Advances in Understanding the Molecular Basis of the Mediterranean Diet Effect

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ABSTRACT

Increasingly, studies showing the protective effects of the Mediterranean diet (MedDiet) on different diseases (cardiovascular, diabetes, some cancers and even total mortality and aging indicators), are being published. The scientific evidence level for each outcome is variable and new studies are needed to better understand the molecular mechanisms whereby the MedDiet may exercise its effects. Here we will present recent advances in understanding the molecular basis of MedDiet effects, mainly focusing on cardiovascular diseases, but also discussing others. There is heterogeneity in defining the MedDiet, and it can, due to its complexity, be considered as an exposome with thousands of nutrients and phytochemicals. We will review MedDiet composition and assessment as well as the latest advances in the genomic, epigenomic (DNA-methylation, histone modifications, micro-RNAs and other emerging regulators), transcriptomic (selected genes and whole transcriptome) metabolomic and metagenomic aspects of the MedDiet effects (as a whole, and for its most typical food components). We will also present a critical review of the limitations of studies undertaken and propose new analyses and greater bioinformatic integration to better understand the most important molecular mechanisms whereby the MedDiet as a whole, or its main food components, may exercise their protective effects.

INTRODUCTION

In recent years the Mediterranean diet (MedDiet) has been consolidating as a healthy pattern of food consumption (Shen et al, 2015). Moreover, the MedDiet represents a sustainable dietary pattern, in which nutrition, food, environment, culture and sustainability all interact giving rise to a new model of sustainable diet that not only takes human health into account, but also the environment (Dernini and Berri, 2015; Dernini et al, 2017). Focusing here on human health, many observational epidemiological studies have shown that MedDiet adherence is associated mainly with a lower incidence of cardiovascular diseases (Gardener et al, 2011; Hoevenaar-Blom et al, 2012; Tognon et al, 2014; Tektonidis et al, 2015; Tong et al, 2016). There are also other observational studies that have found inverse relationships between greater MedDiet adherence and lower mortality due to cancer (Mitrou et al, 2007) as well as a lower cancer risk in different locations: gastric (Stojanovic et al, 2017), breast (van den Brandt et al, 2017) and colorectal (Fassanelli et al, 2017). However, the consistency of the results is much lower than for cardiovascular diseases and, therefore, the protective role of the MedDiet against cancer has not definitely been established (D'Alessandro et al, 2016).

Although intervention studies with the MedDiet are those that provide a higher level of evidence, it is expensive to undertake a long-term follow-up with a large sample size to detect incident events. The PREDIMED study (Estruch et al, 2013), an intervention trial with MedDiet compared to a control diet, has demonstrated that intervention with MedDiet reduces the incidence of cardiovascular diseases (Estruch et al, 2013), as well as the incidence of diabetes (Salas-Salvado et al, 2014), and breast cancer incidence in women (Toledo et al, 2015). In contrast to hard end-points (final disease phenotypes), for intermediate phenotypes such as inflammation markers, changes in blood pressure, changes in lipid concentrations, etc., there are more clinical trials (Davis et al, 2017; Rallidis et al, 2017). Recent meta-analyses, including both observational studies and clinical trials, have

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gathered the evidence on the favorable effects of the MedDiet on various intermediate and final phenotypes of disease (Bloomfield et al, 2016; Grosso et al, 2017). From an integrated point of view, the great advantage of the MedDiet is that its protective effects are observed for a large number of the most prevalent diseases, so that recommending it would favor the prevention of a wide range of pathologies. A recent meta-analysis (Dinu et al, 2017) used this integrated perspective and showed that a greater adherence to the MedDiet t is associated with greater protection against multiple health outcomes including overall mortality, diabetes, cardiovascular diseases, coronary heart disease, myocardial infarction, overall cancer incidence and neurodegenerative diseases. The authors included 13 meta-analyses of observational studies and 16 meta-analyses of randomized controlled trials, assessing 37 different health outcomes. Although most studies focus on negative health outcomes (disease), other researchers have also analyzed the effects of the MedDiet on the improving quality of life (Bonaccio et al, 2013), on cognitive function (Petersson and Philippou, 2016) and on parameters related with ageing (Bocardi et al, 2013).

Likewise, the concept of healthy ageing has been used. Although there is no single definition (Kiefte-de Jong et al, 2014; Assmann et al, 2016; Ma et al, 2017), healthy ageing has been defined as the absence of the most important chronic diseases (cardiovascular diseases, cancer and diabetes). In parallel, this absence of disease has also to be associated with good physical and mental condition, independence in instrumental activities of daily living, absence of depressive symptoms, good self-perceived health, good social functioning, and no function-limiting pain. The MedDiet has also shown its favorable effects on improving healthy aging (Assmann et al, 2016).

This epidemiological evidence on different health-disease phenotypes needs to be supported by studies that can go deeper into the mechanisms through which the MedDiet exercises its protective effects, whether specifically for each of the diseases, or generally through several shared common protective mechanisms.

From the outset we are faced with the difficulty of heterogeneity in defining the MedDiet, given that there may be important differences in the instruments used for measuring it and in the composition of the dietary interventions. Hence, in this review, we will begin with a more detailed definition of the MedDiet. Later, we will review the most recent studies on the molecular mechanisms through which the MedDiet may exercise its protective action, both in an overall way and through its most typical foods. We will analyze both cellular mechanisms and the advances made in the different omics (genomics, epigenomics, transcriptomics, metabolomics and metagenomics, among other) that are contributing to a better understanding of those mechanisms. Similarly, we will present the need to understand and integrate bioinformatics and computation in the assessment of the MedDiet effects.

WHAT THE MEDITERRANEAN DIET IS AND HOW TO MEASURE IT

Before going deeper into the basis of the mechanisms through which the MedDiet may exercise its effects, we must first pause to define the MedDiet, as that definition will largely condition the observed effects. Generally speaking, it can be stated that the MedDiet is the typical dietary pattern of the populations that live on the coast of the Mediterranean Sea. However, there are differences between the diets consumed in each of the counties on the Mediterranean coastline, the intake of virgin olive oil being the common nexus among all of them. Apart from olive oil, the MedDiet is very rich in fruit and vegetables in these countries, there being more differences in other foodstuffs. In general, the traditional MedDiet consumed in Greece and other countries in the Southern Europe, such as Spain is characterized by (Bach-Faig et al, 2011): a) high consumption of vegetables, fruits, cereals, legumes, nuts, and olive oil; b) moderate to high fish consumption; c) low consumption of red meats, and meat products; d) poultry and dairy products in moderate to small amounts; and e) moderate alcohol intake, habitually in the form of red wine. The inclusion of red wine

consumption (richer in phytochemical compounds than other alcoholic beverages) in the characteristics of the MedDiet pattern is the most debated point. That is because there is still a controversy about the favorable and unfavorable effects of alcohol consumption in general, or red wine in particular, on different disease phenotypes, even in in moderate quantities (Toma et al, 2017; Rehm et al, 2017). Moreover, the possible religious and social determinants that restrict alcohol consumption can be added to that (Bach-Faig 2011).

The degree of adherence to that definition of the MedDiet is easily measured in Mediterranean countries, as it is the typical dietary pattern that unfortunately people are moving away from. However, it is more difficult to measure pure adherence to this diet in non-Mediterranean countries, as some foods, for example the use of olive oil for cooking, has not formed part of their usual diet. Furthermore, the number and composition of the servings of some food groups (e.g. vegetables) and the way of cooking them may also vary widely between Mediterranean and non-Mediterranean countries (Hoffman and Gerber, 2013). Despite these limitations, certain tools have been developed for measuring the degree of adherence to the MedDiet adapted to each population.

Scales for assessing adherence to the MedDiet

In the epidemiological studies carried out on large populations, the most widespread way of measuring adherence has been to develop specific scales. These scales contain various questions on the intake frequency of the most typical foods/nutrients of the MedDiet, the results of which can be added up, in an additive way, to obtain a score that indicates higher or lower adherence. Outstanding among these is that proposed by Trichopoulou et al in 1995 (Trichopoulou et al, 1995), which was later updated by the same group (Trichopoulou et al, 2003). In this score, also called t-MED, nine components of the MedDiet are taken into account. Instead of establishing several fixed values of food/nutrient intake as cutoffs, what it does is to use sex-specific population medians as the respective cutoffs for each component.

Depending on the intake that each individual has, a value of 0 or 1 is assigned to each of the nine components. Six beneficial components are considered: high consumption of vegetables; fruits and nuts; legumes; unprocessed cereals; fish; and the high ratio of monounsaturated fatty acids (MUFA) to saturated fatty acids (SFA). In addition, there are two components that are considered harmful and are scored inversely (meat and meat products, also including dairy products with the exception of some cheeses). The last component is moderate alcohol intake which gives one point precisely for moderate alcohol consumption (different fixed values being considered for men and women), with a zero being awarded for both a lower and higher consumption (Trichopoulou et al, 2003). A score of nine points represents highest adherence to the MedDiet. This scale has, as its greatest limitation, the fact that it does not represent an independent instrument, administered by itself. Given that it is necessary to know the amount of MUFA and saturated fatty acids, as well as alcohol consumption, this instrument requires the prior administration of another type of food intake questionnaire on the complete diet, as well as its transformation into nutrients.

A couple of years later, the same group published another score called the modified MedDiet index (m-MED) (Trichopoulou et al. 2005). In this score, the MUFA/SFA ratio is replaced by the [MUFA+ polyunsaturated fatty acids (PUFA)]/SFA. Still later, the alternate MedDiet index (a-MED) (Fung et al, 2006) was proposed, which introduced new changes: The vegetable group excluded potatoes, separated fruit from nuts and considered whole grains products only. The groups of inverse scoring excluded dairy products and included red and processed meats only in the meat groups. With regard to alcohol, it modified the classification cutoff in g/d. Some years later, another modification was introduced, this score being called the relative MedDiet index (r-MED) (Buckland et al. 2010). The 9 components of the MedDiet were considered, but using intake tertiles and awarding 0, 1 or 2 points depending on the corresponding tertile. Several foods were also changed in the groups (for example in fruit, nuts and seeds were included, but fruit juices excluded), and the consumption of fresh

fish and seafood, as well as olive oil, were considered. Alcohol consumption was also considered as three groups. Apart from these variations to MedDiet scores, other many authors have proposed other scores, that we are not going to detail, both for adults (Knoops et al. 2004; Tognon et al, 2012; D'Alessandro et al, 2015) as well as for children and adolescents (Serra-Majem et al, 2004), among others.

What we wish to illustrate through all this is the difficulty of comparing effects between the different studies undertaken on different populations, using, besides, different instruments for measuring adherence to the MedDiet. This limitation in measuring instruments is also an important factor to be taken into account when it comes to trying to better understand the mechanism through which the MedDiet exercises its effects. What is more, the different above-mentioned scores, based on population data for classifying high or low food intakes instead of specific servings, have the added limitation that the quantity consumed may be very different between populations and a very small quantity of a food consumed in one population may receive a high score in another population if the mean intake in the second population is much smaller than that in the first. To resolve these problems of the same food quantity being considered high in one population and low intake in another, it is better to work with cut-offs based on specific food quantities rather than means or tertiles.

At this point, we should highlight the 14-point adherence to the MedDiet validated in the PREDIMED study (Schröder et al, 2011). The 14-item PREDIMED score consisted of 14 questions on food consumption frequency/habits. Each question was scored 0 or 1. Specific values of tablespoons, servings/day, pieces, or glasses of olive oil, vegetables, fruits, red meat or sausages; butter and other animal fats, sugar-sweetened beverages, red wine, pulses, commercial pastries and nuts ,were used as measurement units in order to establish a high or low intake fitted to the Mediterranean pattern. If the condition was not met, 0 points were recorded for the category. This score also includes the frequency of using so-called "sofrito" (sauté), which we can define as a traditional sauce of tomatoes, garlic, onion, or leeks sautéed

in olive oil. The final score ranged from 0 to 14. Another advantage of the PREDIMED score is that it is an independent instrument given that it is not necessary to evaluate total energy intake or nutrients in order to obtain a score of adherence to the MedDiet. The fact that it is easy to use and quick to administer has led to this scale being widely employed to the extent that validations of the same have been published in other countries (Hebestreit et al, 2017).

Interventions with MedDiet

These scales provide an operational means of measuring adherence to the MedDiet. However, in order to obtain a higher level of scientific evidence on the effects of the MedDiet, it is necessary to undertake experimental dietary intervention studies with the MedDiet [one group of participants has to be advised to follow (or administered) the MedDiet and another group not administered and told not to follow the MedDiet]. Here, we must have a very clear pattern of the food intake that forms part of the MedDiet so as to give advice which foods to consume (or provide them), both in quantity and frequency. These standardized interventions are essential for getting to know the molecular basis of the effects of the MedDiet. However, taking into account their complexity and cost, there are few studies that have used interventions with the whole MedDiet on humans. Most studies are short term trials (Estruch et al, 2006; Davis et al, 2017; Rallidis et al, 2017), so the effects analyzed are basically intermediate phenotypes (plasma lipid concentrations, inflammation markers, blood pressure, fasting glucose, weight loss, etc.) rather than final phenotypes of disease (myocardial infarction, stroke, dementia, cancer, etc.). One exception is the PREDIMED study, a long-term intervention trial with the MedDiets [one supplemented with extra virgin olive oil and the other supplemented with nuts (30g/d)] versus a control diet on primary prevention of cardiovascular diseases (Estruch et al, 2013). Likewise, the still ongoing CORDIOPREV study, also tests the intervention with MedDiet versus a low fat diet on the secondary prevention of cardiovascular diseases (Delgado-Lista et al, 2016).

Regarding mechanistic studies in animal models, there are several studies on mice and rats, testing the effect of specific Mediterranean foods/nutrients (Charles et al, 2014; Kalaiselvan et al, 2016; Osman et al, 2017; Rodriguez-Rodriguez et al, 2017) on several phenotypes. However, there are very few animal studies analyzing the MedDiet as a whole (Mizowaki et al, 2017; Kochmanski et al, 2017). In studies on animals we find the same problem as in humans: The definition of the composition of the MedDiet, as well as of the typical foods administered vary widely among studies and that leads to the conclusions varying too. As an example, we could mention the study of Mizowaki et al (2017), in which the effect of a traditional Japanese diet was compared with the effect of what was defined as a "a modern MedDiet" on the lipid metabolism. After administering each of the diets over four weeks, the authors concluded that the Japanese diet reduced the accumulation of lipids in the white adipose tissue and liver by suppressing fatty acid synthesis and promoting catabolism of fatty acids and cholesterol more than the MedDiet. However, after analyzing in detail the composition of the MedDiet administered, it can be observed that it does not match a MedDiet, so the authors have used another diet, not the Mediterranean one to make the comparison. What they considered to be the MedDiet included breakfasting on doughnuts with tea and lemon on one day, jelly toast, banana and caramel latte on another; and the following day pound cake, fruit (grape and apple) and coffee were administered. For lunch, the diet began with seafood pizza, soda pop, chocolate covered almond and affogato. Really, this would appear to be more of a Western diet than a MedDiet.

Typical foods of the MedDiet: extra virgin olive oil

Faced with the difficulty of measuring and administering a MedDiet well, many studies have focused on typical foods of the MedDiet, as this is easier to administer and study for specific effects. Nevertheless, this also has a limitation: one of the advantages that the MedDiet pattern is precisely recognized for having is that "the whole is greater than the sum

of its parts", i.e. separate food items do not have the same protective effect as when they are consumed with others because a synergy is created when consumed together that boosts their protective effects.

Although there are differences in the definition of the MedDiet, in general we can say that it is a diet very rich in foods of plant origin (vegetables, fruit, legumes, whole grain cereals, nuts and olive oil), to which can be added fish, mainly from the sea. Dairy product intake is low and mainly in the form of cheese and yoghurt, given that, apart from factors concerning the production and conservation of milk, in the Mediterranean there is a high prevalence of people who cannot digest lactose in milk (lactase non-persistence) during adulthood (Smith et al, 2016). Greater details on typical foods of the MedDiet can be found in other reviews (Hoffman et al, 2013; Shen et al, 2015). Although it is not the purpose of this review to enter into details on the typical foods of the MedDiet, we do consider it necessary to mention the most representative characteristic of that diet, which is olive oil.

Olive oil is used not only to dress salads and other foods, but also to cook with. The use of olive oil for cooking has beneficial effects over other foods, as it can increase the bioavailability of some nutrients [for example for the more polar phenolic compounds, among which is narigenin, the main polyphenol in tomatoes (Vallverdu-Queralt et al, 2014)], as well as reduce the formation of toxic compounds in high-temperature frying compared with other fats (Rangel-Zuñiga et al, 2016). Moreover, olive oil contributes to make other Mediterranean foods more attractive to the consumer because of improved texture and taste (Hoffman and Gerber, 2015). It is often said that the consumption of vegetables in the MedDiet could not be so high without the use of olive oil for increasing palatability.

Although up till now we have referred to olive oil without calling it virgin olive oil, it must be said that there are big differences between the commercial names of "olive oil" and "virgin olive oil". This difference, although well known in Mediterranean populations, is unclear in others. Virgin olive oil is that obtained from the fruit of the olive tree solely by

mechanical means and which has not undergone any treatment other than washing, decantation, centrifugation and filtration. Depending on the quality of the product achieved in the process, virgin olive oils are classified into: "Extra virgin olive oil" (having the best quality, expressed in objective terms of component analysis) and a "Virgin olive oil", when it does not reach the level of quality to be called "extra" (Aparicio-Soto et al, 2016). Virgin olive oil is *per se* considered as a functional food [as stated by the European Food Safety Authority (EFSA)] due to its content in healthy compounds (Parkinson and Cicerale, 2016). Its composition consists of major compounds (more than 98%, having a high content of MUFA) and minor compounds (about 2%), including more than 250 chemical compounds, among which are antioxidants). The main antioxidants of virgin olive oil comprise carotenes and unique bioactive phenolic compounds, mainly hydrophilic phenols, the composition of which is characteristic of virgin olive oil (Vitaglione et al, 2016). Outstanding among them are hydroxytyrosol (mainly present as a secoiridoid derivative) followed by tyrosol and its secoiridoid derivatives, such as oleuropein, etc. (Reboredo-Rodríguez et al, 2017; Collado-González et al, 2017).

However, when refining of olive oil is used to eliminate non-beneficial compounds that are generated during the production of low quality olive oil, the above mentioned healthy antioxidant compounds are eliminated. Non-virgin olive oil (refined) olive oil has practically no polyphenols or any other of the favorable compounds. This is hugely important because the effects on health will be different depending on whether extra virgin olive oil or simply olive oil is used in the interventions or in habitual intake.

GENERAL MECHANISMS THROUGH WHICH THE MEDDIET MAY EXERCISE ITS EFFECTS

Traditional experimental studies have given us insights into the effects of some of the components of the MedDiet [mainly MUFA, fiber, vitamins, minerals and phytochemicals (a

complex group of thousands of plant metabolites including: phenolic compounds, alkaloids, carotenoids, anthocyanins, glycosides, saponins, etc.] on cardiovascular risk reduction (Trichopoulou and Lagiou, 1997; Simopoulos 2001; Covas et al, 2015; Islam et al, 2016). The first mechanistic studies to explain the inverse relationship between MedDiet and cardiovascular risk focused on the high MUFA (and low SFA) composition of this diet (Sanders et al, 1994; Sacks and Katan, 2002; Covas et al, 2015). These studies also focused on the so-called traditional risk factors, outstanding among which are plasma lipid concentrations [reduction of atherogenic low-density lipoprotein cholesterol (LDL-C), and increases in the concentration of high-density lipoprotein cholesterol], blood pressure, glucose metabolism, decreased prothrombotic environment and endothelial protective capacity (Serra-Majem et al, 2006; Estruch and Salas-Salvado, 2013). More recently, a deeper understanding of these traditional mechanisms has been achieved. At the PREDIMED study, we have contributed interesting results on these mechanisms (Zamora-Ros et al, 20013; Perez-Heras et al, 2016; de la Torre et al, 2017; Hernaez et al, 2017). Our recent findings (Hernaez et al, 2017) that the MedDiet can improve the functioning of HDL particles on reverse cholesterol transport and increase cholesterol efflux capacity by decreasing cholesteryl ester transfer protein activity, so increasing HDL ability to esterify cholesterol, paraoxonase-1 arylesterase activity, and HDL vasodilatory capacity, are of particular interest. In addition to the lipid mechanism, the MedDiet produces other protective effects on cardiovascular diseases by reducing proinflammatory plasma biomarkers and several markers of oxidative stress, as well as providing protection against the vascular aging process (Marin et al, 2013; Ceriello et al, 2016; Fito et al, 2016). Thus, earlier PREDIMED results showed that MedDiet exerts an anti-inflammatory effect on cardiovascular system since it down-regulates cellular and circulating inflammatory biomarkers related to atherogenesis (i.e. serum C-reactive protein and endothelial and monocytary adhesion molecules and chemokines) (Estruch et al, 2006; Urpi-Sarda et al, 2012). Likewise, in PREDIMED participants, 1-year intervention with MedDiet

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was associated with a decrease in systolic and diastolic blood pressure, as well as with the concomitant increase in the total polyphenol excretion in urine and plasma nitric oxide production (Medina-Remon et al, 2015).

Although much progress has been made on research into the mechanisms involved in cardiovascular disease, we do not know the specific through which the MedDiet may exercise its specific protective effects on the different pathologies for which a protective association has been found. There is a wide consensus that it may do so through its modulation of a common underlying mechanism in cardiovascular diseases, diabetes, metabolic diseases, aging and cancer, namely chronic inflammation (Ostan et al, 2015; Welty et al, 2016; Eming et al, 2017). Chronic inflammation is defined by the persistence of inflammatory processes beyond their physiological function, resulting in tissue alteration that can lead to loss of function and organ failure (Eming et al, 2017). Cytokines are involved both in inflammation and anti-inflammation. Inteleukin-1 (IL-1), IL-2, IL-6, IL-12, IL-15, IL-18, IL-22, IL-23, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are considered as pro-inflammatory cytokines, whereas IL-1Ra, IL-4, IL-10, transforming growth factor (TGF)-β1 and lipoxin A4 are anti-inflammatory cytokines (Pirola L and Ferraz JC, 2017). In the PREDIMED study, we have found that after 3 and 5 years of intervention both MedDiet groups (one supplemented with extra virgin olive oil and the other with nuts) showed lower serum concentrations of IL-6 and IL-8 compared to baseline. Furthermore, the MeDiet+extra virgin olive oil group also had lower levels of IL-1β, IL-5, and TNF-α, IL-7, IL-12p70, and IFN-γ in both assessments. At 5 years, the MedDiet+nuts group showed an improvement in the levels of IL-1β, IL-5, and TNF-α, IL-7, IL-12p70, and IFN-γ. In addition, the control group showed an increase in concentrations of IL-7 and IL-8 at 5 years intervention (Casas et al, 2017). Imbalances between specialized proinflammatory mediators and proresolving mediators are the key factors in this process. However, mechanisms that drive this imbalance remain largely unknown (Fredman and Tabas, 2017). Moreover, inflammation is closely

connected to oxidative stress. Although reactive oxygen species (ROS) are continuously produced by the cells as a by-product of oxidative metabolism and are needed for several functions and signaling pathways, a high accumulation of ROS may result in oxidative damage to nucleic acids and other molecules, also contributing to the common mechanism of cardiovascular diseases, diabetes, other metabolic diseases, some cancers, and aging (Luo et al, 2017; Tapia-Vieyra et al, 2017), which, in turn, may be counteracted by the MedDiet (Chatzianagnostou et al, 2015).

Diet-induced modification of autophagy activity

Autophagy is an essential cell process that entails the degradation and recycling of cellular components in the lysosome (Mizushima et al, 2011). We will focus on macroautophagy (termed autophagy from here on), whose role in metabolic and cardiovascular pathology has been better characterized. Initially, autophagy was described as an adaptive response to nutrient deprivation that resulted in the 'in bulk" degradation and recycling of cytosolic components to supply the cell with needed macromolecules to survive starvation (Deretic et al, 2013). Diet can modulate autophagy, and some of the effects of dietary interventions, especially on health-span, have been suggested to relay on the activation of autophagy (Cuervo, 2008).

Studies designed to link any of the beneficial effects of the whole MedDiet to its possible modulation of autophagy are still lacking. However, polyphenols present in some of the staples of the MedDiet have been shown to have a direct effect on autophagy. Resveratrol, present in grapes, wine and some nuts, has been thoroughly characterized as an inducer of autophagy (Morselli et al, 2011). This polyphenol increases the deacetylase Sirtuin 1 activity, which through the regulation of the activity of several ATG proteins may explain the effects of resveratrol on autophagy (Knutson et al, 2008). Similarly, polyphenols present in virgin olive oil, such as oleuropein or oleocanthal, have also been shown to enhance autophagy

(Rigacci et al, 2015). It is tempting to speculate that the effects of the MedDiet on overall health span might be due to the ability of polyphenols or other components of this diet to activate autophagy. Indeed, autophagy malfunction has been proposed to contribute to the progression of pathologies that the MedDiet can help prevent. Autophagy is necessary for efficient cardiomyocyte development and function (Bravo-San Pedro et al, 2017). The relationship between autophagy and atherosclerosis and cardiovascular diseases has also been defined (De Meyer et al, 2015). Furthermore, autophagy also plays an essential role in controlling the inflammatory response of macrophages, possibly by limiting the activity of the inflammasome, and the generation of foam cells, likely through the modulation of lipid turnover (Razanni et al, 2012). However, not only has autophagy a protective role on cardiovascular disease but it has also been shown to regulate other age-associated pathologies, including dysregulation of metabolism and neurodegenerative disease (Madrigal-Matute and Cuervo, 2016; Menzies et al, 2017).Therefore, it is likely that the MedDiet may exert some of its beneficial effects, at least in part, through the regulation of autophagy. However, more research will be required to accurately determine the mechanisms that may account for a potentiating effect of the MedDiet on autophagy.

All the above-suggested mechanisms are only fragments of the whole picture, and they alone cannot explain the important effects of the MedDiet. Therefore, in addition to traditional research, new omics technologies must be incorporated to help us understand the mechanisms. In a recent review (Fito et al, 2016), we commented in detail on the main results obtained from the PREDIMED study on the use of omics together with traditional research, so we will not present detailed results here, but provide a general overview of the most important studies, as well as their limitations and future requirements.

OMICS IN THE MOLECULAR BASIS OF MEDDIET EFFECTS: PUTTING TOGETHER

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THE PIECES OF A MULTIDIMENSIONAL PUZZLE.

Elucidating the mechanisms of cardiovascular disease and of other complex diseases remains a major challenge due to multidimensional alterations at molecular, cellular, tissue, and organ levels. Recent advances in omics and bioinformatics are gradually being incorporated into nutritional studies in general (Nutritional Genomics) and into the MedDiet field, in particular, to help us better understand the basis of the molecular mechanisms and changing paradigms (Corella and Ordovas, 2014; Coughlin et al, 2014, Corella et al, 2017). High-throughput technologies enable omic studies to interrogate thousands to millions of markers using genomics, transcriptomics, epigenomics, metabolomics, proteomics, etc. However, a single layer of omics capture only a fraction of the molecular mechanisms. The integration of omics -omics data can yield more than the sum of the individual - better reflects the interactions that take place between the different molecules and cellular processes (Sun and Hu, 2016). However, this integration of omics is multidimensional and, although the computational techniques and bioinformatics have developed considerably, there are still methodological limitations to their full integration, so it remains an area of increasing development and research (Sun and Hu, 2016; Arneson et al, 2017). **Figure 1** represents the huge complexity of studying the molecular basis of the MedDiet effects. It is so as we are not dealing with a single exposition, but a complex set of foods, nutrients and phytochemicals in which each of them may act on the genomic, epigenomic, transcriptomic, proteomic, metagenomic level, etc. Apart from their separate effects, all these individual components can have synergetic effects at all the levels. We are, therefore, faced with an extremely complex and difficult to solve multidimensional puzzle that requires the help of bioinformatics of systems biology (Badimon et al, 2017). As the computational techniques that will provide an efficient integration of all this information are still being worked on, we will limit ourselves to presenting the main results that are being obtained in the different omic fields that have analyzed the effects of the MedDiet and/or their most important foods/nutrients on

intermediate and final phenotypes of cardiovascular disease and, in parallel, on aging.

An exposome called MedDiet

In integrating omics, not only do we have to consider the technologies of genomic, transcritomic, metabolomic, etc. analysis, but also consider MedDiet as a complex exposition with perhaps tens of thousands of constituent molecules coming from foods, ways of cooking, different quantities, synergetic effects, and additional modulations through other environmental effects such as food additives, pollutants, physical activity, tobacco, stress, hours of sleep, meal times, drugs, etc. To integrate omics well, therefore, the MedDiet must be considered as an "exposome" and new methodologies of integration and analysis have to be applied at this level (Van Breda et al, 2015; Patel, 2017).

Effects of MedDiet on Transcriptome

Transcriptomics allows us to analyze the effect of a specific food or diet on gene expression, and this leads to a better understanding of the mechanism of how foods affect specific gene expression (up-regulation or down-regulation). The effects of the MedDiet or its most representative foods on the transcriptome have been analyzed in humans, both for selected candidate genes and for the whole transcriptome (arrays containing all the genes). Due to the difficulty of undertaking studies on the MedDiet as a whole and also the lack of standardization in the composition of the MedDiet, there are very few studies at the whole transcriptome level that allow the results obtained to be compared and to check consistency. Additionally, the duration of the intervention, the characteristics of the participants included as well as the genes analyzed (selected genes or use of whole transcriptome arrays) have also been heterogeneous (van Dijk et al, 2012; Camargo et al, 2012; Castañer et al, 2013; Marlow et al, 2013). As main results we could mention those obtained in a sub-sample (n=34) of participants in the PREDIMED study, distributed randomly into three groups (MedDiet+extra

virgin olive oil; MedDiet+nuts and control diet). These diets were administered to each group over three months and three-month changes in whole genome peripheral blood mononuclear cells (PBMC) were assessed by using whole transcriptome microarray analyses (Castañer et al, 2013). We examined changes in cardiovascular system canonical pathways. Nine pathways were modified by the TMD+VOO and 4 pathways were modulated by the TMD+Nuts. Overall, we detected that key pathways in the physiopathology of cardiovascular risk, such as atherosclerosis, renin-angiotensin, nitric oxide and angiopoietin signaling, hypoxia and eNOS signaling pathways were modulated by MedDiet, adding evidence that one of the mechanisms by which the MedDiet can exert beneficial effects is by changes in gene expression related with cardiovascular diseases. Interestingly, we detected that the atherosclerosis signaling pathway was significantly downregulated after the MedDiet+virgin olive oil intervention. The main downregulated genes within the pathway were IL1β, IL1RN, TNF-α, and ICAM1. These results agree with those of previous studies where the effect of the MedDiet on candidate genes related with inflammation and oxidative stress was mainly examined (Camargo et al, 2012; Herrera-Marcos et al, 2017) and with studies where the effect of administering olive oil with different polyphenol content was being examined, either on selected gene expressions or using the whole transcriptome approach. All those studies on humans, as well as those undertaken in parallel on animals, have been analyzed in an extensive systematic review on the MedDiet and transcriptomics (Herrera-Marcos et al, 2017). The conclusion of this review is that there appears to be consistency in that the MedDiet and virgin olive oil are capable of producing changes in gene expressions related with inflammation and oxidative stress, although, due to the lack of MedDiet/food administered standardization, dosage, duration, type of cells analyzed, they recommend that new transcriptomic studies, with a more uniform methodology and greater sample size, be undertaken to increase statistical power.

MedDiet and epigenomics

Epigenomics describes a diversity of modifications to the genome that do not involve changes in DNA sequence and can result in alteration of gene expression (Corella et al, 2014). Various studied have reported epigenomic profiles associated with higher cardiovascular risk or aging (Keating et al, 2016; Pal and Tyler, 2016). The epigenetic marks are reversible, and may allow a quick adaptation to the exposome. There are three main categories of epigenetic biomarkers based on the epigenetic regulators involved: DNA methylation, histone modification, and non-coding RNAs. Unlike the genome, which is the same in all somatic cells, the epigenome is specific to each cellular type so adding even more complexity to the study and making the origin of the sample that has been taken for analysis very important. In general there are very few published studies that have investigated the influence of the MedDiet on the epigenome.

MedDiet, DNA-methylation and histone modifications

Despite the importance of discovering whether intervention with MedDiet can modify DNA methylation, especially on those methylation marks associated with higher cardiovascular risk or aging, results on humans for the overall intervention with MedDiet are still very scarce and undertaken with very small samples. There is, therefore, a need to increase them. In an initial study carried out on 36 participants, we investigated whether intervention over 5 years with MedDiet produced changes in the methyloma of peripheral blood cells (Arpon et al, 2017). We detected changes in the methylation of genes related with inflammation and immunocompetence (EEF2, COL18A1, IL4I1, LEPR, PLAGL1, IFRD1, MAPKAPK2, PPARGC1B). Although this was a very small sample study, the results are consistent with an influence of the MedDiet on inflammation also at the epigenetic regulation level. Other studies that have analyzed the influence of specific foods or groups of foods on methylation profiles (hypermethylation or hipomethylation) in humans have also found

several associations (Barrès and Zierath, 2016; Di Francesco et al, 2015), but the consistency among studies is very low and we need to continue investigating this mechanism in a more prolonged and dynamic way over time (considering both the complete MedDiet as an exposome and its specific foods) in different populations in order to better understand the modulations.

The modification of histones, although being quantitatively regarded as important in regulating expression (Nie et al, 2017), has been the least analyzed in epidemiological studies on humans. The main posttranslational modifications that can take place in histones include, among others: acetylation, phosphorylation, methylation or ubiquitinization. However, owing to their complexity, the effects of the whole MedDiet have not been examined in them. New studies are, therefore, required to discover the influence of the MedDiet or its main components on this possible regulating mechanism.

MedDiet and regulation by non-coding RNAs

There are a large number of non-coding RNAs whose function has been unknown for years, but which we are now beginning to understand (Huang and Zhang, 2014; Bayoumi et al, 2016; Rotini et al, 2017). They can be classified into short-non-coding RNAs (less than 200pb) and long-non-coding RNAs (more than 200 pb). MicroRNAs are the smallest in size (some 20-25 pb), and are the most studied. Recent evidence shows that there is also a complex interaction between microRNAs and long-non-coding RNAs. This is a complex process and is still not fully understood, but it is believed that long-non-coding RNAs exert "sponge-like" effects on some miRs, which, in turn, act by inhibiting the function of the microRNAs involved (Cora et al, 2017; Rotini et al, 2017). However, as this regulating function is very complicated and its modulation with diet also less well known, we shall briefly, for reasons of space, focus on microRNAs. The implication of microRNAs (mainly through negative regulation of target gene expression) in the regulation of different process

related with cardiovascular diseases (Ding et al, 2017; Viereck and Thum, 2017) and in general with aging has been widely described (Bu et al, 2017). The expression of miRNAs is tissue specific, but they may also pass into the circulation and so the profiles of circulating microRNAs associated with each disease can be analyzed (Huang, 2017). For example, the miR-1, miR-133a, miR-133b and miR-499-5p are high in plasma following an acute myocardial infarction (Paul et al, 2017). Although there are various studies on animal models showing that the components of the diet may have an influence on the microRNA expression and on their effects (Isac et al, 2017; Matboli et al, 2017; Zhao et al, 2017), studies on humans on the influence of dietary modulation in general on microRNA expression or profiles are still scarce (Desgagné et al 2016; Desgagné et al, 2017; Malcomson et al, 2017; Pan et al, 2017). This lack of studies is more notable when the MedDiet is taken as an exposome (Marques-Rocha et al, 2016; Piroddi et al, 2017) and, therefore, more studies are required to discover how the MedDiet may modify the microRNA profiles associated with the different pathologies. Nevertheless, just as with the definition of the MedDiet, research into MicroRNA regulation needs greater standardization of techniques and processes, as it is subject to very strong variations. Aside from these considerations, another aspect that has generated debate is whether the microRNAs contained in foods (Xi et al, 2016; Golan-Gerstl et al, 2017; Javet et al, 2017) can pass into the circulation of humans and so exert regulatory effects as in the case of vitamins, polyphenols, etc (Zhang et al, 2012; Jiang et al, 2012; Mico et al, 2016).

MedDiet and genomics

The study of the influence of variations in the genome, mainly of single nucleotide polymorphisms (SNPs) in the different intermediate and final phenotypes of cardiovascular and other diseases was the first omic to be developed. Firstly, the influence of SNPs on candidate genes in disease risk was researched, then, with the development of dense genotyping arrays, genome-wide association studies (GWAs) were undertaken and, more

recently, technology is allowing us to directly sequence DNA with de next generation

sequencing techniques (NGS). Even more recently, we have begun to study how the diet may

modulate the genetic risk of disease and our group was a pioneer in the development of

nutrigenomics, mainly in lipid metabolism (Corella and Ordovás, 2005). The first

nutrigenomic studies analyzed the effect of nutrients, fundamentally fatty acids on

cardiovascular risk (Corella and Ordovas, 2009). As the MedDiet concept became more

popular, nutrigenomic studies began to incorporate the MedDiet pattern as a whole, or its

most representative foods in order to analyze how diet interacts with variations in the genome

(individual SNPs or combinations of SNPs through so-called "genetic risk scores")

modulating the risk phenotypes. Recently, our group has published an extensive review on

genomic influence on the effects of the MedDiet (Fito et al, 2016), where greater detail on this

subject can be found. Here, for reasons of space, we are unable to give further details.

Likewise, in a recent review (Corella et al, 2017), our group explained the present situation of

nutrigenomics and the limitations that still exist to its application in so-called Precision

Nutrition within the framework of Precision Medicine. Currently, research into gene-diet

interactions provides us with a better understanding of the heterogeneity of responses to the

same dietary intervention. That is to say that we cannot observe the same effects of MedDiet

intervention or its most representative foods in all individuals. Some of the most relevant

genes for which we have found gene-diet interactions among their main SNPs and

intervention with MedDiet that determine intermediate and final phenotypes of cardiovascular

disease in the PREDIMED study are: TCF7L2 (Corella et al, 2013); MLXIPL (Ortega-Azorín

et al, 2014); LPL (Corella et al, 2014) y CLOCK (Corella et al, 2016), the latter adding

another piece to the puzzle, namely the influence of circadian rhythm on the effects of

MedDiet, including meal times, sleep habits, etc. (Brown, 2016).

Knowledge of inter-individual differences in the risk of disease has led us to

incorporate the study of the most relevant gene variants when we are analyzing the other

omics. So, for example, we know that genetic polymorphisms also have a great influence on DNA methylation (Corella et al, 2017), that there may exist genetic polymorphisms in the genes of microRNAs, which have an influence on their function. Likewise, there may be SNPs in the binding sites of a microRNA and its target RNA, and that have a great influence. An example of that are the results analyzing the gain-of-function microRNA-410 target site polymorphism (rs13702T>C) in the 3'untranslated region of LPL and the MedDiet in triglycerides concentrations and stroke risk, perhaps showing the presence of a gene-diet interaction in such a way that the favorable effects of the CC genotype were increasing when the MedDiet was administered, but got lost in a control diet (Corella et al, 2014).

MedDiet and telomere length

Telomeres are DNA-protein structures that form protective caps at the end of chromosomes. Leukocyte Telomere length (TL) has been associated with multiple diseases related with aging (Haussmann and Mauck, 2008). In general, a smaller TL is associated with faster aging and higher cardiovascular risk and metabolic diseases (Révész et al, 2014; Mazidi et al, 2017). Where there is a greater discrepancy is between TL and cancer, given that several studies have even reported an direct relationship. A recent meta-analysis (Zhang et al, 2017) concluded a greater TL is associated with a higher risk of lung cancer, and with other cancers, mainly in men. Although further studies are required, it seems clear that diet (Vidacek et al, 2017) has an influence on TL and this could be yet another of the mechanisms through which the MedDiet or its components exert their protective effects. Preliminary results in a sub-sample of the PREDIMED study showed that MedDiet does indeed appear to have a favourable effect on TL and that these results may also depend on genotype (García-Calzón et al, 2015). More longitudinal studies and of greater sample size are necessary in order to provide more evidence on the influence of the MedDiet on TL.

MedDiet and metagenomics

Gut microbiota is emerging as a pivotal player in the relationship between dietary habits and health (De Angelis et al, 2017). There are various studies that have analyzed the relationship between the MedDiet or its different components and the microbiota, both on the species level and on the level of analyzing its genetic material by means of metagenomic studies (Espin et al, 2017). Owing to space limitations, we shall not analyze in detail the work published, but, in general, the favorable effects of the MedDiet on the microbiota have been reported (De Filippis et al, 2016; Pastori et al, 2017; Espin et al, 2017), this emerging as one of the relevant mechanisms through which the MedDiet exerts its favorable effects on health, and is worthy of further studies. The added advantage is that there are metabolomic markers in the plasma or urine that indirectly the activity of the microbiota (De Angelis et al, 2017). The integration of metagenomics and metabolomics with exposomics and other omics, for example genomics, will without doubt provide very interesting results on the mechanisms of the MedDiet.

MedDiet and bioinformatics

Bioinformatics and computational methods are crucial for the present and future omic investigation into the effects of the MedDiet. In recent years, the high-throughput data generating methods, as well as the development of sophisticated bioinformatics tools, have allowed us to obtain a huge amount of knowledge and speed in analyzing data. Although, at present, information technology tools (network analyses, pathway analyses, etc.) are being applied separately for a single-omic level, the great complexity of the system (see Figure 1) and the availability of using various omics will generate data sets of greater complexity, requiring multi-omics mechanistic modelling approaches to capture enough of the complex situation at the systems biology level (Badimon et al, 2017). Although work is being carried out on the development of tools for integrating omics, among which we may mention "Mergeomics: a web server for identifying pathological pathways, networks, and key

regulators via multidimensional data integration" (Arneson et al, 2016), developments are limited in this incipient field and there is still much to do (Sun and Hu, 2016; Arneson et al, 2017). However, we shall soon witness important advances that will allow us to make progress on solving the multidimensional puzzle of the molecular bases of the MedDiet's effects.

FUTURES ISSUES

-To continue investigating the molecular mechanisms whereby the MedDiet exerts its protective effects (mainly through experimental studies), using a more homogeneous definition of the MedDiet, standardization of components, servings, etc. to better understand the effects.

-The MedDiet, as a complex exposome, appears to possess common protective mechanisms for various diseases by improving inflammation and oxidative stress. However, more research is required and we postulate that autophagy mechanisms may be also important in this sense.

-In the coming years omics technologies will be widely applied on the MedDiet-health field and better knowledge on the molecular mechanisms will be obtained.

-There is a need to undertake epigenomic studies in order to know how the MedDiet may modify methylation risk profiles and circulating microRNAs or other regulators and whether those effects are short, medium or long term.

-The study of the MedDiet-metagenome and the metabolomics related to the microbiome will be essential in the coming years.

-It will necessary to carry out more nutrigenomic studies in order to know the influence of the genetic variants (single and grouped into profiles) on the effects of the MedDiet to obtain

more information that may be applied to precision/nutrition.

-The use of omics technologies will generate a huge amount of data and require large resources and specific computational tools, as well as the development of bioinformatics methods to integrate omics information.

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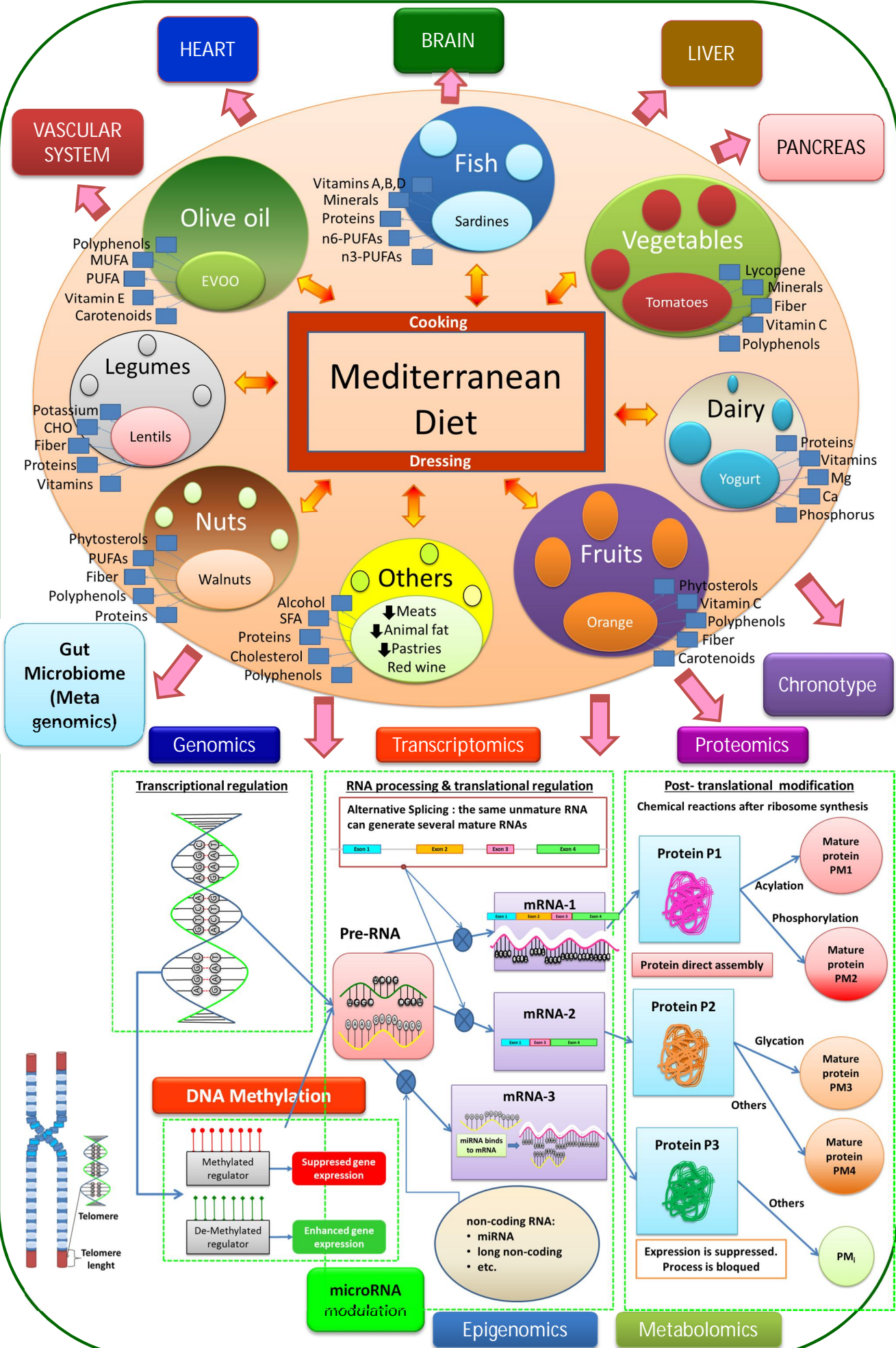
LEGEND TO FIGURE

Figure 1: Huge complexity in the study of the molecular bases of the MedDiet effects. A complex set of foods with different nutrients and phytochemicals in which each of them may act on the genomic, epigenomic, transcriptomic, proteomic, metagenomic level in different tissues, requiring a systems biology approach.