in blood) have been associated with neurological disease (e.g. cognitive impairment, Alzheimer Disease etc.) [42]. CYP46 was found to be highly resistant to most regulatory axes. Oxidative stress was one of the very few factors affecting the expression of the enzyme [43].

25-hydroxycholesterol (25-OHC) is a powerful inhibitor of HMG-CoA reductase, the rate-limiting enzyme in sterol biosynthesis. It is present in all tissues but its physiological function is still uncertain [44]. There is some evidence that ABC transporters may be involved in the transport of 25-OHC,

suggesting an alternate mechanism of cholesterol efflux, similar to HDL-independent 27-OHC efflux.

Limited information is available on the plasma concentration of oxysterols in relation to diet. Some authors reported a strong correlation between oxysterols consumed with food (e.g. mainly from western diets/fast food type) and their level in plasma [45]. A small trial using different fat-enriched meats showed a decrease of plasm 24-OHC and 27-OHC after the dietary interventions. These modifications were observed after only 10 days of dietary intervention, even if no changes in LDL and total cholesterol were observed [46].

1.2.4 State of the art on nutrigenomics and lipid levels

The physiological effect of a nutrient is determined by multiple biochemical processes occurring at molecular level during: gastro-intestinal digestion and absorption, hematic transport, cellular uptake and metabolism, and finally excretion. Each process is controlled by multiple genes, their polymorphisms and their interactions. All can potentially modify the host's physiological response to diet [47].

Several studies indicate that the genetic background is associated to differences in the ability of digesting a number of nutrients. The variants that create this predisposing genetic background may be the result of evolutionary events, since ethnic groups adapted over the centuries to exploit optimally the foods available locally. In has been repeatedly demonstrated that genetic variants associated to the synthesis of enzymes involved in nutrients metabolism are more or less represented in a population in relation to the regional food history (e.g. "lactase persistence" in adults and regional dairy farming history) [48].

In the past decades, nutrigenomics has emerged as a new discipline, recognizing that the effects of nutrition on health and disease cannot be understood without a profound knowledge of how nutrients act at the molecular level [49].

The concept behind nutrigenomic studies, is that unbalanced diets "*may shift the balance between healthy and diseased conditions, increasing the risk of metabolic and immune disturbances, particularly in genetic predisposed subjects"*, as summarized in the figure below [50].

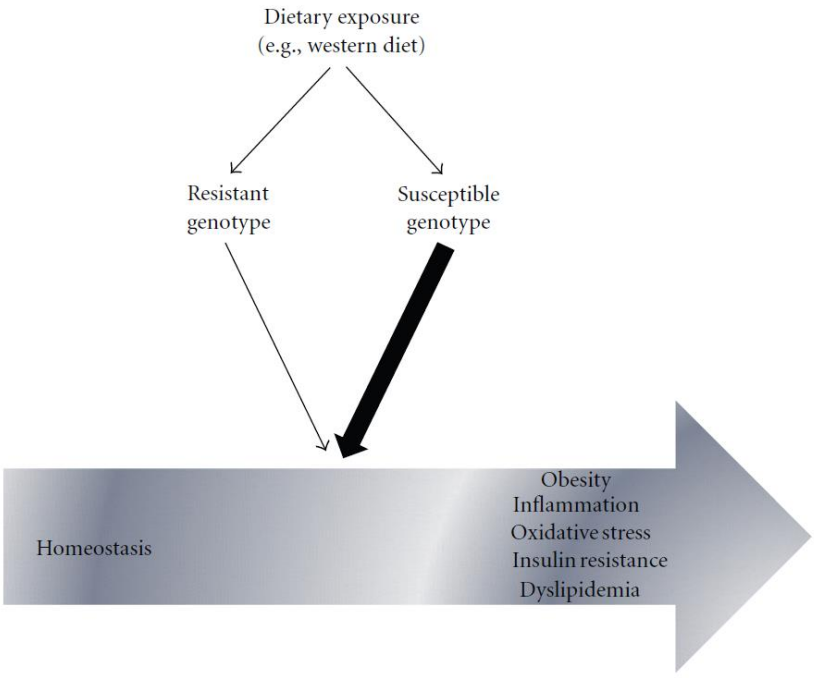


Figure 4: Overview of the concept behind nutrigenomic: dietary exposures may have different effects in relation to the genetic background and may determine the shift between homeostasis and metabolic/immune disturbances only in susceptible individuals (from Curti et al, 2011) [50].

It has been reported by several studies that extrinsic factors, such as pharmaceutical intervention and diet, exert different effects on cholesterol levels depending on the genetic background of the individual and on other factors (e.g. microbiome). For example Herron et al, suggested that men can be categorized in *"hypo-responder, where plasma total cholesterol increases <0.05 mmol/L, or as hyper-responders, where there is an increase of ≥0.06 mmol/L per each additional 100 mg dietary cholesterol, respectively"* [51].

At the same time, data from observational and intervention studies highlighted that genetic heterogeneity was partially responsible of the

marked inter-individual variability in the response of cholesterol metabolism to similar diets [52-53].

A better understanding of the interactions between genetic background and diet has the potential to support tailored programmes for hypercholesterolemia prevention/management via modification of dietary recommendations [54].

The literature related to nutrigenomic of cholesterol (i.e. total cholesterol, LDL, HDL) is modest, a recent review indicates that 69 studies (mostly small size interventional trials) were available in 2015 [53]. In addition most of them concentrated on: the fat content of the diet, on dietary energy intake or/and on characteristics of the fatty acids introduced with the diet. As far as we know there are no nutrigenomic studies published so far, that consider vegetarian diet (vegetarian/no restriction) and just one study (recently published by our group) that consider anthocyanins/polyphenols dietary intake as interaction factor (i.e. environment) [55].

On the contrary there are large GWAS [56-59] reporting in a consistent manner several genes associated with LDL and HDL levels, some of which with clear biological meaning.

In addition a number of nutritional studies reported that diet can modulate the activation/concentration of enzymes/proteins encoded by the same genes reported in the GWAS. For example 1) paraoxanase 1 (PON1) activity and PON1 gene expression are modulated by anthocyanins-rich diets [60-61]; 2) PCSK9 enzymatic activity has been reported to be modulated by several nutrients [62].

1.2.5 State of the art on tools for personalized nutrition for CVD prevention

Mobile health, or mHealth, is defined as the use of mobile tools (e.g. phone, tablets etc.) to foster health promotion or disease management.
New technological approaches (genomics, metabolomics, etc.) have boosted knowledge production in health, nutrition and genetic areas. However such knowledge is only partially available due to the extreme fragmentation of different sources, the lack of operative standards and the lack of correlation/integration between data from different domain (clinical, nutritional, lifestyle, genetic etc.). mHealth is opening up enormous opportunities for the delivery of self-managed, efficient and affordable health care at a time when the demands on health and social care services continue to increase. However, there is still a gap between concept and translation into real health care and lifestyle changes.

Many mHealth interventions have focused on hypertension. A recent review [63] reports that among the 11 trials evaluating the efficacy of different mHealth solutions to support blood pressure control, 7 reported a significantly improved outcome in the group using mHealth. However the interventions were very different in nature and duration.

There are less mHealth apps targeting dyslipidemia control. The same review cited above reports 4 interventional trials using mHealth for the management of hypercholesterolemia, with inconsistent results. Also in this case the type of support provided through mHealth was very different and ranged from reminders to enhance adherence to lipid-lowering therapies to more comprehensive approaches, promoting also lifestyle changes.
The largest trial published so far (TEXTME) was conducted in Australia on more than 700 patients with coronary heart disease, and showed that the

intervention group achieved significantly lower LDL levels, compared to the control group, along with reductions in blood pressure and BMI and significant positive changes in lifestyle (i.e. increase in physical activity, and a reduction in smoking) [64].

In addition there is an increasing number of health Apps on the market, with more than 165,000 mHealth apps available in the Apple iTunes and Android app stores, but it is hard to find scientific validated applications/software/platform able to provide valid suggestions, keeping under consideration different correlation approaches. Furthermore there is lack of evidence for the efficacy and safety for most apps. Possible certification schemas have been proposed, but currently there is no accepted standard.

Finally, despite the good technical quality of several Apps on the market, their use do not foresee a communication between the patients and the care team, and data collected through mHealth are not included in electronic health records (EHRs). Thus mHealth is often a stand-alone intervention, and this approach may limit its efficacy and its adoption in routine practice [65].

As stated above, the **role of nutrition in health promotion and chronic disease prevention** is widely recognized, as well as the need of decision support tools for professionals, able to merge and take into account the large amount of data generated by researcher and updated international guidelines. Also there is the need to increase the awareness towards healthy behaviors in the general society, to allow a life-course approach in CVD prevention.

2 AIMS AND OBJECTIVES

The aim of this thesis is to explore the effects of dietary components, particularly anthocyanins and polyphenols, on CVD risk factors and of possible molecular mechanisms underlying the large individual variability in the response to diet-induced changes in cholesterol levels. This kind of evidences are needed to develop personalized prevention tools. This aim has been addressed through the following specific objectives:

Objective 1: Evaluation of the association between well-established risk factors of CVD (i.e. total cholesterol, LDL, HDL, triglycerides, blood pressure etc.) and markers of atherosclerosis (i.e. Intima Media Thickness) Vs dietary micronutrients/dietary patterns in apparently healthy subjects.

Objective 2: Evaluation of possible mechanism of action of dietary micronutrients/diet on lipid levels, by studying non-cholesterol sterols which are implicated in cholesterol homeostasis.

Objective 3: Evaluation of the possible mechanism of action of dietary micronutrients/diet on influencing lipid levels, considering also the genetic background, by studying Gene*Environment (G*E) interactions. Due to the low number of genes reported in G*E interactions, considering as environment the dietary patterns (i.e. vegetarian diet, adherence to Mediterranean diet) and micronutrients under study, we adopted an exploratory approach. At this purpose we concentrated on four candidate genes (CYP7A1, INSIG2, LPA, PCSK9) for nutrigenetic analysis on “overall diet” and on one candidate gene (PON1) for nutrigenetic analysis on polyphenols/anthocyanins.

Objective 4: Development of tools for personalized nutrition, combining the use of nutritional evaluations and genetic risk scores with established risk scores for atherosclerosis and CVD.

3 MATERIALS AND METHODS

3.1 Recruitment and data collection

In the context of the project ATHENA a multicentre cross sectional study was conducted in Milano. The recruitment started in June 2012 and was completed in December 2013. The dietary follow up was completed in March 2015.

Healthy volunteers were contacted through publication of an invitation letter in the Hospital websites and among those visiting the units for routine controls. Patients were considered eligible if meeting all the following inclusion and exclusion criteria.

Inclusion criteria:

- Age between 18 and 70 years;
- Able to read and understand the study information sheet and to sign the informed consent form;
- Absence of known cardiac diseases.

Exclusion criteria:

- Patients with malignancies or severe psychiatric diseases;
- Patients with severe cardiac disease such as myocardial infraction or cardiac failure;
- Patients with chronic kidney disease higher than stage 3;
- First degree relatives of an already enrolled subject;
- Patients that did not sign the informed consent form.

According to the study protocol, the phenotypic data collection included: 1) laboratory testing for a wide variety of parameters (among which plasma lipid profile); 2) anthropometric measurements (weight, height and waist circumference); 3) blood pressure assessment; 4) renal echography (with

resistance index assessment); 5) echocardiographic examination; 6) carotid doppler.

3.2 Dietary characterization

Dietary habits were collected by trained nutritionists through 24 hours recall interviews, which were repeated 4 times, seasonally. The main tools used in dietary interviews were:

Food Atlas (edited by Scotti-Bassani) including 99 tables with the visual representation of portions/quantities of foods/beverages commonly consumed in Italy, as a support for the volunteer and the nutritionist in understanding the amount (grams) of foods.

Figure 5: Tables from the food atlas, to facilitate the collection of information related to food portion (From Scotti-Bassani Atlas).

Food Composition Table: the “Food Composition Database for Epidemiological Studies in Italy” (IEO) was adopted, with the following additions:

- Anthocyanins contents of several foods from the USDA “Database for the Flavonoid Content of Selected Foods” and from the analysis conducted in ATHENA;
- Foods and food items used by vegans/vegetarians (calculated by professional nutritionists, based on ingredients and recipes);
- Total Polyphenol contents from the “Phenol Explorer 2.0” Database.

Dietary information collected during the interviews were combined with macro and micronutrients data from the Food Composition Table in order to compute the individual daily intake of macro and micronutrients.

In addition, during fall 2014, a subset of the volunteers was also asked to participate to another nutritional assessment (promoted at IEO - Istituto Europeo di Oncologia, Milano, Italy) aimed at validating a questionnaire to evaluate adherence to Mediterranean diet. The questionnaire included 15 items related to the frequency of specific foods consumption. For each question the interviewed had to choose one among 5 possible predetermined answers.

3.3 Laboratory analysis

Blood sample in fasting state were collected during the visit.

The determination of HDL, LDL, total cholesterol and triglycerides was carried out by means of automatic clinical chemistry instrumentation (Abbott Architect 8000). Samples were analyzed in batches after storage at −40 °C for a time not exceeding one month.

For all subjects with sufficient plasma quotes (N=312), non-cholesterol sterols were analysed. To a screw-capped vial sealed with a Teflon septum, 0.25 mL of plasma were added together with 1000 ng of D7-lathosterol, 100 ng of D6-desmosterol, 100 ng of D6-lanosterol, 2500 ng of D7-campesterol and 2500 ng D7-β-sitosterol, 50 ng of D6-24-hydroxycholesterol, 50 ng of 25-hydroxycholestero and and 100 ng D6-27-hydroxycholesterol l as internal standards, 50 µl of butylated hydroxytoluene (5g/l) and 50 µl of K3-EDTA (10 g/l) to prevent auto-oxidation.

Alkaline hydrolysis was allowed to proceed at 40°C for 30 minutes in the presence of ethanolic 1 M potassium hydroxide solution. After hydrolysis, the sterols were extracted twice with 5 ml cyclohexane. The organic solvents were evaporated under a gentle stream of argon and converted into trimethylsilyl ethers with BSTFA.

Gas chromatography mass spectrometry (GC-MS) analysis was performed on a GC equipped with an Elite column (30 m×0.32 mmid×0.25 mm film ;Perkin Elmer, USA) and injection was performed in splitless mode and using helium (1 ml/min) as a carrier gas. The temperature program was as follows: initial temperature of 180 °C was held for 1 min, followed by a linear ramp of 20°C/min to 270 °C, and then a linear ramp of 5°C/min to 290 °C, which was held for 10 min.

The mass spectrometer operates in the selected ion-monitoring mode. Peak integration is performed manually, and sterols are quantified from selected-ion monitoring analysis against internal standards using standard curves for the listed sterols. Additional qualifier (characteristic fragment ions) ions were used for structural identification. Interassay CV was 2.3% for lathosterol, 3.1 % for lanosterol, 4.2 % for campesterol, 3.9 % for β-sitosterols, 3.3 % for 24-S-hydroxycholesterol, 4.6% for of 25-hydroxycholesterol and 3.6 % for 27-hydroxycholesterol. Recovery ranged from 98 up to 103%.

3.4 Genotyping

DNAs were extracted from blood samples periodically shipped from the Recruitment Centres and underwent quality controls, through 1% agarose gel electrophoresis before genotyping.

Samples were genotyped on an Illumina iScan platform, using the Illumina HumanCore array. The HumanCore array contains 200K highly informative genome-wide tag-SNPs including indels and updated exome focused markers. The HumanCore allows a genome-wide approach, as 200K SNPs are directly genotyped, while recovery of a higher number of genotype data is performed through imputation.

Raw intensity data were analysed with the Illumina Software Genome Studio for genotype calling, using the Illumina reference cluster file.

The genotyped sample included 495 individuals who underwent quality controls in accordance with the protocol written by C.A Anderson *et al*. [66]. DNA call rate threshold was set at 0.95. 1 DNA with call rate < 0.95 was excluded from the final data set.

No subjects with sex mismatch (difference between gender reported in demographic data and gender estimated with SNPs mapping in sex chromosomes) were identified.

We removed 8 subjects for heterozigosity rate and using genome-wide Identity by Descent (IBD) estimation in PLINK, we removed 15 related subjects. Using Golden Helix software we performed the principal component analysis and we removed 4 outliers (i.e. individuals that exceed a default number of standard deviations from the whole sample). The final sample included 465 subjects (185 males, 280 females), table 1.

Total Genotyped	495
Call Rate < 95%	1
No phenotype	1
Related	15
Sex Mismatch	0
Heterozigosity Rate	8
PCA	4
GOOD QC SAMPLES	**465**

Table 1: Summary of the quality controls conducted after genotyping.

We used imputation to increase the number of SNPs available for the association analysis. Imputation is a method to predict genotypes at non-directly genotyped SNPs.

In the present study, genome-wide imputation was performed, starting from SNPs experimentally genotyped in our dataset, with >99% call rate and minor allele frequency >1%, in two steps:

1. we used SHAPEIT [67] software to estimate the haplotypes from genotype data,
2. we used MINIMAC software [68] to impute data using as reference the HapMap CEU haplotypes (release 22).

Imputation accuracy was estimated using the R-squared (Rsq), also called coefficient of determination, that measures how well the imputed data fits a statistical model (i.e. the linear regression).

Imputed SNPs with low imputation quality (Rsq<0.8 or MAF<1%) were excluded from the dataset (Table 2).

The results of imputing activities are summarized in Table 2.

	N	%
tot SNPs	2543887	100.00
Rsq>= 30%	2418882	95.09
Rsq>= 30% maf > 0.01	2383468	93.69
Rsq< 30%	125027	4.91
Rsq < 30% maf > 0.01	89567	3.52
Rsq >= 80%	2002145	78.70
Rsq >= 80% maf > 0.01	1991990	78.30

Table 2: results of imputing activities

3.5 Statistical Analysis

The following paragraphs describe the statistical analysis conducted to answer to the first three objectives of the thesis.

Objective 1: Evaluation of the association between well-established risk factors of CVD and dietary factors

The study sample was characterized through descriptive analysis (mean, standard deviations and distributions). For each subject with at least two dietary recalls, the daily intake of macro and micronutrients was computed. To assess the differences in phenotypic and demographic data between males and females we used Ttest for quantitative data and Chi-squared test for qualitative data. As nutrients did not show normal distributions we used Wilkoxon rank sum test to assess the differences in macro and micronutrients between omnivores and vegetarians. For dietary data with marked non normal distributions (i.e. anthocyanins, polyphenols and Mediterranean diet score), we divided the data in tertiles.

To explore the association between CVD risk factors (i.e. Total cholesterol, LDL, HDL, Atherogenic Index of Plasma – AIP-, LDL/HDL ratio, BMI, waist circumference, systolic and diastolic blood pressure and mean IMT, calculated as a mean between left and right IMT) and diet, we used multiple linear regression models adjusting for the main confounders shown to influence the phenotype (i.e. age, sex, BMI, smoking and self-reported sport intensity), using STATA 14.

Objective 2: Evaluation of possible mechanism of action of dietary micronutrients/diet on lipid levels, by studying sterols implicated in cholesterol homeostasis

We characterized through descriptive analysis (mean, standard deviations and distributions) non cholesterol sterols associated to 1) cholesterol absorption (i.e. sitosterol and campesterol); 2) cholesterol de novo synthesis (i.e. lathosterol, desmosterol and lanosterol) and 3) cholesterol turnover (i.e. 27-OHC, 24-OHC, 25-OHC).

We conducted analysis to explore the association between non-cholesterol sterols and specific nutrient/dietary patterns, considering absolute values and their ratios over total cholesterol (which is the most commonly reported data in published studies), using multiple linear regression models in which the main confounders shown to influence sterols levels were used as covariates (i.e. age, sex, BMI), using STATA 14. Data of anthocyanins, polyphenols and Mediterranean diet score were divided in tertiles.

We explored the correlation between non cholesterol sterols and serum cholesterol (i.e. total cholesterol, LDL, HDL) using Spearman correlation.

We computed the ratios between different non-cholesterol sterols and we analyzed their differences among vegetarians and omnivores using Ttest.

Objective 3: Evaluation of the possible mechanism through which dietary micronutrients/diet can influence lipid levels, by studying G*E interactions

We analysed the presence of interactions between selected genes and the following “Environments”:

1. “No-restriction” or “Vegetarian” diet
2. “Low anthocyanin intake” corresponding to the lower tertile and “High anthocyanin intake” corresponding to the higher tertile
3. “Low polyphenols intake” corresponding to the lower tertile and “High polyphenols intake” corresponding to the higher tertile.

To explore the G*E interactions with “overall” diet we tested 4 genes, encoding proteins previously shown to be modulated by diet, and in which map candidate SNPs for LDL, identified in large GWAS [56-58]. Genes CYP7A1, INSIG2, PCSK9 and LPA were assessed. We performed a quantitative trait interaction analysis (G*E analysis) testing each SNP associations with LDL in “vegetarian” and “no restriction” diet as environments.

To explore the G*E interactions with anthocyanins and polyphenols, as no previous nutrigenetic studies were available, we selected PON1 gene, which was associated to HDL in large GWA [59] and which encodes an enzyme strongly modulated by anthocyanins.
We performed a G*E analysis to test the association of the SNPs in PON1 with Total cholesterol, HDL, LDL, Triglycerides and AIP in low and high anthocyanins and polyphenols intake environments.

For all genes we explored the entire gene, including all the SNPs within the gene boundaries and extending the analysis 15 Kb upstream and downstream the 5’ and the 3’ of each gene. We used this last option in order to investigate possible regulatory regions.
All phenotypes were analysed as residuals, adjusted for sex, age, BMI and the first 10 principal components calculated using R software. For each trait, individuals with missing data were excluded from the analysis. To correct for multiple testing for each gene we defined a Bonferroni threshold.

3.6 Development of tools for personalized nutrition in CVD prevention

In the context of ATHENA, together with KOS Genetic and Politecnico di Milano, an integrated platform, called Dietary Monitoring Solution (DMS), was developed to collect phenotypic, genetic and lifestyle information. The DMS includes a Case Report Form (CRF) and a linked mHealth application. The CRF, aimed at collecting information related to cardiovascular diseases and dietary risk factors was designed with the collaboration of domain expert clinicians and nutritionists. A specific software processed nutritional data collected during dietary interviews and computed macro and micronutrients intake from each food/meal. The mHealth application was designed to track subject dietary and lifestyle habit in his/her ecological context.

Genetic information, represented by Single Nucleotide Polymorphisms (SNPs) was organized in PED and MAP files, which constitute standard formats for the most widely used genomic analysis software.

The system can be integrated with a semi-automatic Decision Support System (DSS), which is built on a set of rules that uses as input:

- SCORE risk, is used for risk calculation: the formula is based on few parameters: systolic blood pressure, total cholesterol, high density cholesterol and smoking status. Such endpoints are not the best available predictors of atherosclerosis, but have relevant advantages that make them well suited to be used in a risk score meant for “apparently healthy” subjects: inexpensive, uninvasive, easy to measure, well correlated with atherosclerosis.
- Normality ranges:
    - Available from international guidelines for BMI;
    - Provided by the laboratory for biochemical parameters;

- Food preferences: from the available daily recalls included in the individual records.
- Nutritional Guidelines: from LARN “Reference Levels for the intake of nutrients and energy in the Italian population” tables.
- For LDL and HDL we developed algorithms that propose:
    - optimal cholesterol levels in relation to the presence of concomitant risk factors,
    - dietary or lifestyle interventions

  according with the “American Association of Clinical Endocrinologists’ guidelines for management of dyslipidaemia and prevention of atherosclerosis” [69].

4 RESULTS

The study sample included 465 apparently healthy individuals, divided in vegetarians and omnivores (Table 3). Only 450 individuals, those with at least two dietary interviews, were considered for analysis on dietary aspects and for nutrigenomic. 15 individuals were lost during the follow up (i.e. only one dietary assessment).

Table 3: Number of volunteers recruited in the observational study, number of eligible after dropping subjects not meeting inclusion/exclusion criteria, number of eligible for genetic analysis and those considered for analysis on dietary aspects (having at least 2 dietary assessments).

	Study sample			
	Recruited	Eligible	Genetics	> 1 dietary recall
OMNIVORS	381	374	346	331
VEGETARIANS	122	120	119	119
TOTAL	503	495	465	450

Demographic characteristics of the subjects studied are summarized in Table 4.

Table 4: Demographic and metabolic parameters of the study sample (mean±SD). Abbreviations (BMI=Body Mass Index; WC= Waist Circumference; TGL=triglycerides; LDL=low density lipoprotein; HDL=high density lipoprotein; IMT=Intima Media Thickness)

Phenotypes	**Females**		**Males**		**P-value**	**TOTAL**	
Sex	270		180			450	
Age	49.78	± 13.13	53.19	±14.9	0.011	51.14	±13.96
BMI	24.14	± 5.13	25.51	±4.25	0.002	24.68	±4.84
WC	83.17	± 12.77	94	±11.88	-	86.70	±13.35
Low physical activity	35.51%		27.61%		NS	32.35%	
Smoke (yes)	17.36%		16.57%		NS	17.05%	
Vegetarian	31.23%		19.34%		0.005	26.44%	
TGL (mg/dl)	97.02	± 57.26	120.53	± 69.25	<0.001	106.32	± 60.51
HDL (mg/dl)	58.90	± 12.25	48.55	± 12.36	<0.001	54.63	± 13.27
LDL (mg/dl)	128.03	± 34.72	120.88	± 33.85	0.036	125.18	± 34.51
CHOL (mg/dl)	206.57	± 40.08	193.63	± 36.89	<0.001	201.42	± 39.28
LDL/HDL	2.27	± 0.81	2.61	± 0.84	<0.001	2.40	± 0.84
SBP	120.43	± 19.37	128.21	± 18.15	<0.001	123.57	± 19.24
DBP	74.17	± 10.89	78.44	± 10.77	<0.001	75.89	± 11.03
IMT (mean)	0.58	± 0.1	0.62	± 0.12	-	0.59	± 0.11

Females were more represented than males, being 59.78% of the sample. Female were also more represented in the vegetarian group (70.6%) compared to the omnivores group (see Table 5).

The mean age of our sample was 51 years (±13.96). The average BMI, SBP, DBP, IMT, TGL, LDL and HDL are within normal ranges. Total cholesterol is slightly above of the reference threshold of 200 mg/dl.

Females are slightly younger than males and have slightly lower BMI. Females have higher levels of cholesterols compared to males, but a better LDL/HDL ratio. On the contrary males have higher TG and more elevated systolic and diastolic blood pressure.

Differences between males and females in waist circumference and IMT were not evaluated as they are expected in relation to different average body size.

Objective 1: Evaluation of the association between well-established risk factors of CVD Vs dietary micronutrients/dietary patterns

Table 5 shows the average daily intake of selected nutrients and the adherence to Mediterranean diet, in the total sample and in the groups of omnivores and vegetarians.

Table 5: Average daily intake of selected nutrients and adherence to Mediterranean diet (TP=Total Proteins; AP=Animal Proteins; LT=Lipids Total; SFA=Saturated Fatty Acids; PUFA=Polyunsaturated Fatty Acids; CA= Carbohydrates; ATC=Anthocyanins; PL=Polyphenols; MD=Mediterranean diet). Results from Wilkoxon rank test are indicated: ** P<0.001; *P <0.05

Nutrient	Omnivores		Vegeterians		TOTAL	
Diet type	331 (73.6%)		119 (26.4%)		450	
Females	186 (56.2%)		84 (70.6%)			
TP**	62.14	± 17.72	48.17	± 15.91	58.45	± 18.31
AP**	38.00	± 15.26	5.81	± 6.29	29.49	± 19.58
LT	59.40	± 51.99	56.91	± 22.46	58.74	± 46.05
SFA**	18.80	± 8.25	12.88	± 6.06	17.23	± 8.15
PUFA**	7.82	± 3.75	9.91	± 4.56	8.370	± 4.09
CA**	191.62	± 57.03	212.49	± 57.46	197.14	± 57.82
Starch	115.54	± 42.81	108.74	± 43.50	113.74	± 43.05
Fiber**	18.29	± 9.31	31.22	± 11.59	21.71	± 11.47
ATC*	22.80	± 32.44	40.57	± 69.41	27.50	± 45.84
PL**	1,016.59	± 535.61	1,582.99	± 897.79	1,166.37	± 696.57
MD (Higher tertile)	21.31%		62.79%		32.12%	

The two diets show marked differences for several nutrients: vegetarian diet is significantly higher in fiber, cholesterol, polyunsaturated fatty acids, polyphenols and anthocyanins. On the contrary the “no restriction diet” of the omnivores is higher in proteins, animal proteins and saturated fatty acids.

As expected, a high adherence to Mediterranean diet is more frequent in vegetarians.

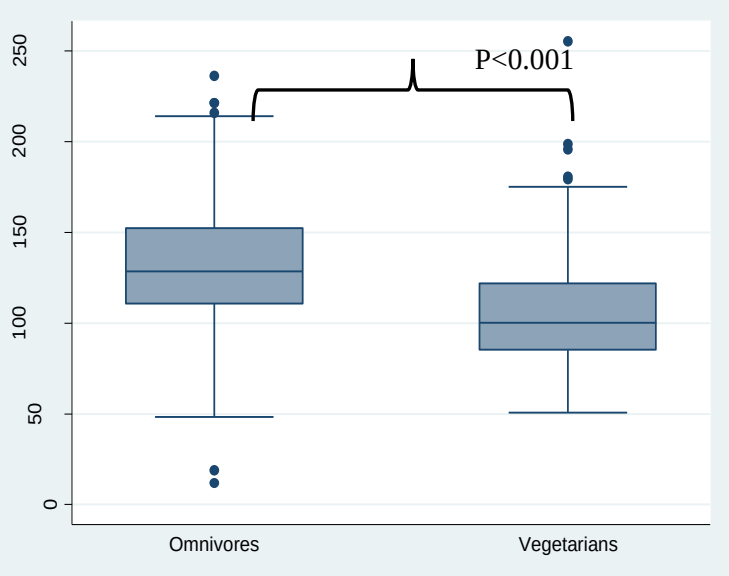
Table 6 presents the results of multiple regression analysis evaluating the associations between markers of CVD and dietary factors.

After adjusting for potential confounders, vegetarians show lower BMI compared to omnivores. This observation is significant also for individuals with high adherence to Mediterranean diet, compared to those with low adherence and for individuals reporting high intake of polyphenols compared to those reporting low intake of polyphenols. For such classes we observe also a lower waist circumference. Systolic and diastolic blood pressures show the same trend: they are significantly reduced in vegetarians (Vs omnivores), in subjects reporting higher adherence to Mediterranean diet (compared to those with low adherence) and in subjects reporting high intake of polyphenols (compared to those with low intake).

As illustrated in Figure 6 and Table 6, vegetarian diet is associated with a strong reduction of LDL, total cholesterol levels and LDL/HDL ratio.

Some individuals in both omnivores and vegetarians groups show LDL and total cholesterol levels higher than normal (200 mg/ml).

Mediterranean diet was found significantly associated with an increase in HDL and with a better (lower) LDL/HDL ratio, but not with a decrease in LDL and total cholesterol.



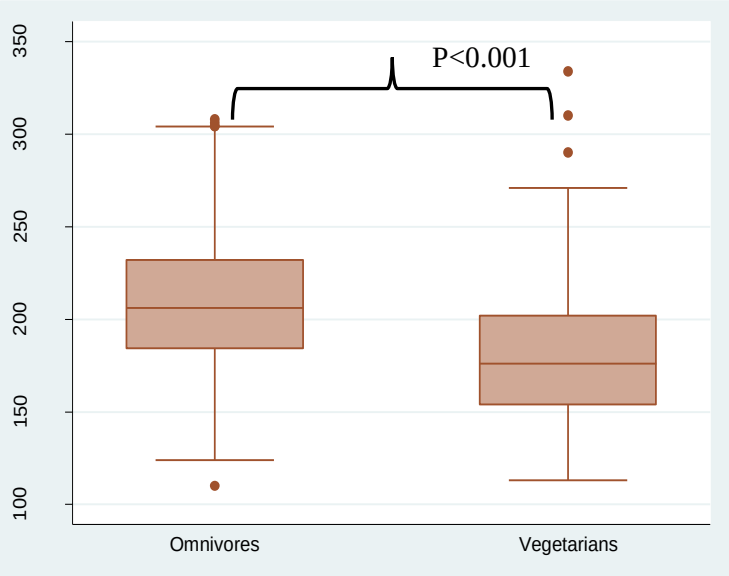


Figure 6: Representation of LDL and total cholesterol serum levels in omnivores and vegetarians.

None of the considered dietary factors influence IMT.

High anthocyanins intake was not associated to any of the parameters considered.

Table 6: Association between phenotypes related to CVD risk and diet/micronutrients (columns). Age, gender, sport intensity and smoke were always used as covariates, while BMI was considered for all phenotypes except for BMI itself. Sample (N=450), * Statistically significant

Phenotype	**Mg Anthocyanins/day (tertiles)**		**Mg Polyphenols/day (tertiles)**		**Diet (Omivorous Vs Vegetarian)**		**Mediterranean Diet Score (tertiles)**	
	Coeff	P	Coeff	P	Coeff	P	Coeff	P
BMI	-0.08 (SE0.29)	0.777	-0.98 (SE 0.28)	0.001*	-2.64 (SE 0.57)	<0.001*	-1.08 (SE 0.32)	0.001*
Waist Circumference	-0.322 (SE 0.46)	0.483	-1.01 (SE 0.44)	0.023*	-1.14 (SE 0.92)	0.216	-1.93 (SE 0.48)	<0.001*
SBP	0.46 (SE 1.05)	0.659	-2.34 (SE 0.99)	0.019*	-5.13 (SE 2.08)	0.014*	-3.06 (SE 1.18)	0.01*
DBP	-1.102 (SE 0.59)	0.064	-0.96 (SE 0.61)	0.119	-4.53 (SE 1.26)	<0.001*	-1.56 (SE 0.71)	0.03*
HDL	0.535 (SE 0.68)	0.435	0.57 (SE 0.70)	0.414	0.45 (SE 1.4)	0.758	1.63 (SE 0.78)	0.037*
LDL	1.75 (SE 2.01)	0.383	0.27 (SE 2)	0.893	-18.24 (SE 4.01)	<0.001*	-1.79 (SE 2.22)	0.423
Total Cholesterol	1.51 (SE 2.31)	0.514	0.28 (SE 2.26)	0.90	-18.40 (SE 4.61)	<0.001*	-0.70 (SE 2.54)	0.783
LDL/HDL	-0.014 (SE 0.05)	0.768	-0.035 (SE 0.05)	0.464	-0.4 (SE 0.01)	<0.001*	-0.11 (SE 0.05)	0.039*
AIP	-0.021 (SE 0.03).	0.508	-0.032 (SE 0.03)	0.313	-0.038 (SE 0.067)	0.567	-0.05 (SE 0.04)	0.159
Triglycerides	-2.83 (SE 3.37)	0.402	-1.83 (SE 3.47)	0.598	-3.51 (SE 7.21)	0.627	-2.56 (SE 4.14)	0.537
IMT_mean	-0.0025 (SE 0.006)	0.651	-0.001 (SE 0.01)	0.794	-0.010 (SE 0.014)	0.406	-0.002 (SE 0.01)	0.723

Objective 2: Evaluation of possible mechanism of action of dietary micronutrients/diet on lipid levels, by studying sterols implicated in cholesterol homeostasis

Table 7 and Table 8 describe mean and standard deviations (SD) of sterol levels measured in our sample, by dietary group and by tertile of anthocyanins/polyphenols daily intake. For each sterol is reported the absolute value.

Table 7: Serum sterols in the different dietary groups (mean± SD). Absolute values in µg/L (N=312).

Phenotype	**Diet (Omivores Vs Vegetarian)**		**Mediterranean Diet Score (tertiles)**	
	Omnivore	Vegetarian	Lowest	Highest
Campesterol	9,999.54	11,757.9	9,604.02	11,411.73
µg/L	±4624.66	±3929.48	±4321.15	±4378.11
Sitosterol	5,026.68	6,398.51	4,628.57	6,335.29
µg/L	± 2631.30	±2400.42	±2248.46	±2702.85
Lathosterol	2,742.16	2,321.68	2,972.75	2,240.67
µg/L	±1,294.75	±1,209.67	±1,284.99	±1,134.24
Desmosterol	98.92	105.58	99.83	101.04
µg/L	±35.24	±33.24	±33.06	±33.72
Lanosterol	147.20	137.37	157.18	140.44
µg/L	±72.56	±61.75	±74.687	±63.149
24OHC	51.18	54.10	51.03	54.58
µg/L	±15.15	±16.95	±14.13	±16.46
25OHC	23.39	24.29	24.07	23.59
µg/L	±12.449	±12.11	±13.76	±10.24
27OHC	147.23	152.89	149.54	158.45
µg/L	±49.16	±40	±43.81	±54.02

Table 8: Serum sterols in relation to anthocyanins and polyphenols daily intake (mean± SD). Absolute values in µg/L (N=312).

Phenotype	**Mg Anthocyanins/day (tertiles)**		**Mg Polyphenols/day (tertiles)**	
	Lowest	Highest	Lowest	Highest
Campesterol	10,077.88	10,634.21	9,993.71±	11,287.40
µg/L	±4687.92	±4236.62	4822.86	±4341.74
Sitosterol	4,961.01	5,722.98	4,720.10	6,222.09
µg/L	±2438.18	±2559.58	±2376.39	±2693.20
Lathosterol	2,742.91	2,619.20	2,912.32	2,473.33
µg/L	±1,306.71	±1,145.12	±1,313.04	±1,115.16
Desmosterol	98.57	104.95	102.43	106.09
µg/L	±36.22	±33.73	±37.14	±30.37
Lanosterol	142.11	151.14	148.42	148.71
µg/L	±64.86	± 68.68	±74.49	±67.31
24OHC	51.25	52.47	52.10	51.97
µg/L	±14.76	±15.98	±15.32	±15.48
25OHC	22.31	24.37	22.43	24.55
µg/L	±0.64	±13.32	±11.15	± 14.93
27OHC	144.61	158.28	141.38	158.39
µg/L	±45.00	±50.20	±43.538	±51.40

Table 9 and Figure 7 show that vegetarians have higher sitosterol concentrations (both absolute values and sitosterol/total cholesterol ratio) and higher campesterol concentrations (only campesterol/total cholesterol ratio) compared to omnivores.

Table 9: Results of association analysis between sterols and dietary factors, adjuster for age, gender and BMI. * Statistically significant

Phenotype	Mg Anthocyanins/day (tertiles)		Mg Polyphenols/day (tertiles)		Diet (Omivorous Vs Vegetarian)		Mediterranean Diet Score (tertiles)	
	Coeff	P	Coeff	P	Coeff	P	Coeff	P
Absorption markers								
Campesterol	227.611 (SE 314.99)	0.470	501.427 (SE 307.50)	0.104	1,197.417 (SE 622.33)	0.055	665.33 (SE 356.31)	0.063
Campesterol/chol	118.02 (SE 147.82)	0.425	279.51 (SE 144.07)	0.053	1176.487 (SE 286.03)	<0.001*	391.70 (SE 164.43)	0.018*
Sitosterol	303.32 (SE 178.25)	0.090	621.06 (SE 171.80)	<0.001*	1,105.529 (SE 350)	0.002*	680.245 (SE 198.54)	0.001*
Sitosterol/chol	161.79 (SE 86.23)	0.062	328.91 (SE 82.84)	<0.001*	865.25 (SE 164.94)	<0.001*	367.80 (SE 95.32)	<0.001*
Synthesis metabolites								
Lathosterol	-18.79 (SE 81.516)	0.818	-130.36 (SE 79.51)	0.102	-49.45 (SE 161.88)	0.76	-213.867 (SE 92.62)	0.022*
Lathosterol/chol	2.21 (SE 37.48)	0.95	-61.66 (SE 36.55)	0.093	74.94 (SE 74.32)	0.314	-91.62 (SE 42.67)	0.033*
Desmosterol	2.69 (SE 2.43)	0.269	1 (SE 2.39)	0.674	11.54 (SE 4.79)	0.017*	4.02 (SE 2.68)	0.134
Desmosterol/chol	1.76 (SE 1.13)	0.12	0.86 (SE 1.11)	0.77	10.71 (SE 2.16)	<0.001*	2.53 (SE 1.2)	0.038*
Lanosterol	4.198 (SE 4.78)	0.381	2.48 (SE 4.69)	0.598	9.81 (SE 9.49)	0.302	-2.45 (SE 5.35)	0.648
Lanosterol/chol	2.28 (SE 2.13)	0.287	1.60 (SE 2.10)	0.446	10.10 (SE 4.21)	0.017*	-0.47 (SE 2.40)	0.845

Phenotype	Mg Anthocyanins/day (tertiles)		Mg Polyphenols/day (tertiles)		Diet (Omivorous Vs Vegetarian)		Mediterranean Diet Score (tertiles)	
	Coeff	P	Coeff	P	Coeff	P	Coeff	P
Turnover markers								
24OHC	0.83 (SE 1.12)	0.460	0.33 (SE 1.10)	0.766	3.15 (SE 2.23)	0.158	1.24 (SE 1.27)	0.332
24OHC/chol	0.418 (SE 0.50)	0.405	0.407 (SE 0.49)	0.407	3.67 (SE 0.97)	<0.001*	0.853 (SE 0.567)	0.134
25OHC	0.98 (SE 0.89)	0.279	1.28 (SE 0.87)	0.144	1.61 (SE 1.77)	0.363	-0.73 (SE 1.03)	0.481
25OHC/chol	0.544 (SE 0.44)	0.219	0.749 (SE 0.432)	0.084	1.85 (SE 0.87)	0.035*	-0.255 (SE 0.514)	0.620
27OHC	5.415 (SE 3.19)	0.091	6.43 (SE 3.12)	0.04*	13.06 (SE 6.33)	0.04*	9.50 (SE 3.67)	0.011*
27OHC/chol	3.029 (SE 1.41)	0.032*	3.87 (SE 1.37)	0.005*	14.40 (SE 2.69)	<0.001*	5.443 (SE 1.623)	0.001*

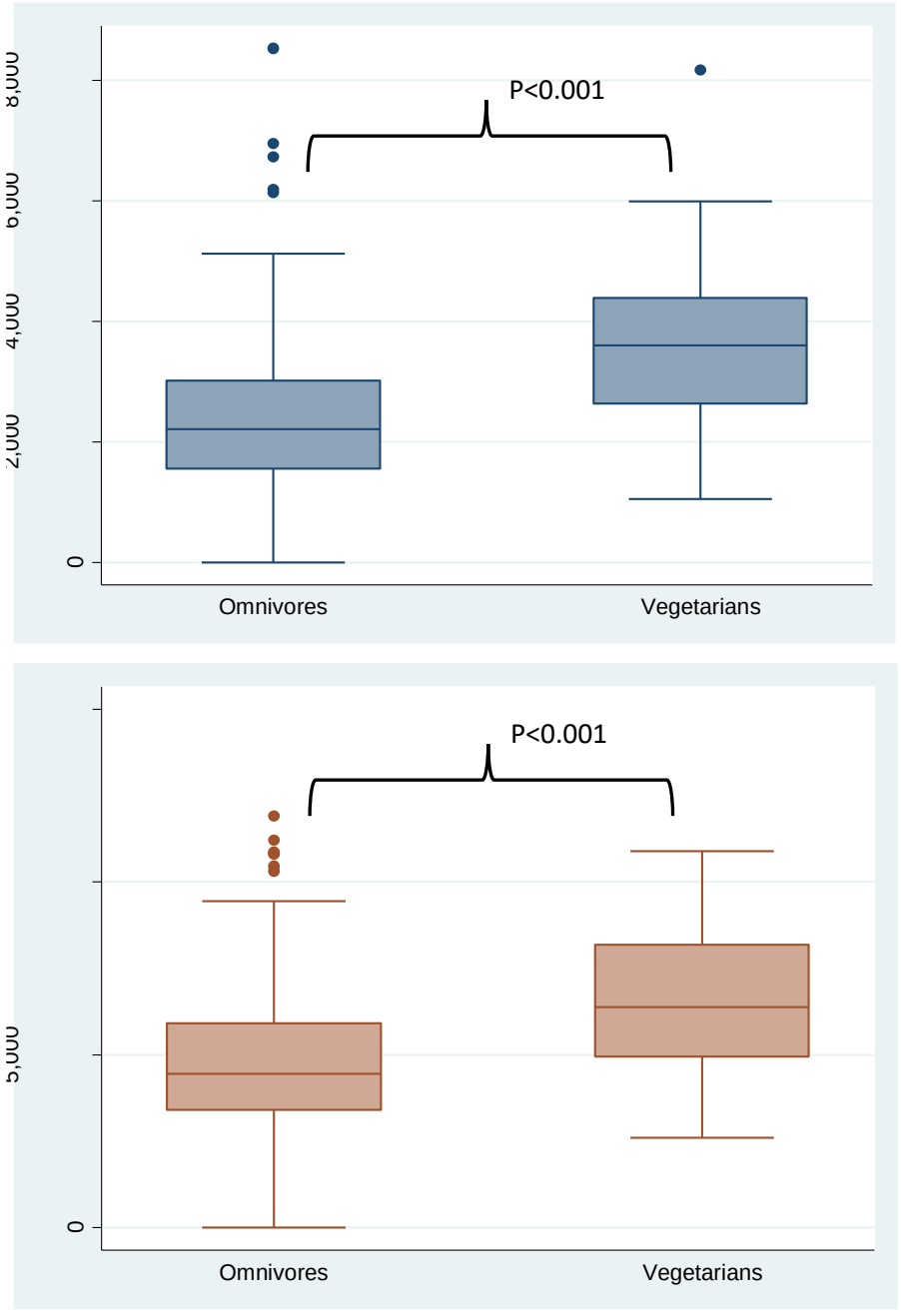


Figure 7: Representation of sitosterol/total cholesterol ratio and campesterol/total cholesterol ratio in omnivores and vegetarians.

Subjects with high adherence to Mediterranean diet also show an increase in sitosterol concentrations (both absolute values and sitosterol/total cholesterol ratio) and in campesterol concentrations (only campesterol/total cholesterol ratio) compared to those with low adherence.

A high consumption of polyphenols with the diet is also associated with increased sitosterol levels (both absolute value and sitosterol/total cholesterol ratio), but not with changes in campesterol levels.

In subjects with high adherence to Mediterranean diet we observe a decrease of lathosterol concentration (both absolute value and lathosterol/total cholesterol ratio) and an increase of desmosterol concentration (only desmosterol/total cholesterol ratio). While in vegetarians we observe higher desmosterol concentrations (both absolute value and desmosterol/total cholesterol ratio).

The ratio between sitosterol: campesterol is not significantly different in vegetarians compared to omnivores (ttest; P=0.49). The ratio between lathosterol: desmosterol (28.66± 13.16 in omnivores Vs 22.95±12.39 in vegetarians) is significantly different in favor of desmosterol in vegetarians (ttest; P<0.001) and in individuals with high adherence to Mediterranean diet compared to those with low adherence (31.48 ± 15.86 in the lower tertile Vs 22.77± 11.57 in the higher tertile) (linear regression; P=0.003).

Table 10: Spearman correlations between sterol levels and cholesterol, for each correlation is reported the rho coefficient and the P-value (P).

	LDL		**HDL**		**Total Cholesterol**	
	rho	P	rho	P	rho	P
Sitosterol	0.152	0.007	0.241	<0.0001	0.1456	0.01
Campesterol	0.198	0.0004	0.1814	0.0013	0.1814	0.0013
Lathosterol	0.389	<0.0001	-0.238	<0.0001	0.3781	<0.0001
Desmosterol	0.356	<0.0001	-0.070	0.2154	0.3713	<0.0001
Lanosterol	0.393	<0.0001	-0.177	0.0017	0.3734	<0.0001
24-OHC	0.400	<0.0001	0.094	0.0987	0.4554	<0.0001
25-OHC	0.299	<0.0001	0.045	0.4244	0.2879	<0.0001
27-OHC	0.363	<0.0001	-0.138	0.0147	0.343	<0.0001

Table 10 indicates that phytosterols (sitosterol and campesterol) are positively correlated with LDL, HDL and total cholesterol levels.

Table 10 shows that intermediates of cholesterol de novo synthesis (lathosterol, desmosterol and lanosterol) are positively correlated with LDL and total cholesterol levels. Oxysterols (24-OHC, 25-OHC and 27-OHC) are positively correlated with LDL and total cholesterol levels. 27-OHC is negatively correlated with HDL.

However if we stratify the analysis for dietary group we observe that the negative association observed between 27-OHC and HDL is not replicated in the vegetarian group (omnivores rho= -0.16, P= 0.0113 Vs vegetarians rho= -0.05; P=0.6).

Table 11, shows that if we stratify the analysis for vegetarians/omnivores, phytosterols show different degrees of correlation with cholesterol levels: the positive correlations between LDL and HDL and phytosterols observed in omnivores are not significant in vegetarians. This suggests that the overall type of diet may be a modifier of the correlation between phytosterols and cholesterol.

Table 11: Spearman correlation between phytosterols (SI=Sitosterol; CA=Campesterol) and cholesterol. , for each correlation is reported the rho coefficient and the P-value (P).

	LDL				**HDL**				**Total Cholesterol**			
	Omnivores		Vegetarians		Omnivores		Vegetarians		Omnivores		Vegetarians	
	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P
SI	0.31	<0.001	0.21	0.06	0.23	<0.001	0.21	0.06	0.27	<0.001	0.26	0.02
CA	0.34	<0.001	0.18	0.11	0.16	0.017	0.20	0.71	0.28	<0.001	0.24	0.03

Objective 3: Evaluation of the possible mechanism through which dietary micronutrients/diet can influence lipid levels, by studying G*E interactions

Overall diet

Table 12 presents the results of G*E interaction considering overall diet as "environment" and LDL as phenotype.

It reports the number of SNPs tested for each gene, the most significant SNP (best SNP), the p-value of the best SNP and the Bonferroni threshold.

Table 12: Results of G*E analysis, with environment as "no-restriction" or "vegetarian" diet

Gene	Nr. SNPs	Phenotype	Best SNP	P-value	Bonferroni threshold
CYP7A1	35	LDL	rs1125226	0.0024	0.00142
INSIG2	47	LDL	rs11679259	0.0004	0.00106
PCSK9	48	LDL	rs11206517	0.0005	0.00104
LPA	84	LDL	rs9365200	0.0005	0.00059

CYP7A1 did not show any gene level significant interaction with vegetarian diet. The candidate SNP rs3808607 showed a borderline significant G*E association if considered alone (P= 0.049).

For INSIG2 besides the best observed SNP (rs11679259) two other SNPs had a significant G*E interaction (rs17047718, rs17047731) and all are in Linkage Disequilibrium (LD).
For PCSK9 two other SNPs beside the best observed SNP had a significant G*E interaction (rs12067569, rs505151) and all are in LD.
For LPA only rs9365200 was above the Bonferroni corrected p-value.

We then analysed the effects of the genotypes at the significant SNPs identified in INSIG2, PCSK9 and LPA on LDL residuals, divided by dietary group (Table 13).

Three SNPs in PCSK9 (rs11206517, rs12067569 and rs505151) showed a gene level significance interaction with the diet. Rs11206517 was previously associated with triglycerides level in Han population [70] and it is in strong LD with rs505151 (also reported as E670G), a SNP that has been repeatedly associated with a “gain of function” mutation of PCSK9, determining higher LDL cholesterol levels [71]. Rs12067569 was previously associated with LDL levels in American Indians, and its mutation was associated with higher LDL [72].

Table 13 shows that for all the three SNPs of PCSK9, individuals homozygous for the wild type exposed to vegetarian diet have lower LDL residuals compared to those exposed to “no restriction” diet. On the contrary heterozygous individuals exposed to vegetarian diet have higher LDL compared to those exposed to “no restriction” diet. In our sample there are no homozygous individuals for the mutated type.

Table 13: For each SNP with a gene level significance are reported: LDL residuals in relation to the genotype, stratified per diet (mean; standard deviation=SD; number of individuals); Minor Allele Frequency (MAF), P of the G*E interaction and region. ** LD

LDL residuals: mean SD and (frequency)									
	No restriction			Vegetarian			MAF	P G*diet	Region
SNP	Homozygous Allele 2	Heterozygous	Homozygous Allele1	Homozygous Allele 2	Heterozygous	Homozygous Allele1			
PCSK9									
rs11206517**	4.68 SD 30.36 (288)	-2.65 SD 50.30 (20)	-	-13.07 SD 24.28 (110)	25.83 SD 55.75 (6)	-	0.03	0.0005214	intronic
rs12067569	4.54 SD 30.40 (289)	-0.84 SD 51.01 (19)	-	-13.07 24.28 (110)	25.83 SD 55.75 (6)	-	0.03	0.0009756	intronic
rs505151**	4.54 SD 30.40 (289)	-0.84 SD 51.01 (19)	-	-13.07 SD 24.28 (110)	25.83 SD 55.75 (6)	-	0.03	0.0009756	coding
INSIG									
rs11679259**	5.98 SD 31.59 (279)	9.37 SD 23.54 (27)	-60 SD 87.68 (2)	-12.91 SD 26.02 (99)	0.125 SD 36.25 (16)	-7 SD 0 (1)	0.06	0.0004389	upstream
rs17047718**	5.98 SD 31.59 (279)	9.37 SD 23.54 (27)	-60 SD 87.68 (2)	-12.91 SD 26.02 (99)	0.125 SD 36.25 (16)	-7 SD 0 (1)	0.06	0.0004389	upstream
rs17047731**	5.98 SD 31.59 (279)	9.37 23.54 (27)	-60 SD 87.68 (2)	-12.91 SD 26.02 (99)	0.125 SD 36.25 (16)	-7 SD 0 (1)	0.06	0.0004389	upstream
LPA									
rs9365200	0.99 SD 32.60 (131)	3.94 SD 29.04 (134)	14.84 SD 36.84 (43)	4.067 SD 33.068 (45)	13.60 SD 22.64 (62)	28.56 SD 21.37 (9)	0.34	0.0005231	intronic

Since rs505151 (also reported as EG70G) has been repeatedly associated with a “gain of function” mutation of PCSK9, determining higher LDL cholesterol levels, we evaluated the levels of cholesterol in relation to rs505151 genotypes (table 14).

Table 14: Variations of cholesterol levels in relation to genotypes of rs505151.

		PCSK9		
	N	LDL	Total cholesterol	HDL
		Rs 505151 (EG70G)		
AA	404	106.05 (SD 53.68)	170.66 (SD 80.25)	66.54 (SD 30.22)
GA	25	110.89 (SD 68.92)	181.8 (SD 91.30)	71.38 (SD 36.88)

As shown in table 14, individuals with the mutated genotype tend to have higher LDL, total cholesterol and HDL levels, although not statistically significant.

One SNP (rs9365200) in LPA shows gene level significant interaction. Rs 9365200 was never reported before. Two nearby SNPs are in strong LD with rs9365200, even if they are not significant for G*E interaction after Bonferroni correction (see Figure 8): rs9365201 and rs9347438. The latter has been reported in a study on coronary heart disease in Han Chines Population [73].

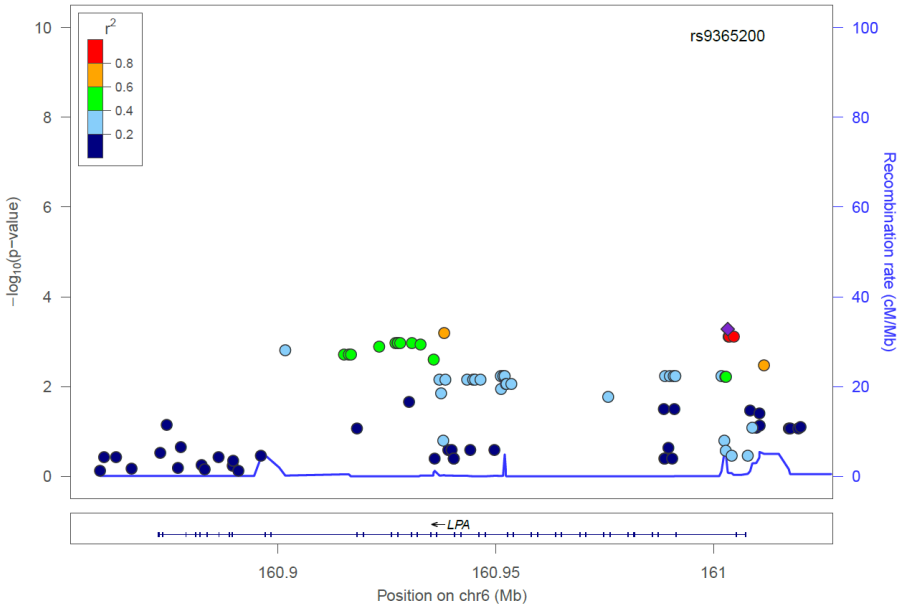


Figure 8: Regional plot of LPA genomic region. In purple it is represented the best SNP (rs9365200)

Figure 9, graphically describes a typical G*E interaction. It presents LDL levels (Y axis) across the three genotypes of rs9365200 (LPA) in vegetarians and omnivores.

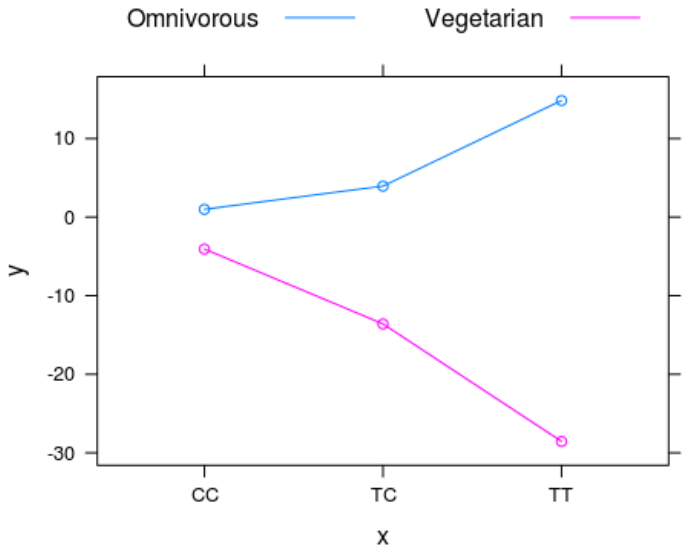


Figure 9: graphical representation of LDL levels (residuals, Y axis), in relation to the genotype at SNP rs9365200, of gene LPA (X axis) in individuals reporting "no restriction" (blue) and Vegetarian diet (pink).

The graph suggests that in TT genotypes of rs9365200 a vegetarian diet is expected to have a much greater efficacy in reducing LDL levels that in CC genotypes.

At the same time it could be speculated that in TT genotype a "no restriction diet" would increase LDL levels.

PON 1

Table 15 presents significant G*E interactions for SNPs in PON1 and selected CVD biomarkers, considering anthocyanins and polyphenols intake as environment.

A significant G*E interaction (Bonferroni threshold is p=0.0028) indicates that the association between the SNP and the phenotype varies in relation to the environment, so it is different in the low Vs high intake group.

Two SNP (rs854549 and rs854552) in PON1 show significant interaction with anthocyanins in the association with HDL. Other two SNPs (rs854571 and rs854572) show significant interaction with polyphenols in the association with HDL. Significant interaction with anthocyanins was also observed for rs854551, in relation to AIP. While an additional interaction with polyphenols intake was observed in the association between rs3917477 and total cholesterol and polyphenols.

For the 3 SNPs showing significant G*E interaction with anthocyanins intake and for the 3 SNPs showing significant G*E interaction with polyphenols we evaluated how the phenotype (i.e. lipid profile) varies in relation to the genotype in the environments considered.

Figure 10 shows "the trend of lipid profiles across the three genotypes for each significant GxE SNP in low or high environment. In particular, in high anthocyanins intake, HDL concentration was higher in carriers of the major CC genotype for rs854549 (p-value=0.001, Beta= 4.7) and in the minor CC genotype for rs854552 (p-value =0.001, Beta=5.6). AIP was lower in minor AA carriers for rs854551 (p-value= 0.034, Beta=-0.07). Considering high polyphenols intake, HDL concentration was higher in minor TT carriers for rs854571 (p-value=0.026, Beta=3.92) and higher in minor CC compared to GG for rs854572 (p-value=0.025, Beta=3.94). Rs3917477 was not significantly associated to cholesterol in high polyphenols intake" [55].

Table 15: Results of G*E analysis, considering anthocyanins and polyphenols intake environments. For each SNP of PON1 is reported the p-value for the interaction for selected phenotypes (HDL, LDL, TC=Cholesterol, TG=Triglycerides and AIP phenotypes). In bold are indicated significant valued (From Rizzi et al, 2016) [55]

	Anthocyanins intake					**Polyphenols intake**				
SNP	**HDL**	**LDL**	**TC**	**TG**	**AIP**	**HDL**	**LDL**	**TC**	**TG**	**AIP**
rs854549	**0.0008**	0.3941	0.1031	0.6548	0.1787	0.0998	0.5160	0.2006	0.5240	0.9975
rs3735590	0.3448	0.1405	0.0397	0.0622	0.3134	0.2444	0.0866	0.0303	0.1277	0.2865
rs854551	0.0042	0.6413	0.8251	0.0103	**0.0022**	0.0206	0.0634	0.0575	0.0064	0.0041
rs854552	**0.0010**	0.6538	0.3149	0.1498	0.0177	0.0071	0.4599	0.6442	0.1186	0.0493
rs3917477	0.9098	0.0364	0.0437	0.4110	0.6409	0.0701	0.0674	**0.0026**	0.0544	0.4517
rs854571	0.1042	0.3670	0.1272	0.3527	0.9355	**0.0021**	0.1417	0.0067	0.4772	0.4889
rs854572	0.0087	0.8026	0.2546	0.6123	0.3915	**0.0020**	0.5219	0.1108	0.8518	0.2163

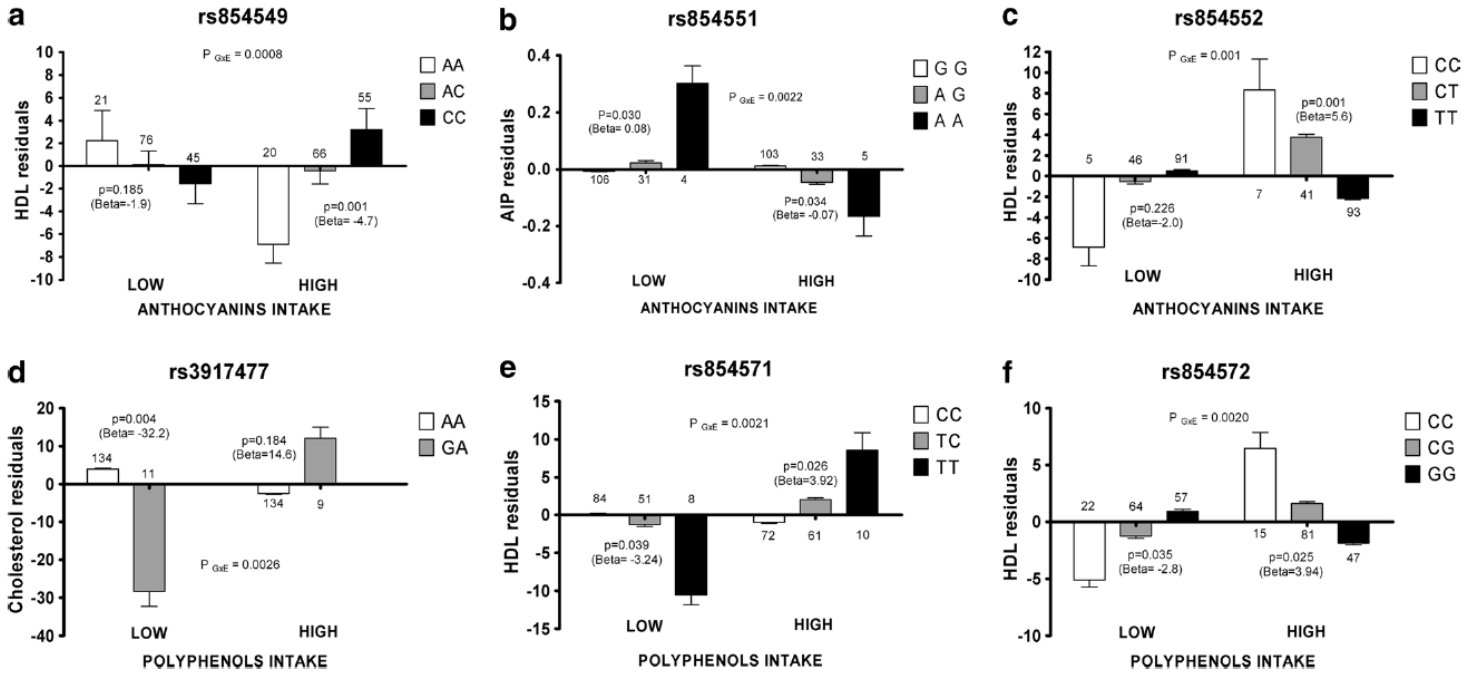


Figure 10: "Phenotypes distribution relative to genotypes at rs854549 (a), rs854551 (b), rs854552 (c), rs3917477 (d), rs854571 (e) and rs854572 (f) according to anthocyanins and polyphenols intakes. Y axis reports the residuals for each phenotype (HDL, AIP and cholesterol). The bars summarize the distribution as mean and standard errors. For each bar, the numbers of individuals per genotype are indicated. P_{GxE} denotes the SNP x environment interaction analysis comparing high and low intake both for anthocyanins (a, b, c) and polyphenols (d, e, f).P indicates the multivariate linear regression analysis comparing genotypes in each environment subgroup (low/high); Beta coefficients refer to minor alleles". (From Rizzi et al, 2016) [55]

Objective 4 - Development of tools for personalized nutrition in CVD prevention

The DMS web-based platform combines validated tools for nutrigenomic research. It provides a configurable CRF to collect patient's data (i.e. personal medical history, physical, laboratory examination, therapies, etc.) and dietary habits, through interviews and food frequency questionnaires. The tool, to support data collection during dietary interviews, includes the possibility to select foods and recipes for each meal. The software then combines input data from dietary interviews and food nutrients composition, from validated food databases, and calculates macro and micronutrients intakes (e.g. daily, monthly etc.).

CRF template can be shared between different research centres, in case of multicenter studies, and used for data collection, ensuring anonymous data processing. The web-based platform in fact stores only patients' unique identifiers and does not contain patients' personal identification information.

Also, the platform manages the direct exchange of data with the mHealth app, that can be used by the patient to upload dietary and lifestyle information (e.g. Diaries).

The following figure presents the working environment of the web platform and the CRF section dedicated to the collection of dietary information.

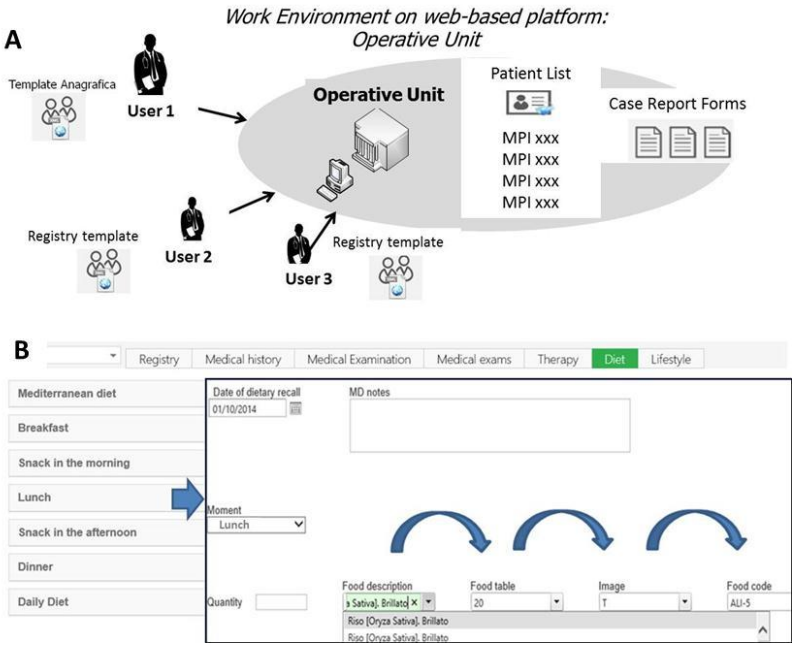


Figure 11: "A. The work environment of the web-based platform in a single operative unit. B. The CRF allows easy data entry by the selection of a food name automatically mapping the selected food to its code in the food database" (From Conti et al, 2015)[74]

Genotypic data are managed exclusively by the healthcare professional and can be uploaded from PED and MAP files, which constitute standard formats for genomic analysis software.

This platform was used to collect information from more than 500 volunteers participating to ATHENA project.

Both the platform and the mHealth app can provide access to a semi-automatic DSS that process data and provide suggestions considering: a) phenotype, b) nutritional habits, c) genetic profile, namely mutations that were associated to G*E interaction with the diet. The logic behind the DSS prioritize the information as indicated in Figure 12, below.

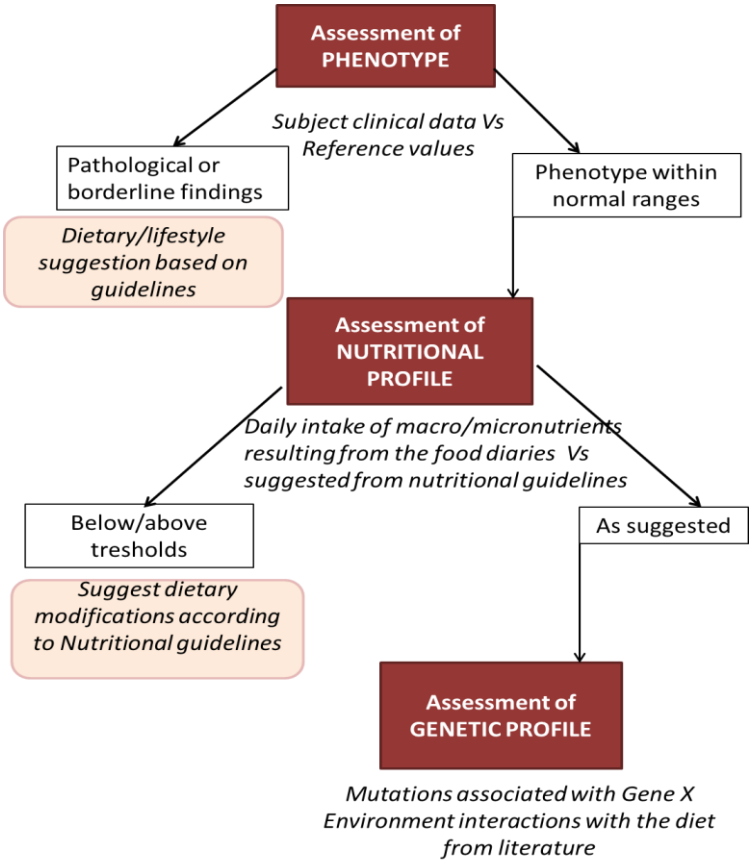


Figure 12: Diagram of the semi-automatic DSS

The genetic information that is processed by the DSS search engine needs to be preselected and presented in a tabular format in which they are related to DSS rules.

All suggestions provided by the DSS need to be validated by healthcare professionals, before being displayed in the patient's mHealth app.

The DMS system was reengineered after the project ATHENA. The current version, named ePhood, is used in research context, to collect data from nutrition and nutrigenomic studies. The mHealth app is provided to subjects

enrolled in observational studies and is used to collect/track their diet and lifestyle.

The integration of CVD risk scores with dietary guidelines (e.g. LARN for Italy) can provide additional information to the healthcare professional and to health concerned citizens.

To overcome limitations typically affecting the usability of mHealth App in a healthcare context, we designed a platform in agreement with standards-based architectures implementing bi-directional communication between mHealth apps and electronic health records, ensuring secure data exchange and technological and semantic interoperability.

Early adopters are suggesting new functionalities and upgrades to the system. In particular we are focusing on developing functionalities to make mHealth more user-friendly.

5 DISCUSSION

Objective 1: Evaluation of the association between well-established risk factors of CVD Vs dietary micronutrients/dietary patterns

In the context of the ATHENA project we conducted a nutritional and nutrigenomic study to analyze the effects of vegetarian diet and of dietary anthocyanins and polyphenols on CVD risk factors.

In our sample of apparently healthy volunteers, the percentage of vegetarians (26.4%) is probably higher than the percentage in the general population of Milano, but we cannot rely on exact statistics. However Milano is known to be the city where vegetarianism is more popular in Lombardy region, and Lombardy is the Italian region with the higher representativeness of vegetarians (18% of the population) [75].

The differences observed in dietary macronutrients and micronutrients composition between omnivores and vegetarians are expected. The average anthocyanin consumption in our sample (22.80 mg/day in omnivores and 40.57 in vegetarians) is slightly lower than the one estimated in a study conducted in Varese in the context of the EPIC investigation (55.48 mg/day in men and 40 in women) [76]. The average polyphenols intake in our sample (1,016.59 mg/day in omnivores and 1,582.99 mg/day in vegetarians) is also slightly lower than the one estimated in Varese by EPIC (~1,300 mg/day for women and ~1,500 in men). However EPIC and ATHENA adopted different anthocyanin and polyphenols dosage methods making comparison very difficult [77].

The observation that all CVD risk factors are decreased in vegetarians compared to omnivores (i.e. SBP, total cholesterol, LDL, BMI, Waist

circumference) except for HDL, triglycerides and IMT is in agreement with previous studies [78-79].

Lower LDL and total cholesterol levels observed in vegetarians could be related to the competitive effect of plant sterols, limiting cholesterol absorption, to low cholesterol and high fibre intake associated to vegetarian diets and/or to differences in the cholesterol synthesis/turnover.

A high adherence to Mediterranean diet is associated with reduced BMI, waist circumference and blood pressure, and with higher HDL levels, but not with changes in LDL and total cholesterol levels.

High intake of dietary polyphenols is inversely associated with BMI, waist circumference and blood pressure. A recent review supports the efficacy of polyphenols in reducing BMI and central obesity in patients with Metabolic Syndrome. However it shows that the result are largely dependent on the type of polyphenols considered. Almost all interventional studies with tea (mainly green tea) as source of polyphenols (catechins) showed a significant decrease of the BMI, while for other foods the results are less consistent. On the contrary the effect on blood pressure reduction was stronger for cocoa and olive oil [80].

Individuals consuming high amounts of anthocyanins do not show protective effects on the CVD risk parameters considered. The effect of anthocyanins on blood pressure has been recently questioned. A meta-analysis including 427 patients from 6 interventional trials, administering anthocyanins supplements (from 162 mg/day to 640 mg/day), indicated that anthocyanins do not decrease blood pressure [81].

However it could be postulated that in our population the consumption of anthocyanins is below the efficacy threshold, which has never been established in pharmacokinetic/pharmacodynamics studies. In facts, studies

reporting effects of anthocyanins on LDL and HDL plasma concentrations are interventional trials, using anthocyanins extracts or pure anthocyanins [82].

Considering the observational nature of the study, it may be difficult to dissect the specific contribution of diet on the reduction of the risk factors considered, since dietary habits are often associated with other lifestyle behaviors. For example, Serdula *et al* have shown a positive correlation between the intake of fruit and vegetables with other negative habits: fruit and vegetables consumption was lower in sedentary persons, heavy smokers and drinkers [83].

Objective 2: Evaluation of possible mechanism of action of dietary micronutrients/diet on lipid levels, by studying sterols implicated in cholesterol homeostasis

Mean campesterol levels in omnivores and vegetarians in our sample, respectively 9,999.54 µl/L (± 4,624.66) and 11,757.9 (± 3,929.48), are higher than in previously published studies. Reported mean campesterol levels varies from 5,400 µl/L ± 2500 [28] to 2,355 in omnivores and from 3,500 µl/L (±1,300) to 3,780 µl/L [29] in vegetarians. In our sample also mean sitosterol levels, respectively 5,026.68 µl/L (± 2,631.30) in omnivores and 6,398.51 (±2,400.42) in vegetarians are higher than previously described. Reported mean sitosterol levels varies from 3,500 µl/L (± 1,300) [28] to 1,246 µl/L in omnivores and 3,310 µl/L to 2,500 µl/L (±1,300) [29] in vegetarians. It should be noted however that such results are likely to be related to different methodologies/instruments used in the different laboratories. The internal validity of our results is supported by fact that all studies observe similar campesterol: sitosterol ratios. In addition none of those studies was

conducted in Italy, and differences in dietary habits may vary across populations.

Sitosterol and campesterol ratios over total cholesterol are higher in vegetarians compared to omnivores. The absolute value of sitosterol is also significantly higher in vegetarians compared to omnivores while there is no difference in the absolute value of campesterol. The stronger association of sitosterol with diet, compared to campesterol, is supported by a recent review indicating that sitosterols plasma concentrations increase in dose-dependent manner as sitosterol dietary intake increases, while no dose-dependent association is observed for campesterol [84].

Interestingly, in vegetarians the observed increase in phytosterols is not correlated with an increase in LDL or in HDL, whereas such correlation was statistically significant in omnivores. This might suggest that the increase in phytosterols in vegetarians mainly reflects an increased phytosterol consumption with the diet, not an increased cholesterol absorption, as previously suggested by Sarkinnen *et al* [27].

For what concerns markers of de novo cholesterol synthesis: we observed lower concentrations of plasma lathosterol in vegetarians, in subjects with high polyphenols intake, in subject with high anthocyanins intake and in subjects with high adherence to Mediterranean diet, but only for the latter group the association was statistically significant. This finding is accompanied by an increase in desmosterol concentration in vegetarians and in subjects with high adherence to Mediterranean diet, which is statistically significant only for vegetarians. Such variations, together, determine differences in lathosterol: desmosterol ratio, which is significant between vegetarias and ominivores and also between group of high and low adherence to Mediterranean diet. This might suggest that diet has a different effect on the two pathways of cholesterol synthesis. The increase in

desmosterol could even be beneficial, given its effect in stimulating cholesterol efflux [37].

The effects of plants-rich diets on lathosterol levels is inconsistent across studies. In 1998, Sarkinnen *et al* reported an increase of lathosterol in individuals receiving diets characterized by low cholesterol/high polyunsaturated fatty acids intake, and suggested that this finding could be related to a feedback reaction, in relation to reduced dietary cholesterol absorption [27]. However most recent studies on vegetarians or vegans indicate a reduction of lathosterol in subjects receiving plants-rich diets [28].

In the present study, we observe an increase in all the considered oxysterols (24-OHC, 25-OHC and 27-OHC) over total cholesterol ratios in vegetarians. However the result for 24-OHC and 25-OHC, could be due to a distortion due to the lower total cholesterol levels observed in vegetarians. In fact, if we consider the absolute values of these sterols there are no significant differences among groups.

The most significant result is recorded for 27-OHC. Its ratio over total cholesterol is significantly higher in vegetarians, in subjects with high polyphenols intake, and in subjects with high adherence to Mediterranean diet. Its absolute values are also significantly higher in vegetarians and in subjects with high adherence to Mediterranean diet.

Interestingly, 27-OHC plasma concentrations are increased in vegetarians despite the fact that these individuals have lower total cholesterol and lower LDL. In addition, in omnivores 27-OHC is inversely correlated with HDL levels as expected, being a compensatory mechanism for cholesterol elimination from tissues [41] when the reverse cholesterol mechanism is not sufficient, while in vegetarians 27-OHC is not correlated with HDL levels.

This findings could suggest that cholesterol turnover is enhanced in vegetarians. Clearance through 27-OHC could contribute to the decreased cholesterol levels observed in individuals consuming plats-rich diets, even if changes on HDL levels are not evident.

Our results on 27-OHC suggest a new possible mechanism through which plant-rich diets reduce total cholesterol and LDL levels: by means of mechanisms that enhance cholesterol turnover and removal from peripheral tissues.

Objective 3: Evaluation of the possible mechanism through which dietary micronutrients/diet can influence lipid levels, studying G*E interactions

We observed that SNPs in genes PCSK9, LPA and INSIG2 modulate the effect of overall diet (omnivore Vs vegetarian) on LDL levels.

PCSK 9 gene encodes for protein convertase subtilisin/kexin type 9, a protease that regulates cholesterol metabolism by promoting LDL-receptor degradation, thus rendering it unavailable and leading to elevated levels of circulating LDL. Overfunctional mutations (“gain of function mutations”), such as those reported in familial hypercholesterolemia are associated with high serum LDL. Several studies are currently studying PCSK9 as a potential candidate for cholesterol lowering therapies.
Serum PCSK9 concentrations appear to be influenced by dietary nutrients: both PUFA and MUFA-enriched diets decrease serum PCSK9 concentrations; increased dietary cholesterol downregulates hepatic PCSK9 mRNA expression; dietary fructose may increase the concentration of serum PCSK9 [62]. The following figure summarizes the findings related to the nutritional control of PCSK9.

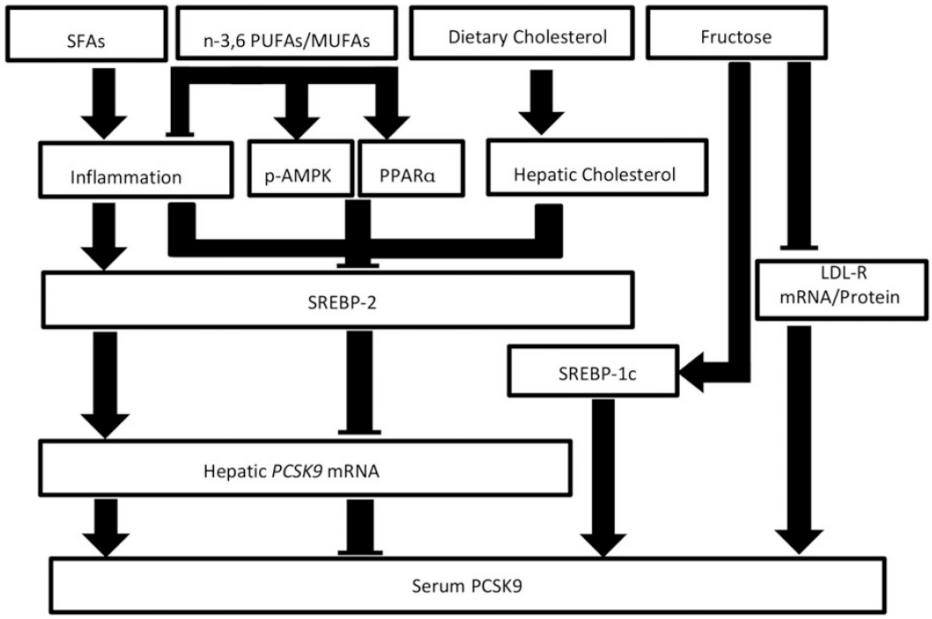


Figure 13: Mechanisms associated with nutrient regulation of hepatic and serum PCSK9 (From Krysa et al, 2017) [62]

PCSK9 showed a G*E interaction, with overall diet. The three SNPs that show gene level significant interaction are in LD and one of them (rs505151) is a strong candidate SNP for LDL, reported in several populations [71]. In our sample the variation in rs505151 was associated with increased LDL levels, as reported in previous studies ("gain of function mutation"). In our sample there are no homozygous individuals for the mutated genotype, in line with the low MAF of this SNP.

A recent study revealed signatures of positive selection in the gain of function mutation of rs505151. However the forces driving this selection remain unclear [85].

The effect of diet on LDL is modulated by rs505151, in particular vegetarian diet is beneficial for subjects homozygous for the wild type allele, but not for

those carrying the mutated allele. In fact, in individuals with the wild type genotype vegetarian diet lowers LDL compared to omnivores, whereas individuals carrying the mutated genotype behave in the opposite way, showing an increase of LDL residuals in vegetarians. Our observation could be associated with higher dietary fructose intake, in the vegetarians, which was previously associated with increased plasma PCSK9 concentrations and with higher cholesterol intake in omnivores which was associated with a downregulation of hepatic PCSK9 [62]. This G*E interaction could also be associated with an increased activity of PCSK9 in individuals that carry a mutation that induces a gain of function. However PCSK9 is regulated by several nutritional factors and it is not clear how they exert their functions or how they interact.

LPA showed to modify the effect of “overall” diet on LDL. In fact, LDL changes in relation to diet were very modest in individuals homozygous for the wild type genotype (similar LDL levels in the two dietary groups) while vegetarian diet exerted a marked beneficial effect in individuals carrying the mutated genotype (stronger in homozygous). However it is difficult to understand the biological plausibility behind this observation, since it was observed only in one SNP (rs9365200) (i.e. the only SNP reaching a gene level significance).

LPA gene encodes an apolipoprotein that is part of lipoprotein A (Lp(a)). Lp(a) is a plasma lipoprotein, synthesized by the liver, containing a LDL particle, one molecule of apolipoprotein B-100 and apolipoprotein (a). Elevated plasma levels of Lp(a) are independent risk factors for atherosclerosis and CVD. It is reported that approximately 30% of variance in Lp(a) levels is determined by the kringle IV type 2 (KIV-2) copy number variant in LPA, which encode variability in the size of apolipoprotein(a). The rs10455872 SNP in LPA was found associated with LDL response to statin

treatment in two GWAS [86-87]. The possible mechanism of action is not elucidated. The Omni Heart Trial studied the effects of three different "healthy" diets (i.e. a protein-rich diet, an unsaturated fat-rich diet and a carbohydrate-rich diet) on Lp (a) levels. All diets were reduced in saturated fat, cholesterol, and sodium, and rich in fruits, vegetables, fish, potassium, and other minerals. Compared with baseline, all "healthy" dietary interventions increased mean Lp(a) plasma levels. Among dietary interventions, the unsaturated fat-rich diet increased Lp(a) plasma levels less than the protein-rich diet and the carbohydrate-rich diet [88].

INSIG2 gene encodes the insulin-induced gene 2, a endoplasmic reticulum protein that binds to oxysterols. In 2006, a SNP in the promoter region of INSIG2 was associated to obesity [89], but only in 2013 it was associated with total cholesterol and LDL [58]. The association of INSIG2 with LDL was then confirmed by a fine-mapping study and by exome sequencing [90].
Binding oxysterols it inhibits the activity of sterol regulatory element binding proteins (SREBP), thus "*preventing the transcription of genes involved in both cholesterol uptake and synthesis. Additionally, INSIG2 binds HMG-CoA reductase, the rate-limiting enzyme in endogenous cholesterol synthesis, and targets HMG-CoA reductase for degradation, further slowing cholesterol synthesis"* [91].
In an observational study conducted on Samoans, studying G*E interactions, considering different type of diets classified as "modern" (western diet), "traditional 1" and "traditional 2", SNP rs9308762 showed an interaction with the type of diet, with homozygotes for the C allele resulting to be more susceptible to the increase in blood triglycerides associated to modern dietary pattern.
In a trial on obese children undergoing a 1 year intervention program which promoted healthy diet (plus other interventions), children homozygous for the

C allele in the rs7566605 SNP of INSIG2 lost significantly less weight than children carrying the G allele. Unfortunately the study does not report the effect of the intervention program on lipid profile, in relation to genotype [92]. In another study evaluating INSIG1 haplotypes and progression of atherosclerosis in women, no G*E association with diet was observed [93].

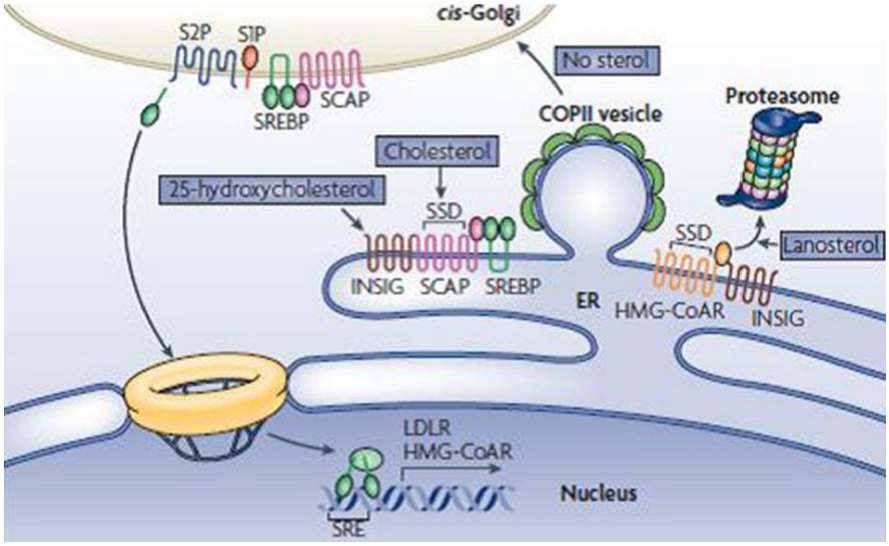


Figure 14: Schematic representation of the biological functions of INSIG2 (insulin-induced gene 2) in relation to cellular cholesterol metabolism. When there is abundant sterol in the endoplasmic reticulum (ER) INSIG undergoes conformational changes and prevents SREBP transport from ER to Golgi, which reduces the production of lipid in cells (From Kalatarian et al, 2014) [93].

The significant G*E interaction shown for INSIG2 gene is difficult to interpret since:

- a very limited number of individuals homozygous for the minor allele were available (2 in the omnivorous group; 1 in the vegetarian group);

- previous published nutrigenomic studies concentrated on BMI and triglycerides, as they were performed before that evidences on the association of INSIG2 with LDL and total cholesterol were available;
- two of the SNPs showing gene level significance, rs17047718 and rs17047731, were previously associated with glucose homeostasis traits, in the Hispanics of the Insulin Resistance Atherosclerosis Family Study (IRASFS) [94]. Reference LDL values for the different genotypes of the two significant SNPs are not published.

However it will be interesting to assess G*E interactions considering oxysterols (24-OH cholesterol, 25–OH cholesterol, 27-OH cholesterol) as phenotypes. In facts INSIGs are oxysterol-binding proteins and have been shown to play a role in the effect of oxysterols as feedback regulators of cholesterol homeostasis. In particular 25-OH cholesterol binds to INSIG2 and induces the binding of INSIG1 to SCAP, and blocking SREBPs [95].

Our result indicate that vegetarian diet does not show significant gene level interaction when considering **CYP7A1** and LDL. However we confirm the SNP level G*E significance in SNP rs3808607, which was reported in several studies.

CYP7A1 gene encodes cholesterol 7-alpha-hydroxylase associated to cholesterol excretion, regulating a rate limiting step in the bile acid synthesis, which is the primary mechanism for the removal of cholesterol from the body. Polymorphisms in the promoter of this gene are associated with defects in bile acid synthesis.

In the MONICA cohort, that considered as protective diet 8 years of low intake of red meat, animal fat and eggs, carriers of the SNP rs3808607-C allele showed a greater reduction in plasma total cholesterol levels than the AC and AA [96]. Several interventional trials confirmed these results. Abdullah *et al* [53] reports that: 1) in a study that employed a Mediterranean

style diet, carriers of the CYP7A1 SNP rs3808607 C allele showed greater reductions in plasma total cholesterol levels than the AA homozygotes; 2) in another trial with crossover design, SNP rs3808607 CC homozygotes showed higher serum total cholesterol and higher LDL levels after 3 weeks of a high-fat (40% energy) compared with a low-fat (22% energy) diet; 3) in a study considering 174 normolipidemic subjects, the presence of the C allele, when compared with the AA genotype, was associated with about 63% higher plasma total cholesterol concentrations, when the subjects were exposed to an increased intake of dietary cholesterol. Another randomized trial, investigating the impact of dairy products consumption on cholesterol levels, showed that, after the intervention, total cholesterol concentrations were higher among carriers of the CYP7A1 rs3808607 C allele, whereas LDL concentrations were higher among rs3808607 heterozygous in comparison to AA homozygotes [97].

It would be interesting to conduct further analysis considering oxysterols as phenotypes, as they are markers of cholesterol turnover, and are related to the activity of CYP7A1 in the synthesis of bile acids.

We identified 5 independent SNPs in **PON1 gene** (rs854549, rs854551, rs854552, rs854571, rs854572) showing a gene level significant interaction between the selected CVD risk factors and anthocyanins/polyphenols intake. PON1 encodes for paraoxonase 1 (PON1) enzyme, a glycoprotein which associates with HDL in the circulation. PON1 is a primary determinant of the antioxidant and anti-inflammatory activity of HDL, promoting the HDL-mediated macrophage cholesterol efflux, in the “Reverse Cholesterol transport”. PON1 activity is genetically regulated and different SNPs in PON1 gene were associated to the strength of arylesterase and paraoxanase

activities [98-99]. In addition several external factors are known to modulate PON1 activity and HDL (e.g. dietary factors, lifestyle, statins, etc.).

Without taking in consideration PON1 genetics we could not observe any association between high and low antioxidant intakes and target phenotypes, in line with previous interventional studies that showed discordant results on the effect of anthocyanins.

In individuals reporting high anthocyanins consumption, "*carriers of the C protective allele at rs854549 showed an increase in HDL levels of 4.7 mg/dl while carriers of the C protective allele at rs854552 showed an increase of 5.6 mg/ dl. In individuals reporting high polyphenols consumption, HDL levels were 3.92 mg/dl higher in T carriers for rs854571 and 3.94 mg/dl higher in C carriers for rs854572"*. […]. "*AIP was lower in A carriers for rs854551, with a decrease of 0.07 in high anthocyanins intak*e" [55].

Rs854549 is a 3′flanking variant that has been repeatedly reported as modulator of PON1 activities [100-101]. Rs854552 was reported as significantly associated to paraoxonase activity of PON1 [101].

Rs854571 and rs854572 map in the promoter region of PON1 gene and have been independently associated to a significant change in PON1 expression levels in human hepatoma cell line HepG2 [102]. In particular for rs854571 it was reported that T allele of rs854571 determined a significant increase in activity of PON1 [103].

Objective 4 - Development of tools for personalized nutrition in CVD prevention

A number of factors contribute to the inter-individual variability observed in responses to diet, including gender, age, metabolic status, physical activity, genetic background, microbiome as well as environmental exposures.

Some of these factors (e.g. age, gender, BMI) are routinely considered when a nutritionist develop personalised nutritional recommendations; however more complex factors such as genomics, microbiome and environmental exposures are not taken into account.

This simplistic approach is much more evident when considering CVD risk scores. It is amazing that despite the strong evidences available on the protective effects of “healthy diets” on CVD risk factors, available risk scores do not even take in consideration dietary behaviours.

As suggested by Lampe *et al*, the best way to guide personalized dietary interventions would be to develop a thorough understanding of the metabolic changes occurring in relation to dietary factors under different conditions, adopting a “multi-omics” approach [104].

Such degree of knowledge is not currently available, but the use of genetic tests to evaluate an individual response to a wide variety of nutrients is becoming more and more common, indicating that at least a health conscious niche of the population is interested on this approach.

The integration of personalized dietary suggestions, based on validated G*E interactions seems promising. In fact, the magnitude of the effects observed in G*diet interactions is much higher than those observed in genetic studies [53], indicating this approach could be useful for stratified prevention.

The DMS addressed these gaps and proposed additional features that make it suitable to be used as a tool in nutritional and nutrigenomic studies. It can be further implemented to support tailored prevention.

As previously discussed, there is urgent need of increasing the efficacy of existing prevention programmes, and several studied indicate that concerted actions (programmes targeting different contexts and using different tools) are more likely to be successful. mHealth could be one of the tools used in a stratified prevention programme, to engage patients. In this context a mHealth app that is used by the patient in agreement/communication with his/her healthcare provider seems an ideal solution. In fact, from one side data gathered by the patient can be transferred to electronic medical records and become available to the healthcare provider when needed, and from the other hand patients/citizens receive information validated by health professionals. The DSS, taking into account research data and updated guidelines, elaborates dietary and lifestyle suggestions by mean of inferential algorithms built to recreate the complexity behind the multiple effects and interactions between nutrients and cholesterol metabolism, considering also evidences from “omics”, when available. The choice of using a semi-automatic DSS increases the trust of healthcare providers and facilitate the adoption of the tool.

The mHealth solution will be further implemented to take into consideration user’s behavioral patterns and machine learning behavioral analysis. Moreover, it will guarantee a smart and quick data entry (e.g. photo capturing).

Strengths and Limitation of the study

The cross sectional study conducted in Milano, in the context of ATHENA project, offers the possibility of studying the effects of long term exposure to

vegetarian diet on a number of established risk factors of atherosclerosis, since the study population (healthy volunteers) involved both omnivores and vegetarians (i.e. lacto-ovo and vegans).

ATHENA has some intrinsic limitations which are characteristic of nutritional studies. In facts it is widely recognised that available methodologies cannot capture precisely the dietary intake.

On the other hand, the finding observed in this type of studies, conducted in an ecological context are not due by artefactual conditions, typically observed in intervention trials. As suggested by Azqueta et, "w*hen investigating the role of phytochemicals in normal human nutrition, the aim should always be to study concentrations close to those likely to be present in humans as a result of dietary intake"* [105].

Typically interventional trials use very high concentration of bioactive components. For example, studies used to define the efficacy of plant sterols interventions in reducing blood cholesterol concluded that 2mg/day of plan sterols are effective, even if this amount is more than 10 times higher than the amount normally introduced with the diet, implicitly paving the way for nutraceutical interventions.

Our healthy and relatively young population may not be appropriate to detect endpoints such as IMT, which indicates a subclinical atherosclerosis.

Nutrigenomic is a young discipline and our results, as most of the results published in this field, warrant validation.

6 CONCLUSIONS

Our study brings new evidences to elucidate possible molecular mechanisms underlying inter-individual variability in the susceptibility to diet induced changes in cholesterol levels and to elucidate effects of vegetable rich diets in modulating cholesterol homeostasis.

Our results on 27-OHC suggest a new possible role of plants-rich diets in reducing total cholesterol and LDL levels, through mechanisms that enhance cholesterol turnover and removal from peripheral tissues.

Results of PCSK9 interaction with vegetarian diet warrant replication, as one of the SNPs found to modulate the effects of diet on LDL levels, is a well-known “gain of function” mutation.

This study is the first exploring G*E interactions considering anthocyanins and polyphenols as “environment”. Different SNPs in PON1 were found to modulate the effects of anthocyanins/polyphenols on cholesterol levels. These results need validation in an independent sample.

In addition, in the context of ATHENA we implemented a platform for nutritional and nutrigenomic studies including a mHealth app to collect dietary and lifestyle data. The use of mHealth tools is synergic to the development of personalized/stratified nutrition interventions, since they facilitate dietary and lifestyle data collection. The use of a DSS that through machine learning and cognitive computing techniques process data and develop inferences is needed to understand the complex effects of dietary components on the homeostasis. Algorithms and risk prediction models can be refined periodically to include the best available research evidences thus fastening the introduction of personalized nutrition, which is a promising, but young field of investigation.

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SCIENTIFIC PRODUCTS

Papers

- Marceglia S, Conti C. A technology ecosystem for chronic pain: promises, challenges, and future research. Mhealth. 2017 Feb 21;3:6
- Rizzi F, Conti C, Dogliotti E, Terranegra A, Salvi E, Braga D, Ricca F, Lupoli S, Mingione A, Pivari F, Brasacchio C, Barcella M, Chittani M, D'Avila F, Turiel M, Lazzaroni M, Soldati L, Cusi D, Barlassina C. Interaction between polyphenols intake and PON1 gene variants on markers of cardiovascular disease: a nutrigenetic observational study. J Transl Med. 2016 Jun 23;14(1):186
- Aletti F, Conti C, Ferrario M, Ribas V, Bollen Pinto B, Herpain A, Post E, Romay Medina E, Barlassina C, de Oliveira E, Pastorelli R, Tedeschi G, Ristagno G, Taccone FS, Schmid-Schönbein GW, Ferrer R, De Backer D, Bendjelid K, Baselli G. ShockOmics: multiscale approach to the identification of molecular biomarkers in acute heart failure induced by shock. Scand J Trauma Resusc Emerg Med. 2016 Jan 28;24:9
- Conti C, Rossi E, Marceglia S, Tauro V, Rizzi F, Lazzaroni M, Barlassina C, Soldati L, Cusi D. An integrated Diet Monitoring Solution for nutrigenomic research. Stud Health Technol Inform. 2015;210:632-6

Oral presentations

- Interactions of dietary factors with candidate genes modify levels of cholesterol in healthy subjects – DISS Congress 2015 (13th November 2015)

- Oral Communication to the Division of Genomic Medicine\NIH Journal Club: “Diet Monitoring Solution for nutrigenomic research” (14th April 2015)

Poster

- Conti C, Rossi E, Marceglia S, Tauro V, Rizzi F, Lazzaroni M, Barlassina C, Soldati L and Cusi D. Towards Personalized Nutrition. AMIA (American Medical Informatics Association) Congress November 14-18 2015.

AKNOWLEDGEMENTS

I thank my tutor Cristina Barlassina for her support, for her suggestions and for her guidance. I also wish to thank my former tutor Daniele Cusi for his advices.

I thank Laura Soldati and her team for conducting dietary assessments, Valerio Leoni and Monica Lazzaroni for their collaboration in generating biochemical and non-cholesterol sterols data and Sara Marceglia for her genuine interest on the project and her collaboration on IT aspects.

A special thank also to the team of the “Genomic and bioinformatics platform” which always supported my activities during these years.

The research that produced the results presented in this thesis was founded through the ATHENA project, co-founded by the European Commission (GA 245121).