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Diet, genetic and epigenetic signatures in women of
childbearing age from a Mediterranean population:
perspectives for public health

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INTRODUCTION

1. Human nutrition: a global perspective

Diet constitutes one of the major environmental factors that exert a profound effect on many aspects of health and disease risk (Jiménez-Chillarón et al., 2012), playing an important role in the prevention and causation of multifactorial diseases, which account for more than half of the deaths worldwide, having a huge impact on national economies (World Health Organization, 2003).

The nutritional quality and quantity of foods, and therefore nutritional status, are major modifiable factors in promoting health and well-being, in preventing and treating diseases, as well as in improving quality of life.

It was only in the second half of the eighteenth century that nutrition started to experience its first renaissance with the observation that intakes of certain foods, later called nutrients, and eventually other substances not yet classified as nutrients, influence the function of the body, protect against diseases, restore health, and determine people's response to changes in the environment. It is now accepted that nutritional status influences health and risk of both infectious and non-communicable diseases (NCDs).

The NCDs that are related to diet and nutrient intakes are obesity, hypertension, atherosclerosis, ischemic heart disease, myocardial infarction, cerebrovascular disease, stroke, diabetes mellitus (type 2), and nutrition-induced cancers of the breast, colon, and stomach.

Together with tobacco use, alcohol abuse and physical inactivity, an unhealthy or inappropriate diet is an important modifiable risk factor for NCDs. There are solid evidences, coming from ecological, numerous epidemiological studies and interventions placebo-controlled trials that established the associations between diet and specific nutrient deficiencies and/or excesses with the development of NCDs, which may therefore be used in their prevention and treatment. Last, molecular and genetic research has elucidated many mechanisms through which diet and nutrients affect genetic mutation and expression, increasing our knowledge of how nutrition influences NCD development (Gilbeny et al., 2009).

This body of knowledge has led to several sets of international dietary recommendations and guidelines to reduce the burden of nutrition-related NCDs (i.e. guidelines from the WHO), to be used as the basis for the development of country-specific strategies and food-based guidelines for dietary prevention of NCDs.

On a genetic level it is now accepted that nutrients dictate phenotypic expression of an individual's genotype by influencing the processes of transcription, translation, or post-translational reactions. Nutrients can directly influence DNA expression and also the synthesis of structural and functional proteins.

Nutrients also act as substrates and cofactors in all of the metabolic reactions in cells necessary for the growth and maintenance of structure and function.

The study of human nutrition therefore seeks to understand the complexities of both social and biological factors on how individuals and populations maintain optimal function and health, how the quality, quantity and balance of the food supply are influenced and the way that diet affects health and well-being.

We now have the opportunity to obtain a much better understanding of how specific genes interact with nutritional intake and other lifestyle factors to influence gene expression in individual cells and tissues and this integrated approach has led to a better understanding of the relationship between nutrition and health.

At the population level, molecular epidemiology is opening up much more incisive approaches to understanding the role of particular dietary patterns in disease causation. The sequencing of the human genome has highlighted the narrower range of genes controlling human biology, emphasising the critically important role of the environment including diet in human health. Moreover, we now recognize the important role that diet plays in interacting with our genome both *in utero* and in the immediate period of postnatal development (Gilbeny et al., 2009).

1.2 Obesity

Obesity constitutes a major public health problem that, in current years, evolved into a worldwide epidemic (WHO, 2000; WHO, 2003). A recent study (von Ruesten et al., 2011) conducted in the Diogenes cohort (Diet, Obesity and Genes Dietary Study European), shows an increase in obesity prevalence since the 1990s and predicts a further increase in European populations of about 30% in 2015.

It is a multifactorial disorder, reflecting complex interactions of genes, environment and lifestyle (Newel et al., 2007), associated with a high risk of chronic diseases such as diabetes, cardiovascular disease and certain cancers (Couto et al., 2011).

A number of candidate genes have been implicated in the pathogenesis of obesity in humans and screenings of those candidate regions as well as genome-wide scans have helped to

identify Single Nucleotide Polymorphisms (SNPs) that increase the risk of overweight or of obesity (Peeters et al., 2009).

General recommendations for obesity related dietary factors are available, but these diseases affect individuals and at risk subsets (i.e., minorities, elderly) of the population differently. Some of this variability is explained by genetic variation, and in this regard the genetics of obesity, have been explored by several studies, but results are inconsistent and heritability was only partially explained. Part of that inconsistency and unexplained heritability could be attributable to complex gene-environment and particularly to gene-diet interactions (Corella D and Ordovas JM, 2009). While most environmental factors are discretionary and transitory (e.g. smoking and exercise), nourishment is a necessary, lifelong and universal environmental factor. Gene-diet interactions reflect the fact that genetic variations can predispose individuals to disease while diet can decrease or exacerbate this risk (Ordovás Muñoz JM).

Nutrigenetics is the emerging discipline studying the different physiological responses to diet depending on the genotype(s) of each individual. From a nutrition research standpoint, gene-diet interactions likely explain some of the inconsistencies of the diet-disease associations reported in different populations. From a genetic research standpoint, a meaningful gene-diet interaction can neutralize genetic effects, resulting in a null genetic effect. From a public health standpoint, it is critical to distinguish between genetic susceptibility, diet impact, gene-diet interactions, and to be able to quantify their relative importance as risk factors for morbidity and mortality in an aging population. The proportion of the excess incidence of disease risk that can be reduced by altering the environmental (i.e., dietary) agent can then be estimated and acted upon. Therefore, nutrigenetics could reveal risks and benefits of specific diets or dietary components to the individual and thus assist the development of personalized dietary recommendations instead of generalized ones. Nutrigenetics offers substantial and prudent direction in the translation of nutrition research into public health recommendations by contributing to the definition of optimal dietary and behavioral (i.e., physical activity and biorhythms) recommendations aimed at preventing disease and promoting optimal health and aging (Ordovás Muñoz JM, 2013).

In addition, other lifestyle factors also have an important role in modifying the onset, development and severity of obesity and obesity-related complications. The observed clinical and molecular heterogeneity in the obese phenotype that can vary both within and across populations stems from genetic, lifestyle and behavioral factors (Stryjecki and Mutch, 2011).

1.2.1 Relationship between inflammation, obesity and lipid metabolism

Obesity is characterized by the accumulation of lipid in white adipose tissue (AT). The AT is a specialized connective tissue composed of a number of different cell types each with specific functions. AT can be roughly divided into two fractions: the adipocyte fraction (AF) and the stroma vascular fraction (SVF). The AF is composed of mature adipocyte cells that are primarily involved in lipid storage, thereby having a major role in the regulation of whole-body energy homeostasis (Di Girolamo et al., 1998). In contrast to the cellular homogeneity of the AF, the SVF is composed of numerous cell types, including preadipocytes, mesenchymal stem cells, macrophages, endothelial cells and fibroblasts - all of which can play a role in regulating adipose tissue inflammation (Nair et al., 2005).

Indeed, obese AT was previously shown to be infiltrated by numerous types of inflammatory cells, and may promote the increase in extracellular components and fibrotic regions seen in adipose tissue biopsies from obese patients (Mutch et al., 2009; Divoux et al., 2010). Furthermore, it is now appreciated that AT is a metabolically active endocrine organ, in which both the AF and SVF secrete molecules, generically described as “adipokines” (Wang et al., 2008), influencing a wide range of biological functions, such as immunity, insulin sensitivity, inflammation, blood pressure, lipid metabolism, energy homeostasis and appetite (Trayhurn and Wood, 2004). Therefore, the identification of factors that regulate their secretion has become a primary interest in obesity research (Wang et al., 2008).

The macrophages present in the SVF are the primary source of obesity-induced inflammation (Gutierrez et al., 2009; Galic et al., 2010). The number of macrophages present in the SVF is directly correlated with the level of adiposity and adipocyte size (Xu et al., 2003). Adipocyte hypertrophy, results in increased chemokine secretion and subsequent increased infiltration of macrophages, which in turn secrete cytokines such as IL-6 and TNF α . Since AT expansion is characterized by increased macrophage infiltration, these cells are responsible for almost all TNF α and significant amounts of IL-6 secreted by AT (Weisberg et al., 2003).

Genetic sequence variants within the promoter region of inflammatory genes influence gene transcription, altering protein production. Functional polymorphisms have been reported in the *TNFA*, *IL-1*, *IL-6* and *lymphotoxin-a (LTA)* inflammatory genes, altering cytokine production (Burzotta et al., 2001; Santtila et al., 1998; Wilson et al., 1997).

Figure 1 illustrates the development of obesity-associated low-grade inflammation and the impact of diet–gene interactions on obesity and dyslipidemia.

1.3 Dietary fatty acids (FAs)

Dietary FAs have received considerable attention for their ability to regulate inflammatory gene expression and secretion. It has been proposed that dietary FAs affect inflammatory processes through the modulation of transcription factors such as kappa-light-chain-enhancer of activated B cells (NF κ B) and peroxisome proliferator-activated receptor gamma (PPAR γ) (Calder P.C 2003 and 2006). PPAR γ inhibits NF κ B, and both transcription factors are sensitive to dietary FAs (Van den Berghe et al., 2003).

Although the molecular mechanisms by which dietary fats regulate adipokine production remain unclear, one proposed link between dietary FA and inflammation may be via the toll-like receptor 4 (TLR4) pathway. The TLR4 pathway is expressed in both SAT and VAT, and has been shown to be activated by saturated fatty acids (SFAs), inducing inflammatory cytokine production and signaling (Fessler et al., 2009). This results in a localized inflammation in adipose tissue that propagates an overall systemic inflammation (Shi et al., 2006; Poulain-Godefroy et al., 2010).

The *n*-6 polyunsaturated fatty acid (PUFA), linoleic acid (LA), constitutes the majority of PUFA intake in the western diet. LA is the precursor of the *n*-6 PUFA arachidonic acid (AA). A high LA intake is considered proinflammatory, however the evidences are contradictory and not conclusive (Baum et al., 2012; Bjermo et al., 2012). AA intake in the diet is low relative to LA intake, its metabolic precursor. However, AA is the most prevalent *n*-6 PUFA in inflammatory cell membranes and is the substrate for the synthesis of the proinflammatory eicosanoids, including prostaglandin E2 (PGE) and 4-series leukotrienes, associated with inflammatory processes (Calder et al., 2011). Despite this, studies investigating the impact of AA on inflammatory markers are inconclusive, and few human intervention studies have reported on the effect of dietary intake of AA on low-grade inflammation (Thies et al., 2001a; Thies et al., 2001b).

The *n*-3 PUFA, alpha-linolenic acid (ALA) is an essential FA common in canola, soybean oil and some nuts, but in greatest concentrations in flax seed and flax seed oil (Erkkila et al., 2008). ALA is elongated and desaturated to eicosapentanoic acid (EPA) and further to decosahexanoic acid (DHA), however the efficiency of this conversion has been debated (Williams et al., 2006). Association studies between dietary intake of ALA and inflammatory markers suggest a modest anti-inflammatory effect of ALA (Stulnig T.M, 2003; Zhao et al., 2007).

The long chain *n*-3 PUFAs, EPA and DHA are found in seafood, especially oily fish and in some algal oils. It is proposed that *n*-3 PUFAs affect inflammation mainly through altered

eicosanoid production, but potentially also impacting cell signaling and gene expression (Calder P.C, 2011; Stulnig T.M, 2003). When EPA and DHA are incorporated into human inflammatory cells, this is partly at the expense of AA, providing less substrate for eicosanoid production (Calder P.C, 2011). Culture systems, animal models and human intervention studies have been generally consistent in recognizing the anti-inflammatory actions of *n*-3 PUFAs (Calder P.C, 2009; Itariu et al., 2012).

Emerging research shows that dietary FAs and SNPs in the genes encoding TNF- α , IL-6 and adiponectin can interact to regulate their production and secretion, thus adding an additional level of complexity in the study of obesity. Taken together, nutrigenomics research can provide a more thorough understanding of how changes in dietary FAs intake may contribute to the inter-individual variability in inflammatory status (Stryjecki and Mutch, 2011 ; Yu et al., 2011).

1.4 Tumor necrosis factor- α

TNF α is a pro-inflammatory cytokine whose expression and circulating levels are increased with obesity and decreased with weight loss (Maury and Brichard, 2010). TNF α acts in a paracrine manner, suggesting that circulating TNF α levels may not be indicative of actual TNF α levels (Suganami et al., 2005). The primary cell type responsible for the production of TNF α are macrophages in the SVF (Trayhurn and Wood, 2004). It is hypothesized that TNF α is produced by macrophages in response to chemoattractant signals released by dying adipocytes. TNF α has numerous effects in adipose tissue, including the regulation of apoptosis, adipogenesis, lipid metabolism and insulin signalling (Galic et al., 2010). The effects of TNF α on lipid metabolism occur in different cells, tissues, and organs and include a number of metabolic processes. TNF α induces lipolysis, increasing free fatty acid (FFA) production. In addition, TNF α regulates cholesterol metabolism and other adipocyte-derived adipokines such as leptin and adiponectin, which may also alter lipid metabolism (Chen et al., 2009).

Evidence suggests that TNF α triggers a signalling cascade that induces cell apoptosis, which may be one mechanism by which TNF α regulates adipose tissue mass (Cawthorn et al., 2007).

An increase in TNF α promotes the secretion of other pro-inflammatory cytokines and reduces anti-inflammatory cytokines, resulting in an overall pro-inflammatory state.

A study conducted by Wang and Trayhurn (2006) found that treating human adipocytes with TNF α for 24 h led to significant decreases in adiponectin expression and increases in IL-6

and TNF α expression, showing that TNF α is a powerful regulator of inflammatory molecules, favoring an overall inflammatory state (Wang and Trayhurn, 2006).

1.4.1 *TNFA* gene variants, obesity, serum lipids and TNF α signaling

Recent evidence suggests that individual SNPs in the genes encoding TNF α , TNF α receptor 2 and TNF α converting enzyme can modify an individual's risk for obesity and obesity-related complications.

One aspect that has not yet been studied is whether a haplotype consisting of SNPs in the three aforementioned genes will have an additive or synergistic effect on these complications.

Several SNPs have been identified in the promoter region of the *TNFA* gene, however the *TNFA* -308 G>A (rs1800629) and -238 G>A (rs361525) SNPs are most commonly associated with measures of adiposity, obesity risk and serum lipids. The A allele of the functional -308 G>A SNP results in a 2-fold increase in *TNFA* transcription, with a subsequent increase in TNF α production (Wilson et al., 1997).

Many studies have reported that carriers of the pro-inflammatory -308 A allele (AA and GA genotypes) reported a higher body mass index (BMI) and/or percent body fat than those with the GG genotype (Brand et al., 2001; Nieters et al., 2002; Pihlajamaki et al., 2003). In two large recent meta-analyses it was shown that the -308 GA + AA genotypes were associated with an increased risk of obesity (Yu et al., 2011; Sookoian et al., 2005).

In comparison to the -308 G>A SNP, only few studies have investigated the association between the -238 G>A SNP and obesity, reporting an association with body fat, insulin resistance and circulating free FA (Fontaine-Bisson et al., 2007). Joffe and colleagues (2012) found that black South African women with the -238 A allele had greater body fat % than those with the GG genotype.

However, not all studies have shown the interaction between the -308 G > A and -238 G > A SNPs and obesity (Joffe et al., 2010; Joffe et al., 2011; Hedayati et al., 2011).

Only two papers have found an independent association between the -308 G > A SNP and serum lipid concentrations. In Caucasian men, the -308 A allele was associated with increased triglycerides (Dalziel et al., 2002), and in Polish Caucasian men and women the AA genotype was associated with lower high-density lipoprotein cholesterol (HDL-C) concentrations compared to the GG genotype (Wybranska et al., 2003).

Further, no independent associations have been reported between the -238 G > A SNP and serum lipid concentrations. While the A allele of the -308 G > A and -238 G > A SNPs

appear to be associated with the obese phenotype and serum lipid concentrations, it is highly likely that genetic variation in the *TNFA* gene may provide only a partial explanation with regards the inter-individual variability observed and the heterogeneity of the results in these studies. Other variables such as ethnicity, gender, diet, lifestyle and environmental factors may modulate these associations and contribute to the different results observed.

Although the mechanisms by which these promoter variants affect circulating TNF α levels remain unclear, their significant association with obesity and insulin levels reinforce the importance of conducting further studies (Joffe et al., 2013).

1.4.2 *TNFA* and dietary FAs

The *TNFA* -308 G > A and -238 G > A SNPs have been shown to modulate the relationship between dietary fat intake on obesity and serum lipid profiles in different populations.

Several studies have investigated the effect of dietary FAs on TNF α concentrations and *TNFA* gene expression in cell, animal and human models. Lifestyle factors, including dietary components, such as FAs, interact with genetic variants to regulate the development and progression of obesity and its comorbidities and these interactions may explain the differences observed across populations (Joffe et al., 2013).

The A allele of -308 G > A SNP has been associated with obesity, obesity-related insulin resistance, and altered serum lipid concentrations in some Caucasian populations (Fontaine-Bisson et al., 2007; Brand et al., 2001), but not all (Um et al., 2003; Sookoian et al., 2005). In addition, in some populations the *TNFA* -308 G>A polymorphism changes the relationship between fatty acids (FA) intake and the risk of obesity (Stryjecki and Mutch, 2011).

Indeed, palmitic acid (saturated FA) was found to increase *TNFA* gene expression and protein secretion in a dose-dependent manner. In contrast, incubation with the *n*-9 monounsaturated fatty acid (MUFA) oleic acid (C18:1) and *n*-3 PUFA DHA had no effect on TNF α expression or secretion (Bradley et al., 2008).

In rodent studies, mice that consumed a high fat diet for 5 weeks showed increases in TNF α expression; however, the concomitant administration of EPA prevented this increase (Perez-Matute et al., 2007). In another study, mice fed menhaden fish oil showed a reduced expression of TNF α in kidneys (Chandrasekar and Fernandes, 1994). When considered altogether, these studies indicate that SFA increase and *n*-3 PUFA decrease TNF α expression and/or secretion.

Importantly, these murine results appear to translate to human beings. Subjects consuming a fish oil supplement showed a significant decrease in TNF α production; however, levels of TNF α returned to baseline upon cessation of the supplements (Endres et al., 1989). This was confirmed in another study where subjects who ingested ALA for 4 weeks had a 30% reduction in TNF α production. When these subjects were further supplemented with fish oil, TNF α synthesis was inhibited by up to 70% (Caughey et al., 1996). Finally, men who consumed fish oil supplements for 12 weeks showed differential decreases in TNF α production in accordance with their pre-supplementation levels (Grimble et al., 2002). In addition to the independent influences of genetic variation and different FA on the TNF α signalling pathway, research discovering diet–gene interactions offers a further explanation for the inter-individual variability observed with obesity-related inflammatory status. Grimble et al. (2002) found that men with one A allele at the -308 SNP and who were in the highest tertile for TNF α production had the most significant reduction in TNF α levels after supplementation with fish oil in comparison to homozygotic G/G men. No conclusions could be made about -308A/A individuals owing to the small sample size.

In another study by Fontaine-Bisson and El-Sohehy (2008), a low *n*-6 PUFA intake in subjects who were homozygous for G at the -238 SNP and had at least one A (that is, GA or AA) at the -308 SNP had an increased risk of developing obesity; however, Nieters et al. (2002) found that German Caucasian men and women with the –308 A allele, who were in the highest tertile for intake of the *n*-6 PUFAs LA and AA (%E), had an increased risk of developing obesity. More recently, it has been reported that the odds of obesity for black South African women with the –308 A allele increased with total dietary fat intake (%E) (Joffe et al., 2011). This interaction was not observed in white South African women (Joffe et al., 2012).

Considered together, these newly discovered diet-gene interactions that affect TNF α signalling are an important point to consider in future intervention studies targeting obesity-related inflammation.

1.5 Mediterranean Diet

Since the early 1990s, growing evidence indicates that the Mediterranean diet (MD), a concept first proposed by Keys in the mid-1980s (Keys et al, 1986), has a beneficial influence on health, preventing NCDs, and longevity (Trichopoulou et al, 1995, 2004, 2005, Sofi et al, 2008, 2010).

The Mediterranean-style diet is not a specific diet, but rather a collection of eating habits traditionally followed by people in the different countries bordering the Mediterranean Sea. The diet refers to a dietary profile commonly available in the early 1960s in the Mediterranean regions and characterized by a high consumption of fruit, vegetables, legumes, and complex carbohydrates, with a moderate consumption of fish, and the consumption of olive oil as the main source of fats and a low-to-moderate amount of red wine during meals.

A great deal of attention has been given to tools that estimate the adherence of individuals to the MD because of the usefulness of these tools to identify the whole dietary pattern instead of single foods or nutrients (Bach et al., 2006). Hence, computational scores have been created and used in several large epidemiologic studies to seek whether they could be useful to estimate the risk of disease in the general population (Kourlaba and Panagiotakos, 2009). Some large epidemiologic studies conducted in different cohorts evidenced an association between a greater adherence to MD, a reduced risk of mortality, and the incidence of major chronic diseases (Mitrou et al., 2007). In 2008, a meta-analysis performed by Sofi et al, that included all cohort prospective studies that investigated this issue, observed that a 2-point increase of adherence to MD conferred a significant protection against mortality, the occurrence of cardiovascular diseases, and major chronic degenerative diseases (Sofi et al., 2008).

Despite the widely proven benefits of the MD the Southern European countries in which MD originated are rapidly withdrawing from this eating pattern orienting their food choices towards products typical of the Western diet which is rich in refined grains, saturated fats, sugars, red and processed meat (Laccetti et al., 2012).

The reasons why people keep on drifting from one dietary regimen to another remain open to several hypotheses (Bonaccio et al., 2012).

Social changes appear to have contributed to radical reversal in dietary habits in Western and Southern European societies although developing countries are slightly turning to westernised diets as well (Prentice, 2006).

The cost of MD seem to have led people to give up this eating pattern in favour of less-expensive products which allow to save money, but are definitively unhealthy (Lopez et al., 2009). Many studies suggest that diet quality follows a socioeconomic gradient highlighting how disadvantaged people present higher rates of obesity, diabetes, CVD and some types of cancer (Darmon and Drewnowski, 2008).

The abandoning of MD is also considered as a possible cause of the increasing obesity pandemic (Esposito et al., 2011). Several studies took a step forward to see whether there is an association between diet cost and obesity, and found that a higher adherence to healthy dietary patterns is linked to higher monetary costs and is inversely associated with BMI and obesity (Schröder et al., 2006).

In 2010 UNESCO has recognized the MD as an Intangible Cultural Heritage of Humanity (UNESCO, 2010). A number of epidemiological studies have shown that greater adherence to the traditional MD, is associated with a significant reduction in total mortality, and particularly of death due to coronary heart disease and to cancer (Trichopoulou et al., 2003), could reduce overall cancer risk (Couto et al., 2011), and provide a consistent protection for the occurrence of major chronic degenerative diseases (Sofi et al., 2010).

1.5.1 Obesity and Mediterranean diet (MD)

The health consequences of obesity in developed countries represent an economic load of between 2 and 7% of the total health cost, a substantial proportion of the national health cost. Obesity is not a single disorder, but a complex multifactorial disease involving environmental and genetic factors. Among the environmental factors, diet appears to be an important contributor to the development of obesity. Epidemiologic evidence on the association of nutrients, particularly fat, with obesity remains controversial. Because of several short-comings in traditional, nutrient-based diet and disease analysis, the focus has shifted from this type of analysis to one describing food intake patterns. Such analysis takes into account the complex combination of foods in a diet. The effect of such food-based defined dietary patterns might be more closely related to obesity than a single nutrient or food. Hence, food-based dietary patterns may be more useful than nutrient based methods for dietary counseling and in public health efforts (Schröder et al., 2004).

Research interest over the past years has been focused on estimating adherence to the whole MD rather than analyzing the individual components of the dietary pattern in order to consider important interactions between components of the diet.

Several epidemiologic studies examined the association of dietary patterns and excessive weight (Togo et al., 2001; Newby et al., 2003). Identifying palatable dietary patterns that prevent weight gain is an important task for health policy in view of the social and economic burden of obesity.

The MD is an eating pattern that successfully combines pleasant taste with positive health effects. The MD does not stand for a homogeneous exclusive model throughout the

Mediterranean basin; rather, it represents a set of healthy dietary habits including high consumption of vegetables and fresh fruits, with olive oil as the main source of fat. However, whether adherence to this healthy dietary pattern might be protective against weight gain remains unclear (Schröder et al., 2004).

Furthermore, independently of energy and macronutrient quantity intakes, a better adherence to the MD, is associated with lower obesity risk (Mozaffarian et al., 2011; Martínez-González et al., 2012).

1.6 Role of folate in 1-carbon metabolism

Folate is an essential water-soluble vitamin occurring naturally in select foods as well as in the synthetic form (folic acid) used in supplements and in food fortification programs (Crider et al., 2012). According to chemical nomenclature, the difference between folate and folic acid is just one proton. However, the term folic acid is in general applied to the synthetic form of this B-vitamin, which is also the most stable form (Blom et al. 2006; Pitkin RM 2007). There are many critical cellular pathways dependent on folate as a 1-carbon source including DNA, RNA, and protein methylation as well as DNA synthesis and maintenance. Folate can be a limiting factor in all these reactions (figure 2).

Under normal dietary conditions, absorbed folate is metabolized to 5-methyltetrahydrofolate (5-methylTHF) in the intestine and/or liver. 5-MethylTHF is the primary folate constituent taken up by nonhepatic tissues, which then must be polyglutamated for cellular retention and 1 carbon cycle coenzyme function.

Tetrahydrofolate (THF) is the most effective substrate for polyglutamate synthetase; therefore, 5-methylTHF must be converted to THF via the methionine synthase reaction. When folic acid is consumed in fortified foods or supplements, it is metabolized primarily to 5-methylTHF during intestinal absorption and/or first pass in the liver, after which it behaves identically to natural dietary folate. Folic acid is normally first reduced to dihydrofolate by dihydrofolate reductase and subsequently to THF to enter the folate pool. Once the THF coenzyme is formed from either folic acid or dietary folate, it is first converted to 5,10-methyleneTHF by the vitamin B-6–dependent enzyme serine hydroxymethyltransferase and subsequently irreversibly reduced to 5-methylTHF by 5,10 methylenetetrahydrofolate reductase (MTHFR). This reaction is key to maintaining the flux of methyl groups for the remethylation of homocysteine (Hcy) to methionine via the vitamin B-12–dependent methionine synthase reaction. Methionine is the substrate for SAM or AdoMet, a cofactor and methyl group donor for numerous methylation reactions including the methylation of

DNA, RNA, neurotransmitters, and other small molecules, phospholipids, and proteins, including histones (Stover PJ, 2009). A number of SAM-dependent reactions have regulatory roles by affecting both genome stability and gene transcription, localization of protein, and small molecule degradation (Winter-Vann et al., 2003; Stead et al., 2004).

In addition to folate, a number of other dietary nutrients are required to maintain 1 carbon flux, ensuring normal Hcy remethylation, SAM formation, and DNA methylation. These nutrients include vitamin B-6, riboflavin, vitamin B-12 and choline (Shin et al., 2010). The 1-carbon pathway, and thus DNA methylation, functions under tight regulatory controls. SAM is the major regulator of folate-dependent Hcy remethylation because it is a potent inhibitor of MTHFR. Under the condition of high SAM concentration, MTHFR is inhibited, which reduces the synthesis of 5-methylTHF and hence remethylation of Hcy. Conversely, when SAM concentrations are low, remethylation of Hcy is favored (Cridler et al., 2012).

1.6.1 MTHFR polymorphisms

MTHFR activity and thus 5-methylTHF formation may be modified by several SNPs. The *MTHFR* gene maps to chromosome 1p36.3, and plays a central role in folate metabolism, together with other enzymes by irreversibly catalyzing the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulating form of folate and a cosubstrate for Hcy methylation to methionine (Goyette et al., 1994). Many rare mutations of the *MTHFR* gene have been described in individuals, resulting in very low enzymatic activity, whereas the most common polymorphism is a C to T mutation in exon 4 at nucleotide 677, leading to Ala222Val and presenting in healthy individuals with lower enzyme activity (Frosst et al., 1995).

Individuals with the *MTHFR* 677TT genotype have been shown to have only 30% of in vitro MTHFR enzyme activity, which reduces plasma folate levels and elevates plasma Hcy levels, compared with the wild type, whereas those with the heterozygous CT genotype have 60% of wild-type MTHFR enzyme activity (Frosst et al., 1995). Reduction of the MTHFR enzyme activity can increase the pool of 5, 10-methylene-THF at the expense of the pool of 5-methyl-THF and impair the DNA methylation (Guo, 2012).

Because the DNA methylation plays a critical role in regulation of gene expression and maintenance of genomic stability, the aberrations in normal methylation patterns have been associated with the development of cancer by impairing the DNA methylation (Cheng et al., 1997). More importantly, the homozygous variant genotype *MTHFR* 677TT has been

associated with risk for many different types of cancer, including colorectal cancer, gastric cancer and breast cancer (Taioli et al., 2009; Dong et al., 2010; Zacho et al., 2011).

1.6.2 Folate and DNA stability

Biomarkers that are influenced by nutritional factors offer a potentially important contribution to public health programs because of their direct relevance to explain the effects of diet on the risk of disease, and their potential role in monitoring the effectiveness of preventive programs (Boffetta P. 2010). Biomarkers of early events should be related to, and ideally critical for, the development of the adverse effect that is the basis for the risk assessment (Renwick et al., 2003, Renwick and Walton, 2001).

Low folate status (as defined by various measures including blood folate concentrations, folate and/or folic acid intake) has been associated with an increased risk of cardiovascular disease, multiple cancers, and neural tube defects (NTDs) (Erickson JD, 2002; Lamprecht and Lipkin, 2003).

The mechanisms by which low folate status contributes to these disorders remain unclear. During DNA replication, folate depletion can have destabilizing consequences. Inadequate folate availability during cell division can result in the compromised production of thymidine, such that uracil may be substituted in the DNA sequence. This mutagenic event may trigger attempts to repair the defect and increases the frequency of chromosomal breaks. In tissue cultures, it has been demonstrated that low folic acid results in the formation of micronuclei (indicative of chromosome breakage) and that the *MTHFR* TT genotype leads to increased micronuclei formation under low folate conditions (Kimura et al., 2004; Crider et al., 2012).

Mitochondrial DNA (mtDNA) is more susceptible to oxidative damage than nuclear DNA since it is not protected by histones and it reveals limited capacity for DNA repair (Yakes and Van Houten, 1997). Alterations in mtDNA both qualitatively (mutations) and quantitatively (mtDNA copy number) have been associated with many human diseases including neurodegenerative diseases, metabolic diseases and various types of cancer (Chen et al., 2011; Nie et al., 2013). Among several mutations that have been reported to be associated with various diseases, a 4977-bp deletion, occurring between two 13-bp direct repeats at positions 13447-13459 and 8470-8482, has attracted great interests since it has also been shown to accumulate in many tissues during aging and it has been used as a mtDNA damage biomarker (Meissner et al., 2008). The mtDNA 4977-bp deletion has been detected in fast replicating cells such as blood leukocytes (Ross et al., 2002; von Wurmb-

Schwark et al., 2010), but little is known about the relationship between this mutation and folate status (Chou and Huang, 2009).

In an experimental study in rodents, a folate-dependent accumulation of mtDNA deletions in lymphocytes has been reported after folate deprivation, suggesting that accumulated lymphocytic mtDNA deletions may serve as a molecular biomarker responding to dietary folate deprivation (Chou and Huang, 2009). Furthermore, mtDNA deletions and low folate status, proposed characteristics of carcinogenesis, have been suggested in relation to human cancer susceptibility, in a case-control study, an increased frequency of lymphocytic mtDNA 4977-bp deletion has been associated with human hepatocellular carcinoma risk; moreover, in the same study, a high frequency of lymphocytic mtDNA 4977-bp deletion was associated with low levels of lymphocytic folate (Wu et al., 2009).

As suggested from experimental studies in rodents (Chou and Huang, 2009), it has been hypothesised that folate intake and folate status may have an impact on lymphocytic mtDNA 4977-bp deletion levels in healthy humans and thus on mitochondrial genomic instability.

1.6.3 Folate and women's health

The effect of folate status on pregnancy outcomes has long been recognized. Folate is now viewed not only simply as a nutrient needed to prevent megaloblastic anemia in pregnancy but also as a vitamin essential for reproductive health, disease prevention, and health maintenance (Tamura and Picciano, 2006). Adequate maternal nutrition during the periconceptual period as well as in pregnancy are key focus of attention in public health because of the increased needs and greater vulnerability of pregnant women to the effects of micronutrient deficiency or imbalance (Ortiz-Andrellucchi et al., 2009).

Since NTDs are caused by the failure of fusion of the neural tube 22–28 days after conception, the public health goal is that each woman could begin her pregnancy with an optimal folate status, estimated to be a red blood cell (RBC) folate concentration >906 nmol/l (Daly et al., 1995). Blood folate levels are directly correlated with intake and, when low, are not only associated with NTDs but also with megaloblastic anemia and high blood Hcy levels (Dary O, 2009).

The crucial role of folate in the conversion of Hcy to methionine and, subsequently, to S-adenosylmethionine, has been already demonstrated. Maternal total Hcy (tHcy) concentrations have been linked to a wide range of adverse pregnancy outcomes, including growth retardation in utero (Hogeveen et al., 2012), preeclampsia, abruptio placentae, low birth weight, and other maternal or fetal complications (Ubeda et al., 2011).

Plasma Hcy levels are determined by both dietary and genetic factors, namely the common SNP *MTHFR* C 677T. Individuals with the *MTHFR* 677 TT genotype have been shown to have higher plasma Hcy concentrations and to be at higher risk of coronary heart disease and pregnancy complications particularly in the setting of a low folate status (Fekete et al., 2010; Klerk et al., 2002).

Besides, cigarette smoking has been related to increased levels of Hcy, and smokers have been shown to develop hypofolatemia (Das et al., 2010; Haj Mouhamed et al., 2011).

There is a growing body of epidemiologic evidence that suggests folate deficiency contributes to cancer risk at several sites (Flatley et al., 2009). Epidemiologic and molecular studies on cervical cancer have shown a causal relationship between infection with high-risk (HR) human papillomaviruses (HPV) and this cancer. Nutritional status and food consumption may be important HPV cofactors that increase risk of persistence and progression to cervical intraepithelial neoplasia (CIN); however, there is insufficient evidence to support the association between nutritional status and cervical carcinogenesis (Garcia-Closas R, 2005). Previous research has shown that circulating folate status significantly influences the natural history of infections with HR-HPV and lower the likelihood of developing HR-HPV-associated CIN 2+ (CIN of grade 2 or higher) (Piyathilake et al., 2009). The apparent role of folate in carcinogenesis in several tissues has stimulated investigations on *MTHFR* SNPs. The *MTHFR* C677T SNP can lead to abnormal DNA methylation and DNA synthesis, possibly resulting in an increased risk of cancer. However, the effect of *MTHFR* SNPs on cancer susceptibility remains controversial. In a population of women in Catania, Italy, with high prevalence of HR-HPV infection (Agodi et al 2009), a decreased risk for CIN of individuals homozygous for the *MTHFR* T allele has been previously reported (Agodi et al., 2010). However, some studies have supported the existence of gene-folate status interactions in the etiology of cervical cancer. Specifically it has been reported that the *MTHFR* T allele and reduced dietary folate may increase the risk for cervical squamous intraepithelial lesions (Goodman et al, 2001), and on the contrary, a study conducted after folate fortification reported that *MTHFR* SNP is associated with reduced risk of CIN 2 or 3 (Henao et al, 2005). The above *scenario* suggests a possible role of folate as a modulating factor on the risk of cervical cancer.

1.7 Epigenetic and epigenomic

Classic genetics alone cannot explain the diversity of phenotypes within a population. Nor does classic genetics explain how, despite their identical DNA sequences, monozygotic

twins or cloned animals can have different phenotypes and different susceptibilities to a disease (Fraga et al. 2005; Humpherys et al. 2001). The concept of epigenetics offers a partial explanation of these phenomena (Esteller, 2008).

The term epigenetics, first introduced in 1942 by Conrad H Waddington, to name “the causal interactions between genes and their products, which bring the phenotype into being” (Waddington, 1939), was later defined as heritable changes in gene expression and chromatin organization that are not attributable to any alteration in the DNA sequence (Holliday, 1987).

Until recently epigenomics was considered a black box. Like genetics, epigenetic traits can be mitotically and meiotically inherited (translational inheritance), but unlike genetics they are not conferred by the sequence of bases defining the genetic code. Recently it has been used the analogy of computer hardware and software to describe genetic and epigenetic mechanisms (Brower, 2011). Thus, while the genetic code can be considered the hardware for life, the epigenetic code is considered the software that determines how the hardware behaves. Fundamentally, ‘faulty’ software can potentially be re-written. This notion in particular has led to a frenzy of research activity in the field of onco-epigenetics, as exemplified by a substantial increase in the number of scientific publications regarding cancer and epigenetics over the past decade (Widschwendter et al., 2013).

Presently, epigenetics is highlighted in many other fields, such as aging, embryo development, inflammation, obesity, type 2 diabetes mellitus, cardiovascular - neurodegenerative - and immune diseases (Choi and Friso, 2010).

The predominant epigenetic mechanisms are DNA methylation, modifications to chromatin structure, loss of imprinting, and noncoding RNA (Gibney and Nolan, 2010).

DNA methylation is described as the covalent attachment of a methyl group to specific nucleotide residues within the DNA sequence, and occurs frequently, though not exclusively, at cytosine residues within a cytosine–guanosine (CpG) dinucleotide context (Jones and Baylin, 2007). CpG dinucleotides are unequally distributed throughout the genome but tend to cluster within and around gene promoter regions. These clusters are referred to as CpG islands, which vary in size from 500 up to 2000 base pairs in length. CpG islands usually remain unmethylated, with the exception of those occurring in or close to genes required for normal embryonic development, including X-chromosome inactivation and genomic imprinting (Baylin and Jones, 2011).

Histones are proteins that package DNA into structural units or nucleosomes to enable higher-order chromatin formation. Protein modifications to the histone ‘tails’ that are

exposed outside of the nucleosome affect gene accessibility by influencing an ‘open’ or ‘closed’ chromatin formation (Greer and Shi, 2012).

Imprinted genes are monoallelically expressed from either the maternally or paternally inherited allele. The crucial nature of this regulation is reflected in phenotypic manifestations in individuals exhibiting improper imprinted gene expression. The maintenance of proper parent of origin gene expression is often aberrant in cancer leading to loss of imprinting. This alteration is observed in many different types of cancer cells and frequently influences the timing of key developmental events or potentially the loss of imprinting in stem cells leading to the genesis of cancer.

Non-coding RNAs are RNA transcripts that are not translated into protein but can demonstrate functional regulation of gene expression by blocking transcription or enhancing mRNA degradation (Esteller, 2011).

1.7.1 DNA methylation

Regarding nucleic acid modification, DNA methylation of cytosine nucleotide at the carbon 5 position of a cytosine (5-mC) is a common epigenetic mark involved in epigenetic regulation of gene expression in many eukaryotes and is often found in regions of the genome rich in sequences of a cytosine preceding a guanine (CpG islands), or in CpHpG (H: A, T, C) (Lister et al., 2009, Mansego et al., 2013).

CpG islands exist in the promoter regions of approximately half of all genes and are usually unmethylated in normal differentiated cells, while CpG islands located in intergenic regions are methylated (Esteller, 2007; Taby and Issa, 2010). Promoter methylation is typically associated with repression, whereas genic methylation correlates with transcriptional activity. In the particular case of cancer, promoter CpG islands of numerous tumor suppressor genes (TGSs) are found to be densely methylated, resulting in transcriptional silencing, with these “epimutations” being cancer type-specific and tumor stagespecific (Rodriguez-Paredes and Esteller, 2011).

DNA methylation has many roles in various cellular processes and may impact the transcription of genes by preventing the binding of key transcriptional factors (Altaf et al., 2007). Transcriptional silencing due to DNA methylation is thought to occur by the recruitment of methyl CpG-binding transcriptional repressors and by interfering with the DNA binding of transcriptional activators, which results in a condensed chromatin state. In addition, methylated DNA may be bound by methyl-CpG-binding domain proteins, which are essential for binding to 5-methylcytosine (Meeran et al., 2010).

DNA methylation is catalyzed by enzymes known as DNA methyltransferases (DNMTs), using the cofactor S-adenosylmethionine (SAM) (Gibney and Nolan, 2010) and hypermethylation of CpG dinucleotides or CpG islands by DNMTs usually results in transcriptional gene silencing and gene inactivation (Herceg, 2007). The human genome contains four DNMT genes, *DNMT1*, *DNMT2*, *DNMT3A* and *DNMT3B* (Stresemann et al., 2006). Altered DNMT expression and activity is seen in numerous diseases including autism, cardiovascular diseases, obesity, Type-2 diabetes and cancer (Grafodatskaya et al., 2010; Milagro et al., 2011; Maier and Olek, 2002, Chowdhury et al., 2003). Finally, global hypomethylation is associated with nearly all human cancers (Irizarry et al., 2009; Tollefsbol T, 2008).

1.7.2 Gene-environment versus epigene-environment

The interplay between the environment and human genome has been traditionally presented under the framework of gene-environment interactions (also indicated as genotype-environment) (Baccarelli and Bollati, 2009).

In gene-environment interactions, the genetic polymorphisms that modify the effects of environmental exposures are transmitted transgenerationally according to Mendelian genetics, and the trait determining effect modifications is generally assumed to follow the same genetic model (dominant, co-dominant, recessive) as that of the levels of expression or function of the protein coded by the locus of concern. A second well-established area of interplay includes the direct effects of environmental exposures on the genome, e.g., DNA damage and/or mutations induced by environmental exposures.

In environmental health, the recognition that exposures could produce DNA mutations represented a major landmark for risk assessment and prevention. Consequently, genotoxic agents have been categorized according to their capability to alter DNA sequence and thus increase disease risk (Siemiatycki et al, 2004). Such information has been fundamental to determine environmental risks and shape current regulatory efforts for exposure reduction. Specifically, potential carcinogenic agents have been carefully tested in in-vitro and in-vivo models of mutagenicity. In human subjects, some of these molecular events may represent early events along the pathways linking carcinogen exposure to cancer.

In principle, the effect-modification model should apply to epigene-environment interactions as well as to gene-environment interactions (figure 3). Similarly to the effect modifications demonstrated or postulated for genetic polymorphisms, epigenetic differences determining disease risk could make individuals less or more vulnerable to environmental.

In environmental studies, the flexibility of epigenetic states has generated a growing interest in evaluating the direct alterations that environmental exposures may produce on epigenetic states, including changes in DNA methylation and histone modifications (Bollati and Baccarelli, 2010).

1.7.3 Environmental effects on the epigenome

Supporting evidence implicates the epigenome as the interface between the genome and the environment (Feil and Fraga, 2011; Jirtle and Skinner, 2007) a broad definition of the environment includes nutrition, lifestyle, external environmental exposures and reproductive history. For this reason epigenetics is now considered an important mechanism in the unknown etiology of many environment-associated diseases, with growing interest in associating epigenetic regulation with lifestyle and risk of disease (Choi and Friso, 2010).

Numerous environmental exposures have linked altering epigenetic patterns during a lifetime, and subsequent risk of disease.

Environmentally triggered epigenetic alterations are often tissue-specific and are established during a phase of epigenetic ‘hypersensitivity’ during development, but can also accumulate as a function of age. The epigenome can be thought as a mechanism of cellular memory that records environmental exposures which accumulate during life-time and which potentially explain predisposition to late-onset disease such as cancer. Such induced epigenetic changes can be inherited during cell division, resulting in permanent maintenance of the acquired phenotype (Widschwendter et al., 2013). Moreover, the reversibility of epigenetic alterations stimulates the development of novel therapeutic approaches with an open field for development of early cancer detection and prevention, namely through chemoprevention (Nogueira da Costa and Herceg, 2012).

In the nutritional field, epigenetics is exceptionally important because nutrients and bioactive food components can modify epigenetic phenomena and alter the expression of genes at the transcriptional level (Choi and Friso, 2010) (figure 4).

An interesting study showed how diverse environmental exposures may alter the epigenome by looking at the methylation and histone modifications in monozygotic twins at different stages in life. It was demonstrated that, early in life, their epigenetic profiles were nearly identical, but, by age 50, considerable changes were detected (Feinberg AP, 2008). These studies indicate that inherited genetic factors make only a minor contribution to susceptibility in most types of neoplasms, indicating that the environment has a principal role in influencing disease predisposition (Lichtenstein et al., 2000).

In general terms, environmental and dietary carcinogens, also known as epimutagens, are capable of inducing tumoral development by deregulating the epigenome and can be divided into two groups: (a) those that induce, both directly or indirectly, changes to genomic DNA and (b) those that affect key cellular processes such as gene transcription, DNA damage and repair, cell cycle control and apoptosis (Herceg, 2007; Herceg and Vaissiere, 2011).

Examples of these epimutagens include tobacco smoke, alcohol, viruses and bacteria and dietary contaminants such as aflatoxin B1 (Lambert et al., 2011; Zhang et al., 2012).

Although extensive and novel studies were conducted in the past half a decade, there is still no clear-cut casual relationship between epimutagens and changes to epigenetic signatures (Bollati and Baccarelli, 2010; Herceg and Vaissiere, 2011). The main limitation of these studies still reside in the fact that the epigenetic changes are thought of as being mostly subtle, cumulative and requiring a long “timeframe” until full manifestation is detectable.

1.7.4 Effects of bioactive food components on DNA methylation: the field of nutriepigenomic

Bioactive dietary components consumed by ingesting natural products, including fruits and vegetables, can act as sources of vitamins and minerals. While this is an invaluable role, these agents have high potential for application to oncogenesis, partially owing to their anticarcinogenic properties (Shu et al., 2010; Link et al., 2010).

A growing body of evidence suggests that dietary agents as well as non-nutrient components of fruits and vegetables can affect epigenetic processes and are involved in processes, including the reactivation of TGS, the initiation of apoptosis, the repression of cancer-related genes and the activation of cell survival proteins in different cancers (Hardy and Tollefsbol, 2011).

Folate, vitamin B-12, methionine, choline, and betaine can affect DNA methylation and histone methylation through altering 1-carbon metabolism. Two metabolites of 1-carbon metabolism can affect methylation of DNA and histones: SAM, which is a methyl donor for methylation reactions, and S-adenosylhomocysteine (SAH), which is a product inhibitor of methyltransferases. Thus, theoretically, any nutrient, bioactive component or condition that can affect SAM or SAH levels in the tissue, can alter the methylation of DNA and histones. Other water-soluble B vitamins like biotin, niacin, and pantothenic acid also play important roles in histone modifications. Biotin is a substrate of histone biotinylation. Niacin is involved in histone ADP-ribosylation as a substrate of poly (ADP-ribose) polymerase as well

as histone acetylation as a substrate of Sirt1, which functions as histone deacetylase (HDAC).

Pantothenic acid is a part of CoA to form acetyl-CoA, which is the source of acetyl group in histone acetylation. Bioactive food components directly affect enzymes involved in epigenetic mechanisms. For instance, genistein and tea catechin affects DNMT. Resveratrol, butyrate, sulforaphane, and diallyl sulfide inhibit HDAC and curcumin inhibits histone acetyltransferases (HAT). Altered enzyme activity by these compounds may affect physiologic and pathologic processes during our lifetime by altering gene expression (Choi and Friso, 2010).

While most natural dietary products have shown beneficial effects on the epigenome, not all dietary components share this characteristic. In fact, alcohol consumption is associated with harmful epigenetic modifications as well as the development/progression of several human cancers: colorectal cancer patients with high alcohol consumption had a prevalence of promoter hypermethylation of numerous genes when compared with patients with low alcohol consumption (Van Engeland et al., 2003; Giovannucci et al., 1995).

Interestingly, one of the most widely used models of diet-induced obesity in animals is the intake of a high-fat diet, and various reseraches have analyzed the epigenetic modifications induced by this dietary approach in rodents (Lomba et al., 2010). However, it is still unknown the capacity of the different types of FAs to induce epigenetic modifications. Few studies have evidenced the role of *n*-3 and *n*-6 PUFA on DNA methylation, although there are examples concerning effects of EPA (Ceccarelli et al., 2011), DHA (Kulkarni et al., 2011) and AA (Kiec-Wilk et al., 2011) FAs on DNA methylation.

It has been also reported that MUFA can modulate other epigenetic mechanisms such as histone acetylation. Thus, more studies must be designed as it is well known that the dietary FA composition is one of the main factors in the development of obesity and metabolic syndrome (Milagro et al., 2013); particularly in relation to the beneficial effects associated to high long-chain *n*-3 PUFA intake, whose anti-inflammatory properties are being studied to reduce obesity-related low-grade inflammatory condition (Calder et al., 2011).

1.8 Cancer epigenetic

Epigenetic modifications are often involved in transcriptional regulation and have been implicated both in tumor development and progression (Hardy and Tollesbol., 2011).

These modifications, causing transcriptional deregulation, may result in the inappropriate expression or activation of transcription factors associated with oncogenes and/or the failure

to express genes responsible for tumor suppression. In fact, cancer cells have genomewide aberrations in a number of epigenetic markers, including global hypomethylation, global downregulation of miRNAs, promoter-specific hypermethylation, histone deacetylation and upregulation of epigenetic machinery (Taby and Issa, 2010).

In tumorigenesis, epigenetic aberrations are believed to play key roles in TSG inactivation, oncogene activation, and chromosomal instability, all of which are involved in the deregulation of critical cellular pathways and steps of carcinogenesis including tumor initiation, invasion and plasticity (Carmona and Esteller, 2010; Jones, 2012; Sincic and Herceg, 2011).

The most studied epigenetic change is the DNA methylation, which occurs primarily in CpG dinucleotides and is often altered in cancer cells. Generally, tumours are characterized by sporadic gene-specific hypermethylation and global DNA hypomethylation, which increases according to tumour progression.

Gene-specific hypermethylation can affect many different types of cancers and is often mediated through the silencing of TSG, whereas hypomethylation can contribute to genomic instability frequently observed in cancer, activation of oncogenes, or loss of imprinting which are also causes of oncogenesis (Tollefsbol T, 2009).

Histone modifications are also important in cancer and result in dramatic changes in chromatin structure as well as the accessibility of DNA to transcription factors that mediate gene expression. Histone acetylation has been associated with an increase in gene activity whereas histone deacetylation normally prevents transcription. Other histone modifications such as histone methylation can have varied effects and taken together the many types of histone alterations seen in cancer have a major impact not only on the initiation of cancer processes but also on its progression potentially to malignant cells.

1.8.1 DNA hypermethylation and hypomethylation in cancer

Hypermethylation is characterized by the addition of methyl groups and, if highly specific to the CpG islands in the promoter region of a particular gene, may lead to transcriptional silencing of the gene, with subsequent loss of protein expression.

This mechanism is currently recognized as an alternative to mutations or allelic loss for gene-silencing in TSGs (Herman and Baylin, 2003). A number of TSGs and other cancer-related genes (i.e. the retinoblastoma gene, RASSF1A, VHL, MLH1, CDH1, LKB1, p16 gene, GSTP1 and MGMT), were found to be silenced by promoter hypermethylation (Feinberg and Tycko, 2004).

Hypermethylation of genes has been implicated in carcinogenesis due to its involvement in cell cycle, DNA repair, angiogenesis, metabolism of carcinogens, apoptosis, and cell-cell interaction. Thus, methylation patterns can be considered as biomarkers for early detection, diagnosis, prognosis, prediction and monitoring of therapy response. The identification of these cancer-associated methylation signatures may also provide the foundations for cancer prevention strategies, with DNA hypermethylation being proposed as a source of potential early event biomarkers in carcinogenesis that may precede the neoplastic process (Taby and Issa, 2010).

Hypomethylation is characterized as a genome-wide decrease in methylation and is frequent in CpG sites in all classes of repeated sequence, intragenic and single-copy intergenic sequences, the inactive X chromosome, and imprinted regions, as well as a subset of promoters or CpG islands that show tissue-specific methylation (Jones, 2012).

Measurements of the total level of 5meC initially identified that the genomes of cancer cells and tissues were frequently hypomethylated relative to the DNA of healthy tissues. While hypomethylation at specific sites of individual genes was shown in some instances, it soon became apparent that hypomethylation of repeat DNA sequences was the dominant factor in the overall reduction in methylation levels. Studies of DNA methylation over the past 20 years have led to the prevailing view that development of cancer is accompanied by widespread epigenetic changes involving global hypomethylation, particularly of repeat DNA sequences, accompanied by focal hypermethylation of multiple CpG island gene regulatory regions.

Compared with gene-specific hypermethylation, the role of hypomethylation is less well understood and characterized (Tollefsbol T, 2009). It is proposed that hypomethylation in coding regions of genes is associated with carcinogenesis through the favoring of mitotic recombination which may lead to deletions, translocations, chromosomal rearrangements as well as alterations in mRNA levels. Also, hypomethylation is associated with alterations to signaling cascades influencing proto-oncogenes, such as c-Jun, c-Myc, and c-H-Ras (Calvisi et al., 2007). In addition, repetitive elements spread across the genome and while normally heavily methylated tend to loose methylation (Roberts and Gores, 2005).

1.9 The human mobilome

Our 3 billion base pairs are overwhelmingly non-coding, and 50% or more are recognizable as repetitive sequences. Most of the repeats are interspersed, meaning that they occur discontinuously as singularly scattered copies in the genome (Babatz and Bruns, 2013).

These include Long INterspersed Elements (LINEs) and Short INterspersed Elements (SINEs). Most abundant in mammals are the retrotransposons, which self-replicate through the reverse transcription of RNA intermediates.

Genomes are not static strings of bases, but instead are mutable, evolving, and structurally dynamic. Three families of human retrotransposons have been active in relatively recent genome evolution and continue to generate human genomic variation today. With specific regard to the autonomous LINE-1 element, it is 6 kb long and contains an internal promoter, two open reading frames (ORFs), and a polyadenylation signal. After RNA polymerase II transcription, mRNA processing, and export to the cytoplasm, ORF1 and ORF2 encode two proteins (ORF1p and ORF2p) that preferentially associate with their encoding RNA (Kulpa and Moran, 2006). The resulting ribonucleoprotein (RNP) particles return the RNA to the nucleus, where ORF2p initiates target-primed reverse transcription (TPRT) to insert a copied DNA sequence at a new site in the host cell genome (Cost et al., 2002).

In humans, more than one-third of DNA methylation occurs in retrotransposons, which represent a large portion of the human genome (Bernstein et al, 2006). Among these sequences, Alu and LINE-1 retrotrasposons are the most plentiful families, representing approximately 30% of the human genome (Babushok and Kazazian, 2007) and are heavily methylated (Yang et al, 2004).

A reduced level of methylation in repetitive elements such as LINE-1 has been associated with genome instability and chromosomal aberrations, leading to an increased risk of cancer, such as colorectal and prostate cancers. In fact, hypomethylation in repetitive elements has been reported in many cancer cells and appears to parallel overall genomic hypomethylation (Hoffmann and Schulz, 2005). Hypomethylation and consequent transcription of LINE and SINE elements has the potential to lead to retrotransposition and insertional mutagenesis.

1.9.1 LINE-1 methylation in peripheral blood as potential biomarker for cancer molecular epidemiology

Biomarkers are biological molecules in body fluids or tissues that are quantitatively measured and evaluated as indicators of normal biological processes, pathogenesis, or pharmacologic response to a therapeutic intervention (Wagner et al., 2004).

Numerous types of biomarkers have been developed and used for early detection of cancer and prediction of prognosis and treatment response in cancer patients (Li et al., 2012).

Because DNA is much more stable than other biological materials, such as RNA or protein, DNA methylation is easy detectable in small specimens and may be suitable for large-scale epidemiologic studies accordingly.

Previous studies of the potential of DNA methylation as a cancer biomarker mainly used tumor tissue, however, an increasing number of studies are using body fluids such as urine, bronchial lavage fluid, breast milk, sputum, plasma and serum, and peripheral blood (Shivapurkar and Gazdar, 2010).

Because of their high representation throughout the genome, Alu and LINE-1 have been proposed as surrogate markers for estimating global DNA methylation level (Weisenberger et al, 2005; Yang et al, 2004), although growing evidence indicates that they could have specific and distinct cellular roles (Wallace et al, 2008). Hypomethylation of repetitive elements favours their activity as retrotransposable sequences and has been suggested to have deleterious effects on cells, initially through insertional mutations (Kazazian, 2004), and later by introducing genome instability through deletions and genomic rearrangements (Wallace et al, 2008).

More recently, global DNA methylation has been examined in surrogate tissues such as leukocyte DNA. A lower level of leukocyte DNA methylation has been associated with an increased risk of head and neck squamous cell carcinoma, bladder cancer, breast cancer, gastric cancer, and colorectal adenoma and cancer (Hsiung et al., 2007; Moore et al., 2008; Choi et al., 2009; Hou et al., 2010) after adjusting for known risk factors. This suggests that leukocyte DNA methylation may serve as a surrogate biomarker for systemic genomic methylation, providing an independent risk factor for cancer development (Zhang et al., 2011a).

Li et al., 2012, provides a comprehensive literature review of blood-based DNA methylation as a cancer biomarker and focus on the study of DNA methylation using peripheral blood leukocytes. Although DNA methylation patterns measured in peripheral blood have great potential to be useful and informative biomarkers of cancer risk and prognosis, large systematic and unbiased prospective studies that consider biological plausibility and data analysis issues are needed in order to develop a clinically feasible blood-based assay.

In healthy populations, inter-individual methylation variations at Alu or LINE-1 elements from blood DNA have also been associated with risk factors for cancer, neurological and cardiovascular diseases (Baccarelli and Bollati, 2009; Rusiecki et al, 2008). Also the finding of an age-associated decline in repetitive element methylation in normal tissues of aging

individuals suggests a possible role in a variety of common human age-related diseases (Bollati et al, 2009).

1.9.2 DNA methylation analysis with nutritional applications

Nutrients may induce transient or permanent alterations in the epigenetic marks that regulate the expression of genes involved in metabolic processes and other biological networks, which could be one of the factors leading to the developmental origin of pathological conditions like obesity, insulin resistance, type 2 diabetes, cancer, neurological and cardiovascular diseases, neurodegenerative diseases, and immune diseases (Wilson, 2008; Urdinguio et al., 2009). In fact, there is widespread interest in finding correlations between the genomic 5-mC levels and diet, lifestyle and clinical disorders (Mansego et al., 2013).

In nutritional studies, it would be important to quantify global patterns of DNA methylation in order to evaluate the effect of nutrient intake, and a specific phenotype such as a different dietary pattern (i.e. diet characterized by high intake of vegetables and fruits) may protect against global DNA hypomethylation (Zhang et al., 2011a).

Several studies have shown that DNA methylation pattern changed over time according to various endogenous and exogenous factors, such as demographic and lifestyle factors (age, race, sex, smoking, and alcohol consumption), diet intake (folate, vitamin B, green tea, and phytoestrogen), environmental exposures (arsenic, cadmium, and benzene), and disease status (infection and cancer) (Yuasa Y, 2010). Interestingly, several well-designed epidemiology studies found that obesity, dietary pattern, and physical activity were associated with global methylation in DNA extracted from peripheral blood leukocytes (Milagro et al., 2013).

The field of nutrigenomics involves studying how genes and dietary components interact to influence phenotype and can reveal how one responds to bioactive components based on genetics, nutrient-induced changes in DNA methylation and chromatin alterations, and nutrient induced changes in gene expression, whereas the field of nutriepigenomics involves the lifelong remodeling of our epigenomes by nutritional factors (Davis and Hord, 2005; Gallou-Kabani et al., 2007).

Nutriepigenomic studies focusing on individual responses to bioactive components and individualized epigenetic diets consisting of bioactive dietary factors mentioned herein will be of particular interest in the future. Furthermore, future studies focusing on the clinical relevance and mechanism of epigenetic modification of bio-active dietary factors are needed

to further assess the applicability of dietary factors as cancer preventive and chemopreventive agents (Hardy and Tollesfbol., 2011).

1.9.3 DNA methylation, cancer and folate

It appears that folate is essential for DNA methylation reprogramming during the early embryonic period. Because folate deficiency in early pregnancy is associated with an increased risk of neural tube defects, aberrant reprogramming of DNA methylation by low dietary folate has been suggested as a candidate mechanism (Choi and Friso, 2010). Animal studies indicate that dietary methyl nutrients during the periconceptional period can change DNA methylation patterns in offspring and it may modify adult health-related phenotypes (Sinclair et al., 2007).

The causes of DNA hypomethylation remain elusive. Animal studies provided direct evidence that certain dietary factors such as folate deficiency and alcohol consumption can induce changes in genomic DNA methylation (Keyes et al., 2007; Kotsopoulos et al., 2008). Because epigenetic variation can be influenced by dietary factors, it is reasonable to believe that investigating strategies that utilize dietary compounds to target epigenetic modifications may be worthwhile in preventing and treating diseases including cancer (Hardy and Tollesfbol, 2011).

Folate deficiencies are reported to contribute to the development of several different cancers including: breast, cervix, ovary, brain, lung and colorectal (Kim Y.I, 2007; Duthie, 2011; Chen et al., 2011). Folate regulates the biosynthesis, repair and methylation of DNA, whereas deficiencies in folate can induce carcinogenesis by augmenting these processes.

However, studies on diet and DNA methylation in humans have yielded inconsistent findings. Some found dietary folate restriction or folic acid supplementation resulted in changes in DNA methylation (Pufulete et al., 2005; Rampersaud et al., 2000), whereas others did not observe methylation changes in response to folate depletion or treatment (Axume et al., 2007a; Axume et al., 2007b).

A cross-sectional study was conducted to test the hypothesis that folate status and aberrant DNA methylation show a progressive change across stages of cervical pathology from normal cells to invasive cervical cancer and, additionally, that a gene-specific hypermethylation profile might be used as a predictive biomarker of cervical cancer risk (Flatley et al., 2009). Results of this study report that lower folate status was associated with high-risk HPV infection and with a diagnosis of cervical intraepithelial neoplasia or invasive cervical cancer. Global DNA hypomethylation was greater in women with invasive cervical

cancer than all other groups. A cluster of three TGS, *CDHI*, *DAPK*, and *HIC1*, displayed a significantly increased frequency of promoter methylation with progressively more severe cervical neoplasia.

Only a few studies have focused on precancerous stages in relation to dietary patterns, a point where the development of cancers could be prevented by dietary modifications, as well as biomarkers associated with such dietary patterns. Even though it is logical to assume that dietary recommendations focused on promoting a healthier overall dietary pattern rather than encouraging consumption of certain foods or food categories should be the first line of intervention for prevention of many different types of cancers, use of biomarkers to monitor the effectiveness of these interventions should be an integral part of such efforts. There have been no systematic studies conducted to derive biomarkers of dietary patterns which are also associated with higher risk of developing precancerous lesions (Piyathilake et al., 2012).

In a recent study, it has been shown that the degree of peripheral LINE-1 methylation in peripheral blood mononuclear cells may serve as a biomarker for monitoring the effectiveness of dietary modifications needed for reducing the risk of cervical intraepithelial neoplasia grade 2+ (CIN 2+) (Piyathilake et al., 2012). In this study, women with the unhealthiest dietary pattern were 3.5 times more likely to be diagnosed with CIN 2+ than women with the healthiest dietary pattern. Women at risk for developing CIN 2+ with the healthiest dietary pattern were 3.3 times more likely to have higher peripheral blood mononuclear cells LINE-1 methylation than women with the unhealthiest dietary pattern.

However, folate intake and intake of other micronutrient could be modulate the risk of CIN by the means of a gene-nutrient interaction mechanism, thus further knowledge could integrate and reinforce the efficacy of preventive strategies in a population at risk.

Epidemiologic studies examining the association between dietary folate intake and leukocyte DNA methylation in cancer-free controls reported null associations (Choi et al., 2009; Moore et al., 2008). Previous studies focused on the consumption of specific food items and nutrients and ignored the fact that humans eat diets characterized by different balances of food and nutrients. As such, a recent study, examined the association between dietary patterns and leukocyte DNA methylation in a cancer-free population (Zhang et al., 2011a). Two dietary patterns were identified: the prudent dietary pattern, characterized by a high intake of vegetables and fruits and the Western dietary pattern, characterized by a high intake of energydense foods such as grains, meats, potato, oil, and dairy.

In this study, participants with low DNA methylation had a lower dietary folate intake than those with high DNA methylation, but the association was not significant. Dietary intake of

other one carbon nutrients such as methionine, riboflavin, vitamin B-6, and vitamin B-12 did not differ by levels of DNA methylation. These findings are in line with previous findings (Hsiung et al., 2007; Moore et al., 2008), both of which reported that dietary folate intake and global DNA methylation were not associated among cancer-free controls.

When evaluated individually, none of the 13 food groups except for dark green vegetables was significantly associated with DNA methylation. However, a broader view of dietary patterns revealed an inverse association between the prudent dietary pattern and global DNA hypomethylation in a dose-response fashion. Study of dietary patterns may have improved the power of detecting the effect of diet on DNA methylation than examining individual nutrients or individual food groups. The consumption of multiple nutrients from the prudent dietary pattern, such as 1-carbon nutrients, antioxidants, and others may work interactively to protect against DNA hypomethylation in a complex network. As a result, intervention on the overall dietary pattern rather than on single nutrients or food groups may be a more effective way to protect against cancer risk through epigenetic regulation.

Although 1-carbon nutrients are the focus of most previous studies of nutritional influences on epigenetic regulation, other nutritional factors such as dietary fatty acids have been suggested to affect DNA methylation patterns by epigenetic mechanisms independent of 1-carbon nutrients (Waterland and Rached 2006). This study found that saturated fat intake was higher in participants with low global DNA methylation compared with those with high methylation. The biological mechanism underlying this association is still not understood. Further studies are needed to confirm the association between dietary fat intake and DNA methylation and to further examine how and when it occurs (Zhang et al., 2011a).

However, only a few of the previous observational studies examined associations of dietary and genetic factors related to one-carbon metabolism with global methylation level of leukocyte DNA, and their overall findings showed no association (Hou et al., 2010; Hsiung et al., 2007). These inconsistent findings might be explained by differences in exposure level of nutrients related to one-carbon metabolism, differences in assay methods of global methylation level, and difference in the distribution of genetic factors related to one-carbon metabolism, either alone or in combination. In particular, no study has investigated the interaction of genetic factors such as SNPs and nutrient intake related to one-carbon metabolism with DNA methylation level.

A recent study, carried out a cross-sectional study, using a well-characterized control group of a breast cancer case-control study, to evaluate the associations of dietary and genetic factors related to one-carbon metabolism with the global methylation level of peripheral

blood leukocyte DNA among Japanese women, reported that higher folate intake was significantly associated with a lower level of global methylation of leukocyte DNA, in fact, global methylation level significantly decreased per quartile category for folate level, furthermore alcohol drinking modified the association between folate intake and global methylation level. However, no statistically significant association was observed for intake of vitamins B2, B6, and B12, alcohol consumption. In addition, none of the five candidate SNPs examined in this study, *MTHFR* rs1801133 (C677T) and rs1801131 (A1298C), *MTRR* rs10380 and rs162049 and *MTR* rs1805087 showed a statistically significant association, although rs1801131 and rs1801133 in *MTHFR*, have been reported to be linked to altered enzymatic activity and folate level. Although the data of this study and others suggest that folate intake can modulate the global methylation level of leukocyte DNA, inconsistencies among the studies have been noted, and may reflect the complex and multifactorial nature of individual variation in the global DNA methylation level of peripheral blood leukocytes (Ono et al., 2012).

Recent focus has shifted to concern about folic acid supplementation resulting in progression of existing tumors and altering normal DNA methylation patterns (Smith et al., 2008; Kim Y.I, 2008). Currently, there is no direct evidence of aberrant DNA methylation and change in gene expression in response to “high” levels of folate/folic acid intake. In addition, there is no consensus on a dose of folic acid or a blood folate concentration that would be associated with potential adverse health outcomes (Crider et al., 2012).

Regarding the effect of folate supplementation on the methylation level of leukocyte DNA, a randomized controlled trial of 400 ug folic acid supplementation per day (n = 15) or placebo (n = 16) for 10 weeks in patients with colorectal adenoma showed an increase in leukocyte DNA methylation level (Pufulete et al., 2005). In contrast, supplementation with 2 mg folic acid and 20 ug vitamin B12 for 12 weeks did not change this variable (Fenech et al., 1998).

These intervention studies suggest that the effect of folate on the methylation level of leukocyte DNA might depend on dose, but that a dose–response pattern might not be straightforward. For instance, it has been suggested that folates act as inhibitors of dihydrofolatereductase, and that high folate levels could have the same functional effect as a low folate status under certain circumstances (Smith et al., 2008; Nijhout et al., 2004).

In fact, several animal studies showed that the effect of isolated folate deficiency on genomic DNA methylation in rodent liver and colon was either a decrease or increase (Song et al., 2000). The final message from these studies may be that the mechanisms of individual

variation in the global DNA methylation level of peripheral blood leukocytes are complex and multifactorial in nature (Ono et al., 2012).

While many of the aforementioned studies were conducted using a particular dietary factor, it is reasonable to believe that most may be consumed in combination and over a period of a lifetime. This may provide a rationale for studying nutrient epigenetic modifiers more in combination studies or the proposal of an 'epigenetic diet' focused on consuming products that show the ability to stimulate beneficial epigenetic modifications.

This may be used from a chemopreventive standpoint to incorporate anticancer nutrients into one's daily routine to impede disease mechanisms. From a therapeutic perspective many nutrients have been and are being studied for their ability to prevent and reduce the risk or severity of certain diseases and for their anticarcinogenic properties (Hardy and Tollefsbol, 2011).

2. RATIONALE AND AIMS OF THE STUDY

Knowledge about genetic risk factors of complex diseases may be used to address diagnostic, preventive, and therapeutic interventions based on individual's genetic risk. Implementation of genetic risk prediction in health care requires a series of studies that include all phases of translational research, starting with a comprehensive evaluation of genetic risk prediction (Janssens et al., 2011).

Obesity is a multifactorial disorder, reflecting complex interactions of genes, environment and lifestyle (Newel et al., 2007), associated with a high risk of chronic diseases such as diabetes, cardiovascular disease and certain cancers (Couto et al., 2011). Obesity constitutes a major public health problem that, in current years, evolved into a worldwide epidemic (WHO, 2000; WHO, 2002). Hundreds of genes are known to have minor contributions in defining the obese phenotype; however, the current lack of understanding of the numerous gene–gene and gene–environment interactions underlying obesity poses one of the major obstacles for the development of effective preventative and therapeutic intervention strategies.

Dietary FA are one class of compounds that have garnered considerable attention for their ability to regulate adipose tissue metabolism and secretory function (Oller do Nascimento et al., 2009). Emerging research shows that dietary FA and SNPs in the genes encoding TNF- α , IL-6 and adiponectin can interact to regulate their production and secretion, thus adding an additional level of complexity in the study of obesity. Taken together, nutrigenomics research can provide a more thorough understanding of how changes in dietary FA intake may contribute to the inter-individual variability in inflammatory status.

Despite many studies showing independent association between the *TNFA* SNPs and obesity, only few studies have investigated diet–gene interactions. Nieters et al. found that German Caucasian men and women with the –308 A allele, who were in the highest tertile for intake of the n-6 PUFAs LA and AA (%E), had an increased obesity risk (Nieters et al., 2002).

More recently, Joffe and colleagues reported that the odds of obesity for black South African women with the –308 A allele increased with total dietary fat intake (%E), however this interaction was not observed in white SA women (Joffe et al., 2010, Joffe et al., 2011). Researching these diet–gene interactions more extensively, and understanding the role of ethnicity as a confounder in these relationships, may contribute to a better understanding of the inter-individual variability in the obese phenotype (Joffe et al., 2013).

Furthermore, the MD has long been reported to be the optimal diet for preventing NCDs and preserving good health, and independently of energy and macronutrient quantity intakes, a better adherence to the MD, is associated with lower obesity risk (Mozaffarian et al., 2011; Martínez-González et al., 2012). As such, research interest over the past years has been focused on estimating adherence to the whole MD rather than analyzing the individual components of the dietary pattern in order to consider important interactions between components of the diet.

Diet and lifestyle play a crucial role in cancer etiology. Recent studies provide evidence that dietary components may affect the process of carcinogenesis. Different mechanisms, including antioxidant, anti-inflammatory, and anti-estrogenic processes, have been proposed to explain the protective nature of certain dietary components. Isothiocyanates from cruciferous vegetables, diallyl sulfide, isoflavone, phytosterole, folate, selenium, vitamin E, flavonoids and dietary fibers, may reduce the risk of cancer. However, the exact mechanistic pathways in which these effects are exerted on cells to avoid, delay, or reverse carcinogenesis are not as clear (Supic et al., 2013). In addition, dietary and other lifestyle factors interact with genetic susceptibility, such as polymorphisms, to determine health outcomes i.e. there is phenotypic plasticity in the context of a fixed genotype (Mathers 2002). These observations demonstrate that dietary exposures can have long-term consequences for health and raise questions about the mechanisms through which early life exposures are 'remembered' over long-time periods and how they result in altered disease risk (Mc Key and Mathers, 2011), . Emerging evidence suggests that the protective effects of nutrition and environmental can be mediated through epigenetic mechanisms (Su et al., 2011). Although epigenetic changes are heritable in somatic cells, the notion that these modifications are also potentially reversible as observed through the finding of Agouti mouse model makes them attractive and promising avenues for tailoring cancer preventive and therapeutic strategies (Link et al., 2010).

There is growing evidence that epigenetic mechanisms may mediate the effects of nutrition and may be causal for the development of common complex (or chronic) diseases (Petronis 2010).

Broadly, there are two mechanisms through which nutritional and dietary factors have been postulated to effect DNA methylation. These are: (i) changing the availability of methyl donors and (ii) altering the activity of enzymes involved in the process of DNA methylation, more specifically the DNMT enzymes. The nutrients implicated in this mechanism are the methyl donors and those micronutrients which are co-factors for enzymes involved in one-

carbon metabolism including folate, vitamin B6, vitamin B, choline and methionine (McKay et al., 2012).

The effect of folate status on several outcomes has long been recognized. Folate is now viewed not only simply as a nutrient needed to prevent megaloblastic anemia in pregnancy but also as a vitamin essential for reproductive health, disease prevention, and health maintenance (Tamura and Picciano., 2006). There is a growing body of epidemiologic evidence that suggests folate deficiency contributes to cancer risk at several sites (Flatley et al., 2009), such as cervical cancer (Agodi et al., 2010, Piyathilake et al., 2012).

Aberrant DNA methylation is a recognized feature of human cancers, and folate is directly involved in DNA methylation via one-carbon metabolism. A cross-sectional study was conducted to test the hypothesis that folate status and aberrant DNA methylation show a progressive change across stages of cervical pathology from normal cells to invasive cervical cancer and, additionally, that a gene-specific hypermethylation profile might be used as a predictive biomarker of cervical cancer risk (Flatley et al., 2009). Results of this study report that lower folate status was associated with high-risk HPV infection and with a diagnosis of cervical intraepithelial neoplasia or invasive cervical cancer.

Furthermore, folate insufficiency is thought to influence DNA stability involved in cancer carcinogenesis (Fenech M, 2001a; Choi and Mason, 2000). Several recent studies have demonstrated that folate status could also modulate mitochondrial DNA (mtDNA) stability (Branda et al., 2002; Crott et al., 2005; Chou et al., 2007). Particularly, mtDNA deletions and low folate status, proposed characteristics of carcinogenesis, have been suggested in relation to human liver cancer (HCC) susceptibility in a recent cross-sectional study (Wu et al., 2009).

The studies of nutrigenomics and of nutriepigenomics may offer the opportunity to understand the combined effect of a number of polymorphisms on the same or different genes interacting with the environment. Furthermore, in addition to diet–single polymorphism interactions on obesity risk, it is also important to understand the association with methylation of repetitive elements, as they may provide a global picture of genomic stability (Weisenberger et al., 2005) which has health implications beyond the regulatory function of a single gene.

Animal studies provide compelling evidence that patterns of DNA methylation can be affected by environmental exposures to nutritional factors, leading to the hypothesis that changes in DNA methylation may be an intermediate step between the environment and

human diseases. In humans, previous studies have found that dietary folate restriction or folic acid supplementation can change DNA methylation.

Little is known about the environmental impact on DNA methylation in humans that are amenable to intervention.

Dietary intervention studies aimed to enhance life-long health, or to reduce the risk of common complex diseases, need good biomarkers of the healthy phenotype for use as surrogate endpoints. Given the plasticity of epigenetic marks and their responsiveness to dietary factors, it will be important to discover whether epigenetic marks can be used as biomarkers of health in such intervention studies (McKay et al., 2011)

Nutritional epigenetics may provide a novel way to naturally modulate epigenetic traits that are seen in the progression of particular diseases, such as cancer. Future research is still needed to better discriminate a “healthy” epigenetic pattern from those related to disease state, which may aid the medical field in making earlier and more specific disease diagnoses and prognoses (Dworkin et al., 2009; Levenson and Melnikov, 2012; Tammen et al., 2013).

In this framework, in order to describe and evaluate the role of nutrient intakes, of MD pattern and of gene-diet and gene-nutrient interactions we have conducted an epidemiological cross-sectional study on a population of women in childbearing age living in Catania, Sicily. The main aim was to define the scientific evidences useful for an integrated strategy of primary prevention against multifactorial diseases in terms of translational research from basic molecular epidemiology to effective prevention, with the following specific aims:

- 1) to investigate the distribution of the *MTHFR* C677T polymorphism in relation to women’s year of birth and to assess their folate intake and folic acid supplementation;
- 2) to study associations between folate intake, red blood cell (RBC) folate, total homocysteine (tHcy) and the *MTHFR* 677T allele;
- 3) to characterize the levels of the mtDNA deletion in the lymphocytes of healthy young women, taking into account folate intake, RBC folate levels, and the distribution of the *MTHFR* C677T polymorphism;
- 4) to explore the interactions between *TNFA* -308 G>A polymorphism and adherence to MD pattern or FA intakes, respectively, on overweight/obesity risk;
- 5) to determine whether MD patterns and folate intake are associated with global DNA methylation, using LINE-1 methylation as surrogate biomarker in leukocytes.

3. METHODS

3.1 Study design

Since 2008, all women at childbearing age referred to the Laboratory of the S. Bambino Hospital, Catania, an obstetric center for preconceptional, prenatal and/or postpartum care, were prospectively enrolled in the present cross-sectional study.

All women gave their informed consent to participate in the study. The study protocol was approved by the involved Institution.

Fasting venous blood samples were collected, from each enrolled women, in EDTA-containing tubes and aliquots were stored at -80° C until analysis.

Data were collected by trained interviewers using a structured questionnaire to obtain information on socio-demographic and lifestyle data, including education level, smoking habits, obstetric history and physical activity.

Furthermore, education level was collected and divided into two or three categories: low-medium (primary school) and high medium (high school education or greater) or as ≤ 8 (low), >8 and ≤ 13 (medium) and >13 (high) years of studies. Employment status was also recorded and women were classified as employed, unemployed, student and housewife. Body Mass Index (BMI) as kg/m^2 , was based on criteria from the World Health Organization (WHO, 1995). Pre-pregnancy BMI was based on self-reported pre-pregnancy weight.

3.2 Dietary Assessment

Nutrients intake during the past month was estimated by a validated semiquantitative 46-item food frequency questionnaire (FFQ) using the previous month as a reference period (Agodi et al., 2011). Previously, the validity of folate intake estimates from the FFQ was assessed in a random subsample of 30 women that completed both the FFQ and, afterwards, a 4-day weighted dietary record, obtaining a Spearman's correlation coefficient of 0.59 (Agodi et al., 2011). FFQ was administered by trained epidemiologists, for each of the food items, women were asked to report their frequency of consumption and portion size. The FFQ comprises eight food group items: cereals, bread and snacks; fish and egg; pasta and soup; dairy products; vegetables; fruit; sweets; beverages. An indicative photo atlas showing standard portion sizes (small, medium and large) was used to estimate the amount of each food item and to minimize inaccuracies. Nutrients intake derived from the FFQ by multiplying the frequency of intake for any food item by its respective portion size (g), was

calculated using the table of alimentary composition of the US Department of Agriculture, that had been modified to accommodate the particularities of the Italian diet (<http://ndb.nal.usda.gov/>).

Intake of folic acid from supplements was specifically addressed. Prevalence of folate deficiency was estimated by comparing folate intake with the recommended daily allowance (Institute of Medicine, 2001).

Adjustment for total energy intake is usually appropriate in epidemiologic studies to control for confounding, reduce extraneous variation, and predict the effect of dietary interventions. FA intake was both evaluated as average intakes in grams per day (g/day) and as percentage of energy (%E). Particularly, the “nutrient density” method, in which nutrient intake is divided by total energy intake (Willett et al., 1997), was used. Total daily energy intake was calculated as Kcal of energy provided by macronutrients (total proteins, carbohydrates, and lipids) and alcohol. Folate intake was energy-adjusted ($\mu\text{g}/1,000 \text{ kcal}$) using the general formula: average daily folate intake in microgram multiplied by a common denominator (1,000 Kcal) and divided by total energy intake, to give an estimate of nutrient density.

3.3 MDS and adherence to MD

Adherence to the MD pattern was assessed using the Mediterranean Diet Score (MDS), 9-Unit dietary score proposed by Trichopoulou et al (1995), including fruits and nuts, vegetables, legumes, cereals, lipids, fish, dairy products, meat products, and alcohol. Lipid intake was assessed by calculating the ratio of unsaturated (the sum of monounsaturated and polyunsaturated lipids) to saturated lipids.

A value of 0 or 1 was assigned to each of nine indicated components with the use of the median as the cut-off. For beneficial components (vegetables, legumes, fruits and nuts, cereal, fish and unsaturated-saturated lipids ratio), persons whose consumption was above the median were assigned a value of 1. For components presumed to be detrimental (meat, poultry, and dairy products, which are rarely nonfat or low-fat), persons whose consumption was at or below the median were assigned a value of 1. For ethanol, a value of 1 was assigned to men who consumed between 10 and 50 g per day and to women who consumed between 5 and 25 g per day. MDS components are calculated using median value as cut-off, thus, the score is population-based.

In order to classified women regarding adherence to MD, these were classified into three groups, through a categorical approach. Particularly, $\text{MDS} \leq 25^{\text{th}}$ percentile was defined as low adherence to MD; $\text{MDS} >25^{\text{th}}$ percentile but $\leq 90^{\text{th}}$ percentile as intermediate adherence

to MD; and MDS >90th percentile as high adherence to MD. Further, women were classified as with greater adherence to MD if MDS was >90th percentile of MDS distribution and as with poor adherence to MD if MDS was ≤90th percentile, other specified cut-off value were used when appropriate.

3.4 Blood Sampling and Analyses

Venous blood samples (2 ml) were collected in EDTA-containing tubes after at least 4 h of fasting. Plasma, obtained by centrifugation at 1,500 g for 15 min, was aliquoted and stored at -80° C. RBC folate levels were measured in the whole blood samples using a commercial kit (Elecsys Folate III Test System; Roche Diagnostics, Indianapolis, Ind., USA) according to the manufacturer's recommendations. The intra-assay coefficients of variation for the trilevel controls for RBC folate were 2.6, 2.4 and 1.7, and inter-assay coefficients of variation were 6.4, 5.1 and 5.8, respectively.

Hcy concentration in the plasma samples was determined using ion exchange chromatography. An HPLC system (Perkin Elmer) and a fluorescence detector (Varian Prostar) were employed (Signorelli et al., 2006).

Blood Folate deficiency was defined as RBC folate <305 nmol/L (Institute of Medicine, 2001) and taking into account that the concentration above which women were shown to have the lowest risk of a NTD-affected pregnancy, as RBC folate <906 nmol/L (Daly et al., 1995). Elevated Hcy level was defined as tHcy >10 µmol/L (Zappacosta et al., 2011).

In addition, women were classified according to the distributions across three clinically relevant subgroups defined by clinical or research laboratory tHcy values: >13(hyperhomocysteinemia), 10–13 (elevated tHcy) and <10 µmol/l (values in the desirable range) (Dhonukshe-Rutten et al., 2009).

3.4.1 DNA extraction

DNA was extracted from whole blood samples resuspended in fresh PBS using the High Pure PCR Template Preparation Kit (Roche Applied Science) according to the manufacturer's protocol, as described below, and stored at -80°C.

Procedure

- Into a nuclease-free 1.5 ml microcentrifuge tube add: 200 µl of blood samples, 200 µl of Binding Buffer and 40 µl of Proteinase K (reconstituted).

Then mix immediately and incubate at +70°C for 10 min. After that, add 100 µl of Isopropanol and mix well.

- Insert one High Pure Filter Tube into one Collection Tube and pipet the sample into the upper buffer reservoir of the Filter Tube.
- Insert the entire High Pure Filter Tube assembly into a standard table-top centrifuge and centrifuge 1 min at $8,000 \times g$.
- After centrifugation: remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.
- Combine the Filter Tube with a new Collection Tube.
- Add 500 μ l of Inhibitor Removal Buffer to the upper reservoir of the Filter Tube and centrifuge 1 min at $8,000 \times g$.
- Remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube and combine the Filter Tube with a new Collection Tube.
- Add 500 μ l of Wash Buffer to the upper reservoir of the Filter Tube, centrifuge 1 min at $8,000 \times g$ and discard the flowthrough.
- Remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube and combine the Filter Tube with a new Collection Tube.
- Add 500 μ l of Wash Buffer to the upper reservoir of the Filter Tube and centrifuge 1 min at $8,000 \times g$ and discard the flowthrough.
- After discarding the flowthrough: centrifuge the entire High Pure assembly for an additional 10 s at full speed and discard the Collection Tube. The extra centrifugation time ensures removal of residual Wash Buffer.
- To elute the DNA: insert the Filter Tube into a clean, sterile 1.5 ml microcentrifuge tube, add 200 μ l of prewarmed Elution Buffer ($+70^{\circ}\text{C}$) to the upper reservoir of the Filter Tube and centrifuge the tube assembly for 1 min at $8,000 \times g$.

The microcentrifuge tube contains the eluted, purified DNA, which can be used directly or stored at $+2$ to $+8^{\circ}\text{C}$ or $+15$ to $+25^{\circ}\text{C}$ for later analysis.

3.4.2 DNA quantification

DNA quantification was performed by the Qubit fluorometer (Invitrogen). The Qubit fluorometer uses fluorescent dyes to determine the concentration of nucleic acids and proteins in a sample, where each dye is specific for one type of molecule: DNA, RNA or protein. These dyes have extremely low fluorescence until they bind to their targets (DNA, RNA or protein). Upon binding, they become intensely fluorescent. The difference in fluorescence between bound and unbound dye is several orders of magnitude. The Qubit DNA dye used for the high sensitivity assay, has extremely low fluorescence until it binds

to DNA. Upon binding to DNA, probably by intercalation between the bases, it assumes a more rigid shape and becomes intensely fluorescent. Once added to a solution of DNA, the Qubit DNA dye binds to the DNA within seconds and reaches equilibrium in less than two minutes. At a specific amount of the dye, the amount of fluorescence signal from this mixture is directly proportional to the concentration of DNA in the solution. The Qubit fluorometer can pick up this fluorescence signal and convert it into a DNA concentration measurement using DNA standards of known concentration. The Qubit fluorometer uses DNA standards to derive the relationship between DNA concentration and fluorescence. It then uses this relationship to calculate the concentration of a sample, based on its fluorescence when mixed with the Qubit dye.

The Qubit dsDNA HS (High Sensitivity) Assay Kit was used. The assay is highly selective for double-stranded DNA (dsDNA) over RNA and is designed to be accurate for initial sample concentrations from 10 pg/ μ l to 100 ng/ μ l.

3.4.3 Analysis of TNF A -308 G>A polymorphism

Subjects were genotyped for the TNFA-308 G>A polymorphism (rs1800629) using the PCR-RFLP method, as described previously (Wilson, 1997; Szabò et al, 2011, Agodi et al., 2012).

A reaction volume of 25 μ L was amplified by polymerase chain reaction (PCR). Each reaction mixture contained 10 pmol of each primer (forward primer: 5' AGG CAA TAG GTT TTG AGG GCC AT 3'; reverse primer: 5' GGG ACA CAC AAG CAT CAA G 3'), 20 ng/ μ l of DNA samples, using pureTaq Ready-To-Go PCR Beads. PCR cycling conditions were 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min and 30 s, and a final extension of 1 cycle at 72 for 5 min.

The PCR products were digested by NcoI (Fermantas, Vilnius, Lithuania) at 37°C overnight. Electrophoresis of the digested PCR products was performed on a 5% NuSieve agarose gel (Lonza, ME, USA). Gels were stained with GelRed (Biotium, Inc, Hayward, CA, USA) in order to visualize the DNA fragments.

In particular, in homozygous wild type individuals (GG) fragments length was 87+(20) bps, in homozygous mutated individuals (AA), 107 bps and in heterozygous individuals (AG), 107 + 87 + (20) bps (figure 5).

3.4.4 Analysis of *MTHFR* C677T polymorphisms

Determination of *MTHFR* C677T polymorphism was performed as previously described (Agodi et al., 2011) using TaqMan allelic discrimination Assay, with the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). Allelic discrimination was carried out by measuring fluorescence intensity at the endpoint. The results of the measurement and subsequent genotype were evaluated with SDS software version 2.3 (Applied Biosystems). Allelic discrimination analysis is shown in figure 6.

3.4.5 Real-time PCR analysis of mtDNA deletions

mtDNA 4977-bp deletion was determined, by co-amplifying the mt ND1 gene with a rarely deleted region and the mt ND4 gene, which is commonly absent in the majority of patients with large deletions, according to the method of He et al. (2002), by real-time PCR analysis (figure 7). The PCR primers and the fluorogenic probe for the ND1 region and for the ND4 region were described elsewhere (Wu et al., 2009). PCR amplification was carried out in a 50 µl reaction volume consisting of TaqMan Universal Master Mix, 200 nmol/l of deletion ND4 primer, 100 nmol/l of each ND1 primer, 100 nmol/l of each mtDNA deletion probe, and the ND1 probe primer. The cycling conditions included an initial phase of 2 min at 50°C and 10 min at 95°C, followed by forty cycles of 15 s at 95°C and 0.5 min at 72°C. The fluorescence spectra were monitored using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). The cycle at which a statistically significant increase in normalized fluorescence was first detected was designated as the threshold cycle number (Ct). The Ct values were used to quantify the relative amount of ND4 to ND1 with the equation: $R = 2^{-\Delta Ct}$; where $\Delta Ct = mtCt_{ND4} - mtCt_{ND1}$. A smaller value of ΔCt indicates fewer mtDNA deletions. Furthermore, to quantify the fold change reduction of ND4, the negative inverse of R was computed (Schmittgen, T.D. and Livak, K.J., 2008).

3.5 DNA methylation technologies

Recent developments concerning epigenetic technologies are showing promising results of DNA methylation levels at a single-base resolution and provide the ability to differentiate between 5-methylcytosine and other nucleotide modifications such as 5-hydroxymethylcytosine. A large number of methods and powerful techniques can be used for the analysis of DNA methylation such as, digestion with methylation sensitive restriction enzymes combined with Southern blotting, amplification of bisulfite-treated DNA

with primers selective for unmethylated DNA (qualitative USP by gel analysis or quantitative real time USP), or nonselective amplification of bisulfite-treated DNA followed by determination of methylation by clonal sequencing, COBRA, or pyrosequencing (Mansego et al., 2013; Tollefsbol T, 2009) .

3.5.1 Pyrosequencing

Pyrosequencing is a high-throughput quantitative method which requires the use of bisulfite-converted DNA and has the same limitations derived from this treatment. This method is the gold standard technique for DNA methylation detection, providing also information on the methylation status of single-CpG sites. Using PCR, the DNA is amplified and tagged by a primer that is biotinylated. The pyrosequencing technology is based on the release of pyrophosphate when nucleotides incorporate into the sequencing primer only if it is complementary to the template DNA sequence. It is a method widely used in disease research and it has also been employed in different nutritional studies (Mansego et al., 2013).

3.5.2 LINE-1 methylation analysis

Methylation analysis of LINE-1 promoter (GeneBank accession no. X58075) in lymphocytes was investigated by pyrosequencing-based methylation analysis, using the PyroMark Q24 instrument (Qiagen), after DNA bisulfite conversion.

Location of L1 pyrosequencing sites 1-3. CpG island region of the human L1 transposon is reported in figure 8.

Bisulfite-pyrosequencing LINE-1 analysis

The methylation status of a DNA sequence can best be determined using sodium bisulfite. Incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. Therefore, bisulfite treatment gives rise to different DNA sequences for methylated and unmethylated DNA.

The most critical step for correct determination of a methylation pattern is the complete conversion of unmethylated cytosines. This is achieved by incubating the DNA in high bisulfite salt concentrations at high temperature and low pH.

These harsh conditions usually lead to a high degree of DNA fragmentation and subsequent loss of DNA during purification. Purification is necessary to remove bisulfite salts and chemicals used in the conversion process that inhibit sequencing procedures. Common

bisulfite procedures usually require high amounts of input DNA. However, due to DNA degradation during conversion and DNA loss during purification, such procedures often lead to low DNA yield, highly fragmented DNA, and irreproducible conversion rates.

Furthermore, the bisulfite thermal cycling program provides an optimized series of incubation steps necessary for thermal DNA denaturation and subsequent sulfonation and cytosine deamination, enabling high cytosine conversion rates of over 99%. Desulfonation, the final step in chemical conversion of cytosines, is achieved by a convenient on-column step included in the purification procedure.

The EpiTect Bisulfite procedure comprises a few simple steps: bisulfite-mediated conversion of unmethylated cytosines; binding of the converted single-stranded DNA to the membrane of an EpiTect spin column; washing; desulfonation of membrane-bound DNA; washing of the membrane-bound DNA to remove desulfonation agent; and elution of the pure, converted DNA from the spin column. The eluted DNA is suited for all techniques currently used for the analysis of DNA methylation, including PCR, real-time PCR, MSP-PCR, bisulfate sequencing (direct und cloning), COBRA, and Pyrosequencing.

Protocol: Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA

Bisulfite conversion and cleanup of DNA for methylation analysis of 1-100 ng of DNA extracted from each sample was completed using the EpiTect Bisulfite Kit (Qiagen) and the converted DNA was eluted with 20 µl of Elution Buffer, provided by the kit.

Bisulfite Conversion

Prepare the bisulfite reactions in a final volume of 140 µl, adding each component in the following order.

- DNA solution and RNase-free water (the combined volume of DNA solution and RNase-free water must total 20 µl).
- Bisulfite Mix (85 µl)
- DNA Protect Buffer (35 µl)

Close the PCR tubes and mix the bisulfite reactions thoroughly. DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.

Perform the bisulfite DNA conversion using a thermal cycler with the following parameters:

- Denaturation 5 min at 95°C and incubation 25 min at 60°C;
- Denaturation 5 min at 95°C and incubation 85 min at 60°C;
- Denaturation 5 min at 95°C and incubation 175 min at 60°C;

- Hold Indefinite at 20°C

Cleanup of bisulfite converted DNA

Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.

- Add 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA to each sample, mix the solutions by vortexing and then centrifuge briefly.
- Place the necessary number of EpiTect spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube into the corresponding EpiTect spin column, centrifuge the spin columns at maximum speed for 1 min, discard the flow-through, and place the spin columns back into the collection tubes.
- Add 500 µl Buffer BW to each spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
- Add 500 µl Buffer BD to each spin column, and incubate for 15 min at room temperature (15–25°C).
- Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
- Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.
- Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.
- Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.
- Place the spin columns with open lids into clean 1.5 ml microcentrifuge tubes (not provided) and incubate the spin columns for 5 min at 56°C in a heating block. This step enables evaporation of any remaining liquid.
- Place the spin columns into clean 1.5 ml microcentrifuge tubes. Dispense 20 µl Buffer EB onto the center of each membrane. Elute the purified DNA by centrifugation for 1 min at approximately 15,000 x g. To increase the yield of DNA in the eluate, dispense the eluate to the center of each membrane, and centrifuge for 1 min at maximum speed.

The bisulfite-converted DNA was stored at -20°C until used.

LINE-1 PCR

A reaction volume of 25 μ L was amplified by polymerase chain reaction (PCR), using the PyroMark PCR Kit (Qiagen). According to the manufacturer's instructions, each reaction mixture contained 10-20 μ g of bisulfite-converted DNA, 12.5 μ L of PyroMark PCR Master Mix 2X, containing HotStart Taq DNA Polymerase, 2.5 μ L of CoralLoad Concentrate 10X, 2 μ L of the forward primer (5'-TTTTTTGAGTTAGGTGTGGG-3') and the reverse-biotinylated primer (5'-biotin-TCTCACTAAAAAATACCAAACAA-3') (0.25 mM for each). PCR primers and pyrosequencing primer were reported by Estecio et al., 2007. HotStart PCR cycling conditions were 1 cycle at 95°C for 15 min (for HotStart Taq DNA Polymerase activation), 45 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30s, and a final extension of 1 cycle at 72°C for 10 min.

Electrophoresis of the PCR products was performed on a 2% Seakem Agarose (Lonza, ME, USA). Gels were stained with GelRed (Biotium, Inc, Hayward, CA, USA) in order to visualize the amplified DNA fragment of 290bps.

The biotinylated PCR product was purified and made single stranded to act as a template using the Pyrosequencing Vacuum PrepTool (Biotage, Inc., Charlottesville, VA, USA).

Immobilization of PCR Products to Streptavidin Sepharose HP Beads

For each sample, prepare a solution for DNA immobilization as described as follow.

Prepare a master mix with:

- Streptavidin Sepharose HP beads: 2 μ L
- PyroMark Binding Buffer: 40 μ L
- RNase-free water: 18 μ L

Aliquot the master mix to a PCR plate or strips and then add 20 μ L of PCR product.

Seal the PCR plate using strip caps and agitate the PCR plate constantly for 5–10 min at room temperature (15–25°C) using a mixer (1400 x g).

Proceed immediately with the protocol "Preparation of Samples for Pyrosequencing Analysis". Sepharose beads sediment quickly and capturing of beads must take place immediately once the agitation is complete.

Preparation of Samples for Pyrosequencing Analysis

The biotinylated single-stranded product was annealed to the pyrosequencing primer (5'-GGGTGGGAGTGAT-3', 0.4 mM final concentration) and then subjected to sequencing using an automatically generated nucleotide dispensation order for sequences to be analyzed

corresponding to each reaction. The pyrograms were analyzed using allele quantification mode to determine the proportion of cytosine/thymine and, hence, methylated and unmethylated cytosines at the targeted position(s). The degree of methylation was evaluated at three cytosine followed by guanine (CpG) methylation sites. Reproducibility of the assay was satisfactory with a coefficient variation of 2% to 2.2%.

This protocol is for the preparation of single-stranded DNA and annealing of the sequencing primer to the template before Pyrosequencing analysis using the PyroMark Q24.

Before starting, prepare the PyroMark Q24 Vacuum Workstation (figure 9) filling five separate troughs (supplied with the PyroMark Q24 Vacuum Workstation) with the following solution: ethanol (70%), denaturation Solution, wash buffer and water.

Procedure

- Dilute the sequencing primer to 0.3 μ M in Annealing Buffer and add 25 μ l diluted sequencing primer to each well to be analyzed on the PyroMark Q24 Plate.
- Place the PCR plate and PyroMark Q24 plate in the workstation.
- Apply vacuum to the tool by opening the vacuum switch.
- Carefully lower the filter probes into the PCR plate to capture the beads containing immobilized template. Hold the probes in place for 15 s. Take care when picking up the tool. Sepharose beads sediment quickly. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads.
- Transfer the tool to the trough containing 70 % ethanol (trough 1). Flush the filter probes for 5 s.
- Transfer the tool to the trough containing Denaturation Solution (trough 2). Flush the filter probes for 5 s.
- Transfer the tool to the trough containing Wash Buffer (trough 3). Flush the filter probes for 10s.
- Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes. While holding the tool over the PyroMark Q24 Plate, turn the vacuum switch off.
- Release the beads into the PyroMark Q24 Plate by gently shaking the tool in the wells.
- Transfer the tool to the trough containing high-purity water (trough 4) and agitate for 10s.
- Wash the filter probes by lowering the probes into high-purity water (trough 5) and applying vacuum. Flush the probes with 70 ml highpurity water.
- Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes.
- Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position. Turn off the vacuum pump.

- Heat the PyroMark Q24 Plate containing the samples at 80°C for 2 min using the PyroMark Q24 Plate Holder.
 - Allow the samples to cool to room temperature (15–25°C) for at 15 min.
- Proceed with the protocol “Quantification of CpG Methylation in LINE-1”,

Quantification of CpG Methylation in LINE-1

This protocol describes the loading of PyroMark Gold Q24 Reagents into the PyroMark Q24 Cartridge (figure 10), and quantification of CpG methylation level in LINE-1 using the PyroMark Q24.

- Load the cartridge with the appropriate volumes of nucleotides, Enzyme and Substrate mixtures according to the Pre Run Information.

Open the instrument lid and insert the reagent filled

Open the plate-holding frame and place the PyroMark Q24 Plate on the heating block. Close the plate-holding frame and the instrument lid.

For the set up enter the followings “Sequence to Analyze” and “Dispensation Order”:

TYGATTTTTTAGGTGYGTTYGTTA (Y represents a C or a T);

GTCGATTAGTAGTCAGTCGCT (C represents a control for bisulfite treatment).

The quantification of CpG methylation and quality assessment are displayed above each CpG site in the Pyrogram trace, as shown in figure 11.

To perform control reactions, we used three different types of human DNA: unmethylated genomic DNA as internal control to verify efficient sodium bisulfite DNA conversion, universal methylated and unmethylated bisulfite converted DNAs were run as controls.

Methylation quantification was performed using the PyroMark Q24 2.0.6 software.

We used non-CpG cytosine residues as built-in controls to verify bisulfite conversion. The degree of methylation was expressed for each DNA locus as percentage methylated cytosines over the sum of methylated and unmethylated cytosines.

3.6 Statistical analyses

All data collected in the present study, e.g. socioeconomics data, lifestyle factors, anthropometric data, nutrients consumption derived from the FFQ, MDS, and data from genetic polymorphisms, biomarkers of effect, and methylation analyses, were included in an electronic database designed *ad hoc* and statistical analyses were performed using the SPSS software (Version 14.0, SPSS, Chicago, IL).

Descriptive statistics will be used to characterize the population using frequencies, mean, median, range and standard deviation values.

Furthermore, the χ^2 test was used for the statistical comparison of proportions and to estimate the strength of associations by calculating the crude odds ratios (ORs) and the corresponding 95% confidence intervals (95% CIs).

Continuous variables were tested using Student's t-test and one-way ANOVA. Quantitative data were converted in qualitative data using the median values as the cut-off point or other specified cut-off value as appropriate.

Correlations were assessed by Spearman's rank correlations.

Genotype frequencies were calculated by determining the percentage of individuals carrying the different genotypes. Hardy-Weinberg equilibrium for each of the polymorphisms in study were tested using GENEPOP software, version 1.31.

Multivariate logistic regression analysis (Backward Stepwise - Likelihood Ratio method) was used to adjust for possible confounding effects and to obtain the adjusted OR with the respective CI 95%.

A *p* value < 0.05 was considered statistically significant in all performed analyses.

Gene-environment interaction analyses were conducted in order to evaluate the potential interaction between *TNFA* -308 G>A polymorphism and adherence to MD pattern or FA intakes, respectively, on overweight/obesity risk. The reference group was women without risk factor exposures, such as carriers of the GG genotype and with greater adherence to MD (i.e. >6) or with FA intakes above the 75th percentile of the unsaturated FA intake distribution or below or equal to the 75th percentile of the saturated FA intake distribution, respectively.

The degree of LINE-1 methylation was reported for each locus as well as the average percentage of methylation of the three CpG sites evaluated.

Percentages of lymphocytic LINE-1 methylation was categorized based on median distribution, as well as tertiles distribution.

Low global methylation (i.e. DNA hypomethylation) was defined as DNA methylation below the median value, or the first tertile (T1) of the distribution; high methylation level (i.e. DNA hypermethylation) was defined as DNA methylation above the median value, or the third tertile (T3) of the distribution.

The issue of statistical power and of sample size is critical in epidemiological studies. Our epidemiological study was designed to look at several exposure-outcome relationships (among all, the principal are: folate deficiency and poor MD adherence). Given the lack of

reference data regarding the association between MD exposure and folate deficiency and global DNA methylation, we assessed the statistical power at the end of the study. We have calculated separately the study size necessary to allow us to detect the most important exposure–outcome relationships. Ideally, we would then select the largest of these as our actual study size.

This approach will ensure that the study has enough power (ability to detect a statistically significant result if the true magnitude of the effect is as anticipated).

We have evaluated the power of our study (using EpiInfo version 6), given the observed frequency of outcome in the unexposed group and in the exposed one to obtain at least 80% power for a 95% confidence interval.

4. RESULTS

4.1 Distribution of the *MTHFR* C677T polymorphism in relation to women's year of birth, folate intake and folic acid supplementation

A total of 307 women were enrolled, the mean age was 28.9 years (median 28 years; range: 14–49 years). The majority (94%) of women were born in Italy and 45% were pregnant. Particularly, 43.8% of pregnant women were in the third trimester of pregnancy. The main characteristics of the women enrolled in the study are shown in Table 1.

Folate intake

A total of 43.3% women reported the use of folic acid supplements or of multimineral/multivitamin supplements containing folic acid, particularly, 87% of pregnant women and 7.7% of non-pregnant women ($p=0.000$). Reported use of folic acid supplements increased significantly from 71.4% in the first trimester of pregnancy to 88.1% in the second trimester and up to 95% in the third trimester ($p=0.001$) (table 2).

The mean folate intake, assessed by the validated FFQ, was 208.5 mg per day (median 196.0 mg per day; range: 47.4–939.7 mg per day), 222.8 mg per day for pregnant women (median 204.4mg per day; range: 67.2–939.7 mg per day) and of 196.9 mg per day for non-pregnant women (median 186.7 mg per day; range: 47.4–579.3 mg per day) ($p=0.026$). Mean folate intake increased, but not significantly, during the three trimester of pregnancy (Table 2).

Considering only diet and comparing folate intake with estimated average requirement, the prevalence of inadequate folate intake was 90.2%, 89.9% and 90.5%, respectively, for the overall sample, for pregnant women and for non-pregnant women. The prevalence of inadequate folate intake was 94.3%, 88.1% and 88.3%, respectively, in the first, in the second and in the third trimester of pregnancy.

Taking into account the use of supplements, and thus classifying each woman as taking or not taking supplements, the prevalence of inadequate folate intake decreased to 51.5% overall and during the three trimesters of pregnancy ($p=0.013$).

Following the percentile distribution, the population was divided into four groups according to birth date and prevalence of inadequate folate intake - considering only diet, without taking into account intake from supplements - was compared between groups. An increase of the prevalence of inadequate folate intake (from 88.3 to 91.7%) was observed in the group of women born in 1985–1994 when compared with the older group born in 1959–1973.

Inadequate folate intake was significantly larger among working women (61.7%) than housewives (43.7%; $p=0.002$) who tended to take folic acid supplements (51.1%) more than

women working outside (33.1%; $p=0.002$), also after controlling for educational level. Inadequacy was significantly larger among overweight or obese women (58.5%) than among women with normal weight (45.0%; $p=0.022$).

Furthermore, estimated folate intakes were ranked by tertiles and resulting rankings were compared across baseline characteristics of enrolled women. No statistically significant association was observed (Table 2).

Distribution of MTHFR C677T and A1298C polymorphisms

The distribution of *MTHFR* genotypes for the C677T and A1298C polymorphisms is shown in Table 3. The allelic distribution of both *MTHFR* polymorphisms follows the Hardy–Weinberg equilibrium expectations ($p= 0.26$). The relative frequencies of the 677T- and 1298C-mutated alleles were 43.8% and 30.5%, respectively. Overall, 20.5% of the subjects were homozygous for the 677TT-mutated genotype and 7.8% for the 1298CC-mutated genotype. The heterozygous status for both mutations was similar, 46.6% for the C677T mutation and 45.3% for the A1298C mutation.

Six of the nine possible genotype combinations were found for the two *MTHFR* polymorphisms, whereas no subjects had the *MTHFR* 677CT/1298CC, 677TT/1298CC or 677TT/1298AC genotype combination.

Genotype frequencies and age

Following the age percentile distribution, genotype frequencies of *MTHFR* polymorphisms were compared between groups.

For the C677T polymorphism an increase of the TT genotype (from 14.3 to 28.2%; $p=0.03$) was observed in the group of women born in 1980–1984 when compared with the group of women born in 1959–1973 (Table 3). Furthermore, an increase of the T-allelic frequency was shown from 36.4% in the group of women born in 1959–1973 to 48.1% in the group born in 1980–1984 ($p= 0.04$) and to 47.6% in the group born in 1985–1994 ($p=0.04$).

For the A1298C polymorphism no significant differences were observed in allelic or genotypic frequencies between the different groups (Table 4).

4.2 Dietary Folate Intake and Blood Biomarkers Reveal High-Risk Groups in a Mediterranean Population of Healthy Women of Childbearing Potential

RBC Folate Levels, Plasma Concentrations of tHcy and Folate Intake

In a subgroup of 204 women, with a mean age of 28.9 years (median 28; range 14–49 years), a total of 57.4% of women were pregnant and their mean gestational age was 23.1 ± 10.1 weeks (range: 4–40 weeks), 29.9% of women reported smoking (25.6% of pregnant women

and 35.6% of nonpregnant women), 51% of women reported the use of folic acid supplements (85.5% of pregnant women and 4.6% of nonpregnant women; $p < 0.001$) and the mean daily dose of folic acid from supplements was $477.2 \pm 217.1 \mu\text{g/day}$, RBC folate levels, plasma concentrations of tHcy and folate intake derived from FFQs in all women, and in pregnant and nonpregnant women were assessed (table 5). Subjects who reported consuming folic acid supplements had a significantly higher mean RBC folate level (394.5 nmol/L) and a significantly lower plasma concentration of tHcy ($13.9 \mu\text{mol/L}$) than those who did not take supplements (266.0 nmol/L and $15.2 \mu\text{mol/L}$, respectively). On the contrary, folate intakes were not statistically different ($216.0 \mu\text{g/d}$ versus $208.0 \mu\text{g/d}$). Furthermore, the mean RBC folate level was lower in smokers (317.7 nmol/l) than in nonsmokers (337.4 nmol/l), and the mean tHcy concentration was higher in smokers ($14.6 \pm 2.2 \mu\text{mol/l}$) than in nonsmokers ($14.5 \pm 2.9 \mu\text{mol/l}$), although differences between the two groups were not statistically significant.

Risk Assessment

A total of 49.0% of women had an RBC folate level $<305 \text{ nmol/l}$ (32.5% of pregnant women and 71.3% of nonpregnant women, $p < 0.001$). All women had an RBC folate level $<906 \text{ nmol/l}$. Furthermore, considering only women who did not take supplements, only 12.0% had an RBC folate level $= 305 \text{ nmol/l}$ (5.9% of pregnant women and 13.3% of nonpregnant women).

A total of 91.5% had tHcy concentration $>10 \mu\text{mol/l}$ (90.5% of pregnant women and 92.9% of nonpregnant women), and 8.5% had tHcy concentration $<10 \mu\text{mol/l}$ (9.5% of pregnant women and 7.1% of nonpregnant women), 12.4% had tHcy concentration between 10 and 13 $\mu\text{mol/l}$ (15.5% of pregnant women and 8.2% of nonpregnant women) and 79.1% had tHcy concentration $>13 \mu\text{mol/l}$ (75.0% of pregnant women and 84.7% of nonpregnant women).

Comparing folate intake with the estimated average requirements, the prevalence of inadequate folate intake was 45.1, 13.7 and 87.4%, respectively, for the overall sample, and pregnant and nonpregnant women, respectively ($p < 0.001$).

According to age, a total of 69.8% of women younger than 28 years (i.e. the median age) had RBC folate $<305 \text{ nmol/l}$ against 56.5% of older women ($= 28 \text{ years}$; $p = 0.050$).

Smokers showed a significantly higher mean folate intake ($239.3 \mu\text{g/day}$) than nonsmokers ($200.4 \mu\text{g/day}$; $p = 0.019$). However, among smokers, a total of 55.7% had RBC folate $<305 \text{ nmol/l}$ against 46.2% of nonsmokers.

Furthermore, 96.7% of smokers and 89.4% of nonsmokers had tHcy concentration $>10 \mu\text{mol/l}$.

A total of 47.0% of women with a medium-high level of education (= 13 years) had RBC folate <305 nmol/l against 76.3% of women with a low level of education ($p < 0.001$).

Correlation between RBC Folate Levels, Plasma Concentrations of tHcy and Folate Intake

RBC folate levels were negatively correlated with tHcy concentrations: $r_s = -0.19$ ($p=0.008$) and folate intakes, assessed by the FFQ, were negatively correlated with tHcy concentrations: $r_s = -0.17$ ($p=0.015$). On the contrary, no correlation between folate intakes from diet and RBC folate levels was shown, also controlling for supplement use.

Among smokers, folate intake was negatively correlated with tHcy concentrations: $r_s = -0.32$ ($p = 0.013$) and among nonsmokers, RBC folate levels were negatively correlated with tHcy concentrations ($r_s = -0.32$ ($p = 0.013$) and among nonsmokers, RBC folate levels were negatively correlated with tHcy concentrations ($r_s = -0.17$; $p = 0.043$).

MTHFR C677T Polymorphism

RBC folate levels, plasma concentrations of tHcy and folate intakes, and their correlations were stratified by genotype. Genotype distribution was 28.9, 46.1 and 25.0% for CC, CT and TT, respectively. The allelic distribution of *MTHFR* polymorphisms follows Hardy-Weinberg equilibrium expectations ($p > 0.05$).

A total of 34.4% of women younger than 28 years had the TT genotype against 16.7% of women older than 28 years ($p=0.004$).

Neither mean RBC folate level nor mean plasma concentration of tHcy and mean folate intake varied significantly by genotype, also comparing the carriers of the T allele (TT or CT genotypes) versus the CC genotype and the TT genotype versus the carriers of the C allele (CC or CT genotypes). Mean (\pm SD) RBC folate level, tHcy concentration, and folate intake according to the *MTHFR* C667T genotype are shown in Table 6.

Furthermore, no significant differences were identified stratifying by pregnancy and smoking status (data not shown).

Analysis of correlation between biomarkers levels, stratified by genotype, showed that only in the CC group, RBC folate levels were negatively correlated with plasma concentrations of tHcy $r_p = -0.25$ ($p=0.015$). No other significant correlations stratified by genotype were found (data not shown).

4.3 Levels of mtDNA 4977-bp deletion in lymphocytes of healthy young women, folate intake, RBC folate levels, and distribution of the *MTHFR* C677T polymorphism

In a group of 476 women of childbearing age, with a mean age of 28.4 years (median 27 years, interquartile range: 23 – 33 years; range: 14 - 49 years), a total of 42.1% of enrolled

women (n=199) were smokers. Regarding BMI, only 8.8% of women were underweight (n=42), 56.1% were of normal weight (n=267) and 35.1% overweight or obese (n=167).

Mean folate intake, assessed by the FFQ, thus considering the total folate intake from food excluding folic acid supplements, was 242.9 µg/d (median 219.0 µg/d; range: 47.4 – 972.8 µg/d). Particularly, mean folate intake was 264.0 µg/d (median 239.4 µg/d; range: 49.4 – 972.8 µg/d) in pregnant women, and 222.4 µg/d (median 204.9 µg/d; range: 47.4 – 773.0 µg/d) in non-pregnant women (p=0.001). Mean folate intake did not vary significantly by age (253.0 µg/d in women younger than 27 years - i.e. the median age, and 232.6 µg/d in older women; p=0.093).

A total of 45.6% (n=217) of enrolled women reported intake of folic acid supplements or of multi-mineral/multivitamin supplements containing folic acid. Thus, taking into account the intake of folic acid supplements the prevalence of inadequate folate intake was 45.4% (n=216) for the overall sample of women, 13.2% (n=31) in pregnant and 76.8% (n=185) in non-pregnant women (p=0.001).

Mean RBC folate level was 322.3 nmol/L (median 291.2 nmol/L; range 111.9 - 622.2 nmol/L). Particularly, mean RBC folate level was 372.8 nmol/L (median 364.6 nmol/L; range: 111.9 – 622.2 nmol/L) in pregnant women, and 273.6 nmol/L (median 255.4 nmol/L; range: 114.4 – 542.5 nmol/L) in non-pregnant women (p<0.001). Besides, the mean RBC folate level was 303.9 nmol/L in women younger than 27 years (i.e. the median age) and 338.6 nmol/L in older women (p=0.023). Considering all women, the prevalence of folate deficiency was 52.4%, RBC folate level <305 nmol/L: 33.6% in pregnant women and 70.5% in non-pregnant women, p<0.001.

The relative frequency of the T mutated allele of the *MTHFR* C677T polymorphisms was 43.8%. Overall, 20.5% of the subjects were homozygous for the 677TT mutated genotype and 46.6% were heterozygous.

MtDNA 4977-bp deletions in the study subjects

Mean level of lymphocytic mtDNA 4977-bp deletions of the women in study was $\Delta C_t = 1.24$ (median 1.09, range 0.12 – 2.85). Mean R value was 0.45 (median 0.47 range 0.14 – 0.92). Furthermore, the mean fold change reduction of ND4 was -2.55 (median -2.13; range -7.19 - -1.08).

Mean levels of lymphocytic mtDNA 4977-bp deletions did not differ significantly by age (quartiles distribution and median value as cut-off), smoking habits, pregnancy status, inadequate folate intake, folate deficiency, use of folic acid supplements and homozygous 677TT mutated genotype. However, mean levels of lymphocytic mtDNA 4977-bp deletions

were significantly higher in underweight and normal weight than in overweight and obese women considering both ΔCt value (Table 7) and fold change reduction of ND4 (data not shown).

Correlation between lymphocytic mtDNA 4977-bp deletions and folate levels, as folate intake estimated by FFQ and RBC folate level, were assayed. A significant, albeit weak, correlation between folate intake and lymphocytic mtDNA 4977-bp deletions (ΔCt value) was showed ($r = 0.133$, $p = 0.004$). Furthermore, lymphocytic mtDNA 4977-bp deletions (ΔCt value) were not significantly correlated with RBC folate levels ($r = -0.063$, $p = 0.316$).

Univariate analysis was used to assess the association between lymphocytic mtDNA 4977-bp deletions (using the median value of ΔCt as cut-off) and age (using the median value as cut-off), smoking habits, pregnancy status, nutritional status, inadequate folate intake, folate deficiency, use of folic acid supplements and homozygous 677TT mutated genotype. After multivariate logistic regression analysis, results did not show any significant relationship between the above listed factors and lymphocytic mtDNA 4977-bp deletions (Table 8). The same results were obtained using as cut-off the median value of fold change reduction of ND4 (data not shown).

4.4 Interactions between *TNFA* -308 G>A polymorphism and adherence to MD pattern or FA intakes, on overweight/obesity risk

In a group of 380 women enrolled (main characteristics of the women are shown in Table 9), the mean age was 28.7 years (median 27 years; range: 13–85 years). The majority of women (95.3%) were born in Italy and 53.4% were pregnant. A total of 23.7% of women were current smokers. Furthermore, 58.7% of women were normal weight and 32.6% overweight or obese.

*Distribution of *TNFA* -308 G>A polymorphism*

The distribution of *TNFA* -308 G>A polymorphism is shown in Table 9, 80.5% of women were homozygote wild-type (GG), 18.2% heterozygote (AG) and 1.3% homozygote mutant (AA). Thus, allele G frequency was 89.6%. Genotype frequencies follow the Hardy–Weinberg equilibrium expectations ($p = 0.27$).

Dietary assessment

Mean energy and FA intakes (g/day) between underweight/normal weight women and those overweight/obese, are reported in Table 10. Except for unsaturated/saturated FA ratio, the mean values of FA intakes (unsaturated and saturated FA) and the mean energy intake were statistically significant higher in underweight/normal weight than in overweight/obese

women. However, considering the mean FA intakes as daily %E, differences between the two groups were not statistically significant (data not shown). The mean MDS value was 4.1 (median 4; range 0-9, Table 1). According to MDS, 8.2% of women were classified as with greater adherence to MD (MDS >90th percentile of MDS distribution; i.e. >6) and 91.8% as with poor adherence to MD (MDS ≤90th percentile of MDS distribution; i.e. ≤6;).

Mean energy and FA intakes (g/day) between women with greater adherence to MD and those with poor adherence to MD, are reported in Table 11. Except for some unsaturated FA, i.e. arachidonic acid, docosahexaenoic acid, for total saturated FA and palmitic acid, the mean unsaturated FA intakes and the mean energy intake were statistically significant higher in women with greater adherence to MD than in women with poor adherence. Furthermore, considering the mean FA intakes as daily %E, the mean saturated FA intake was statistically significant higher in women with poor adherence to MD than in the other (data not shown).

A significantly higher proportion of women with poor adherence to MD (34.4%) were overweight/obese than the other (12.9%; $p=0.015$). Therefore, the risk of being overweight/obese due to poor adherence to MD was 3.5 fold increased (OR: 3.54; 95% CI: 1.21 - 10.34).

Following the percentile distribution, the population was divided into four age groups and mean MDS values were compared between groups. A significantly increase of mean MDS values was observed from 3.6 in the age group 13-22 years, to 4.04 in the age group 23-33 years, to 4.24 in the age group 34-41 years, to 4.45 in the age group 42-85 years (one-way ANOVA, $p=0.004$).

Education was positively associated with adherence to MD, i.e. less-educated women showed a lower adherence to MD, although this association was not statistically significant (data not shown).

A significant association between education and overweight/obesity was observed: 39.6% of women in lower (≤ 8), 26.1% in medium (>8 and ≤ 13) and 21.6% in highly (>13 years of studies) educated groups, were overweight or obese (p for trend= 0.003).

Considering employment status, any statistically significant association was shown with adherence to MD, instead, 40.7% of housewife, 30.0% of employed, 21.2% of unemployed and 19.6% of student were overweight or obese (p for trend= 0.042).

Gene–environment interactions

A total of 37.0% of carriers of the A allele (AA or GA genotypes) and a total of 31.6% of carriers of the GG genotype were overweight/obese and this difference was not statistically significant ($p= 0.383$).

Interaction between *TNFA* -308 G>A genotypes and adherence to MD on overweight/obesity risk was assessed. The risk of overweight/obesity was higher in women with both exposures (i.e. carriers of the A allele and with poor adherence to MD) than the reference group (OR: 2.464; 95%CI: 1.079-5.629).

Results of the interaction analysis between *TNFA* -308 G>A genotypes and FA intakes are reported in Table 12 and in Table 13. There were significant interactions between *TNFA* -308 A allele and linoleic acid, γ -linolenic acid, α -linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, poly-unsaturated FA, mono-unsaturated FA and total unsaturated FA intake on overweight/obesity risk (Table 12). Considering total saturated FA intake no significant interactions were found (Table 13).

4.5 Association between MD pattern, folate intake and LINE-1 methylation level

In order to control any confounding effect of pregnancy on epigenetic changes, LINE-1 methylation analysis was carried out in the subgroup of 177 non pregnant women (46.6% of the overall sample).

Mean age was 30.75 years (median 28 years; interquartile range: 23 – 37 years; range: 13 - 85 years), and a total of 75% of enrolled women (n=132) were non current smokers. Regarding BMI, only 6.8% of women were underweight (n=12), 60.4% were normal weight (n=107), 18.1% were overweight (n=32) and 14.7% were obese (n=26).

The main characteristics of the women enrolled in the study are shown in Table 14.

Dietary assessment

The mean crude folate intake, assessed by the validated FFQ, was 245.8 μ g/day (median 228.8 μ g/day; range: 46.3–773 μ g/day). Only 6.8% of women reported the use of folic acid supplements or of multimineral/multivitamin supplements containing folic acid.

The mean MDS value was 4.1 (median 4; range 0-9). According to MDS, 36.7% of women were classified as with poor adherence to MD (MDS \leq 25th percentile of MDS distribution; i.e. 0-3), 53.7% as with average adherence to MD (4-6) and only 9.6% as with greater adherence to MD (MDS >90th percentile of MDS distribution; i.e. 7-9).

In our population, mean folate intake was significantly lower (p=0,000) of the recommended intake value (400 μ g/day, IOM, 2001).

Considering only diet and comparing folate intake with recommended daily intake, the prevalence of inadequate folate intake was 88.1%.

Mean crude folate intake and adjusted values, as nutrient density - considering only diet, without taking into account intake from supplements - were compared across baseline

characteristics of enrolled women (Table 15). Considering age groups, age below or above the median value and quartiles distribution reported no significant differences. Particularly mean crude folate intake was lower in obese women (192.5 µg/day) than in non obese (254.9 µg/day) (p= 0.017). Furthermore, analysis of variance between nutritional status groups reported decreased mean folate values with increasing BMI (p=0,002). Conversely, mean crude folate intake as well as nutrient density folate intake reported a positive trend when compared among MDS groups (176.9 µg/day in MDS 0-3; 273.7 µg/day in MDS 4-6 and 353.0 µg/day in MDS 7-9; 110.9 µg/day in MDS 0-3; 138.5 µg/day in MDS 4-6 and 149.3 µg/day in MDS 7-9, respectively) (one-way ANOVA, p=0.000, both).

Similarly, mean MDS was compared across baseline characteristics of enrolled women (Table 16). A significantly increase of mean MDS values was observed from 3.74 in the age group below or equal to 28 years (median), to 4.53 in the age group above 28 years (p=0.002). Following the age percentile distribution, a positive trend was reported (one-way ANOVA, p=0.001). Considering BMI, mean MDS was lower in obese women (3.27) than non obese (4.27) (p= 0.007).

Furthermore, mean MDS was significant lower in women with low-mean education level (3.82) than in women with mean-high education level (4.36) (p=0.042)

Finally, women who consumed folic acid or vitamin supplements reported higher mean MDS (5.27), compared with others (3.89) (p=0.000).

Taking into account the use of supplements, and thus classifying each woman as taking or not taking supplements, the prevalence of inadequate folate intake was 83.1%. Notably, a significantly higher proportion (36.7%) of women with folate deficiency were overweight/obese than women with no folate deficiency (13.3%; p=0.013). Therefore, the risk of being overweight/obese due to folate deficiency was 3.8 folds increased (OR: 3.8; 95% CI: 1.3 – 11.4) (Table 17). In addition, considering only women with poor adherence to MD (MDS ≤6, i.e. 90th percentile) the risk increased up to 4.1 folds (OR 4.1; 95%CI: 1.2 – 14.4), on the contrary, no association was shown in the group of women with greater adherence to MD (data not shown).

Global DNA methylation

Preliminary experiments of LINE-1 methylation led to the definition of the protocol that was carried out and repeated several times in order to provide data comparable with those from the reference literature.

Mean global LINE-1 methylation level (considering all three loci) was 65.3% (SD±3.3) and range was from 52% to 74%. Mean LINE-1 methylation levels for each locus are shown in table 18.

Methylation levels of the three loci positively correlate with each other ($p=0,000$).

Table 18 shows mean levels of global methylation according to women characteristics. No significant differences were found.

Mean global methylation levels, were lower in folate deficiency group ($p=0.054$, borderline, table 19). However, women with folate deficiency had a 4 folds increased risk to be in the lowest tertile of global methylation level (T1, hypomethylation) compared with women with no folate deficiency (T3 vs. T1: OR = 4; 95% CI: 1.3–11.8) and the association showed a dose-response relationship ($p\text{-trend} = 0.032$) (table 20).

Women with low DNA methylation levels (i.e. \leq median or in T1) did not report any differences on average MDS, compared with those with high DNA methylation levels (i.e. $>$ median or in T3), and no associations were found between LINE-1 methylation, considering all the three loci, and MDS distribution.

However, when all the 9 Mediterranean food components (of the MDS) were individually examined by DNA methylation level, women whose consumption of fruit and nuts was below the median value (201 gr/day) had a 2.4 folds increased risk to be in the lowest tertile of global methylation level (T1, hypomethylation) compared with women whose consumption was above the median value (T3 vs. T1: OR = 2.8; 95% CI: 1.3–5.8) (table 21) and the association showed a dose-response relationship ($p\text{-trend} = 0.019$) (data not shown).

When considering only the methylation level at the locus 1, LINE-1 methylation ($> 75^{\text{th}}$ percentile) was significantly higher in women with greater adherence to MD ($MDS \geq 5$) than women with lower adherence to MD ($MDS \geq 6$) ($p = 0.000$; OR = 4,2; 95% CI: 1.8–9.4).

Consumption of fruit and nuts and consumption of vegetables were associated with DNA methylation at the locus 1. In fact, women whose consumptions of these two beneficial components were below the median values had an increased risk to have methylation level $\leq 75^{\text{th}}$ percentile compared with women whose consumption was above the median value (for fruit and nuts: $p = 0.001$; OR = 4.4; 95% CI: 1.8–10.9, for vegetables: $p = 0.021$; OR = 2.6; 95% CI: 1.1–5.8, respectively) (data not shown).

Multivariate logistic regression analysis was used to assess the association between LINE-1 methylation levels (T1 vs. T3), as independent variable, and age (using the median value as cut-off), smoking habits, nutritional status (using BMI > 25 as cut-off value), folate deficiency, smoking status and the 9 Mediterranean food components of the MDS

individually considered (using the median value as cut-off value), total dietary calorie intake (using the median value as cut-off value) controlling for possible confounders.

A first multivariate analysis was performed including only the two variables (folate deficiency and fruits and nuts intake) significantly associated with global LINE-1 methylation in the univariate analysis. Results showed a significant relationship between low fruits and nuts intakes and low overall methylation level ($p = 0.033$; OR = 2.3; 95% CI: 1.1–4.9). Finally, folate deficiency showed a borderline relationship with low methylation level ($p = 0.053$; OR = 3; 95% CI: 1–3.3) (Table 22).

A second multivariate analysis was performed including all the characteristics in study. Results confirmed the significant relationship between low fruit and nuts intake and hypomethylation ($p = 0.006$; OR = 3.8; 95% CI: 1.5–9.7) (Table 23).

Any significant relationship between the above listed factors and methylation level at locus 1 was observed after multivariate analysis.

In order to assess the association between folate deficiency (exposure) with low global methylation level (risk), given the observations obtained in our study (the observed frequency of outcome in the unexposed group that was 25% and in the exposed one that was 57%) and our sample size, the power of our study was about 95%, with a 99% confidence interval. However, the power of the study, to assess the association between poor adherence to MD (exposure) with global hypomethylation level (risk), was too low, particularly, considering the observed frequency of outcome in the unexposed group (41.7%) and in the exposed one (54.2%), we needed to enroll about 500-530 women to obtain a significant association between poor adherence to MD and global hypomethylation.

5. CONCLUSIONS AND DISCUSSION

In gene-environment interactions, the genetic polymorphisms that modify the effects of environmental exposures are transmitted transgenerationally according to Mendelian genetics, and the trait determining effect modifications is generally assumed to follow the same genetic model (dominant, co-dominant, recessive) as that of the levels of expression or function of the protein coded by the locus of concern. A second well established area of interplay includes the direct effects of environmental exposures on the genome, e.g., DNA damage and/or mutations induced by environmental exposures. In principle, the effect-modification model should apply to epigene-environment interactions as well as to gene-environment interactions. Similarly to the effect modifications demonstrated or postulated for genetic polymorphisms, epigenetic differences determining disease risk could make individuals less or more vulnerable to environmental (Bollati et al., 2007).

Dietary factors have become agents of strong interest in the field of epigenetics. In fact, several naturally occurring dietary phytochemicals have been demonstrated to have anticarcinogenic properties and may play a role in regulating biological processes (Kunnumakkara et al., 2008). Many studies have shown that natural products have epigenetic targets in cancer cells and can act as cancer preventive agents. Compounds found in dietary phytochemical preparations such as teas, garlic, soy products, herbs, grapes and cruciferous vegetables are now generally accepted to defend against the development of many different types of tumors as well as acting as epigenetic modulators that impact not only the initiation, but also the progression of oncogenesis (Ravindran et al., 2009; Shu et al., 2010). While many of the aforementioned studies were conducted using a particular dietary factor, it is reasonable to believe that most may be consumed in combination and over a period of a lifetime. This may provide a rationale for studying nutrient epigenetic modifiers more in combination studies or the proposal of an 'epigenetic diet' focused on consuming products that show the ability to stimulate beneficial epigenetic modifications (Hardy and Tollefsbol, 2011). Therefore, nutrigenetics could reveal risks and benefits of specific diets or dietary components to the individual and thus assist the development of personalized dietary recommendations instead of generalized ones. By contributing to the definition of optimal dietary and behavioral (i.e., physical activity and biorhythms) recommendations aimed at preventing disease and promoting optimal health and aging, nutrigenetics offers substantial and prudent direction in the translation of nutrition research into public health recommendations (Ordovás Muñoz et al., 2013).

5.1 Distribution of the *MTHFR* C677T polymorphism in relation to women's year of birth, folate intake and folic acid supplementation

The *MTHFR* C677T has been a focus of increasing interest worldwide since its genetic identification in 1995, and accurate information on its distribution can contribute to studies of gene–disease associations. Our study, conducted in a sample of the women population in Sicily (southern Italy), reports a TT genotype frequency of 20.5%, consistently with previously published works. Notably, an increase in the prevalence of the TT genotype in the cohort of women born since 1959 was shown, although this finding should be interpreted with caution given the sample size.

A variation in the prevalence of the TT homozygous genotype was previously reported, with the presence of a north to south gradient. Particularly in Europe, this prevalence has been shown to increase from the lowest values in the north (4–7%), to the highest frequencies in southern Italy (20.1 and 19.9% in Sicily; Wilcken et al., 2003; Guéant-Rodriguez et al., 2006). The mechanisms generating this gradient are not clearly known, although it has been hypothesized that they could involve, at least in part, gene–nutrient interactions and that in Europe dietary folate may have influenced the prevalence of the T allele (Guéant-Rodriguez et al., 2006). In fact, some results obtained in subgroups of the European populations confirmed a south to north decreasing gradient of dietary folate intake and of the T-allele frequency: a high frequency of the TT genotype in the presence of a high concentration of folate in plasma and a low frequency in the presence of low folate concentrations have been reported (Guéant-Rodriguez et al., 2006).

Our cross-sectional study was conducted on women of childbearing age in Catania, Italy, in which no folic acid fortification has been introduced but only supplementation in the periconceptual period is routinely recommended since the 1980s. The observed increase of the T-allelic frequency in the group of women in study, born in the last quarter of the last century, has been already associated with some selective advantage in a previous research conducted on a Spanish population (Mayor-Olea et al., 2008). Particularly, it has been reported that selection in favor of the T allele could be due to the increased fetal viability in early stages of embryonic development due to an increase in folic acid and vitamin supplements intake by women in the periconceptual period that began to be established in Spain in the last quarter of the 20th century as well as it started in other European countries including Italy (Mayor-Olea et al., 2008). Previous studies have suggested that the *MTHFR* TT homozygosity may confer a survival advantage in populations with adequate dietary folate consumption (Munoz-Moran et al., 1998; Rosenberg et al., 2002; Guéant-Rodriguez et

al., 2006). The T allele is associated with a greater risk of neural tube defects in those geographical areas or ethnic groups with a high frequency of this genotype, but that risk seems to be neutralized by a diet rich in folate, such as the MD (Zetterberg, 2004). In fact, in southern Italy, the TT genotype is common, but the rate of neural tube defects is not particularly high (International Clearinghouse for Birth Defects Monitoring Systems, 2001), probably because environmental and nutritional factors are likely to modulate the risk (Wilcken et al., 2003).

In our study, using the validated FFQ, taking into account the folic acid supplements and comparing the overall intake with estimated average requirement, the prevalence of inadequate folate intake was 51.5%, significantly higher in non-pregnant women (83.4%) than in pregnant ones (12.3%). Furthermore, the prevalence of inadequate folate intake significantly decreased during the three trimesters of pregnancy (from 25.7 to 5.0%; $p=0.013$). These findings may be the result of the higher use of folic acid supplements in pregnancy (87.0%) compared with the lower use in non-pregnant women (7.7%). Our results show that the use of folic acid supplements increased significantly during the three trimester of pregnancy (from 71.4 to 95.0%; $p=0.001$). Intake of multimineral/multivitamin from supplements during pregnancy is an attractive option to improve the nutritional status of pregnant women. However, although it has been recently reported that the daily use of multivitamin and mineral supplements (mainly folic acid) during any stage in pregnancy is not associated with lower birthweight and taking supplements in the third trimester has been associated with a threefold increased risk of preterm delivery (Alwan et al., 2010). Even if those findings need to be confirmed by other cohorts and/or trials, they suggest that clinicians should be cautious when recommending multivitamin–mineral supplements to women in late pregnancy due to the possible implications in the pregnancy outcome. Nutritional status and food consumption may be important HPV cofactors that increase risk of persistence and progression to CIN. Using the validated FFQ, a high prevalence of folate intake inadequacy has been shown along with an increase in mutated *MTHFR C677T* polymorphism that may modulate the risk of cancer according to folate status (Henaó et al., 2005; Piyathilake et al., 2007; Flatley et al., 2009). According to our study, healthy young women may have higher folate needs due to the increased prevalence of the T allele and reduced folate intake compared with older groups. In interpreting the findings of this study, its limitations should be considered. First, we have not analyzed biomarkers of folate status, as our aim was to measure habitual folate intake over an extended period of time, although biomarkers of folate status could have helped us to better define folate inadequacy and to

validate our FFQ. All dietary assessment instruments are associated with different, and sometimes considerable, random and systematic measurement errors (Kristal and Potter, 2006; Willett and Hu, 2007; Ortiz- Andrellucchi et al., 2009) and thus, when possible, biomarkers have to be incorporated into the validation of dietary assessment methods (Jenab et al., 2009), because folate biomarkers are very robust and sensitive markers of folate status. However, it has been reported that biomarkers are affected by different sources of error than FFQs, such as variation over time probably reflecting changes in intake, and, on the contrary, the advantages of using FFQs include measuring usual food intake over an extended period, allowing for large study samples (Johansson et al., 2010). Besides, this study was not designed to measure other important protective micronutrients, such as other B-vitamins for example, riboflavin) or antioxidants (for example, vitamins C, A, E and carotenes), and this could be an important limitation of the study, nevertheless the FFQ used in our study was designed and validated using WDR, in order to estimate only folate intake, with a correlation coefficient of 0.59, in accordance with other FFQ validation studies (Erkkola et al., 2001; French et al., 2001; Flood et al., 2004; Johansson et al., 2010).

Therefore, future studies, carefully designed to address the above limitations, are needed to further our knowledge about the critical role of maternal nutrition and, in particular, of micronutrients to reproductive health. Furthermore, potential implications for folate requirements are highlighted by our results. Healthy young women may have higher folate needs due to reduced folate intake compared with older groups and moreover they show an increasing prevalence of the T allele, which in turn may require supplement recommendation. Besides, appropriate timing of supplement use should be advised in order to prevent a poor pregnancy outcome, as it has been previously suggested to occur in the late pregnancy (Alwan et al., 2010).

5.2 Dietary Folate Intake and Blood Biomarkers Reveal High-Risk Groups in a Mediterranean Population of Healthy Women of Childbearing Potential

As sources of folate may have changed among younger generations in Mediterranean countries, it is necessary to evaluate the current diet of women of childbearing potential in terms of adequacy of folate nutrition, using dietary intake measures and biomarkers, in order to assess the need of folate supplementation and to address public health interventions.

Our study revealed significant folate deficiency in our Mediterranean population and higher than ideal Hcy concentrations, thus emphasizing that in these groups an improvement in the

folate status is needed via a foodbased approach or supplement. Consequently, public health policy strategies aiming at improved supplementation are required.

The importance of folate in human health is well recognized. Pregnant women are at risk of folate insufficiency because of the increased need for folate for rapid fetal growth, placental development and enlargement of the uterus (Fekete et al., 2010).

RBC folate is a measure of the long-term folate status because it reflects tissue folate stores. An RBC folate level >906 nmol/l has been associated with a significantly decreased risk of NTD pregnancies. Metabolically, increased folate intake first increases serum folate concentrations and then erythrocyte concentrations. Folate is incorporated into erythrocytes during their formation in the bone marrow (Institute of Medicine, 2001). Experts had regarded RBC folate concentrations as better folate status biomarkers because they are integrative measures of folate intake over the 90- to 120-day lifespan of RBCs, whereas serum folate concentrations reflect recent intake (Yetley et al., 2011). An erythrocyte folate value of 305 nmol/l has been chosen as the cut-off point for an adequate folate status for the general adult population (Institute of Medicine, 2001). Maternal tHcy concentrations have been linked to a wide range of adverse pregnancy outcomes, and a recent meta-analysis reported that higher maternal tHcy concentrations are associated with a small increased risk for small-for-gestational-age offspring, which is of little clinical relevance for the individual newborn but of greater importance at a population level (Hogeveen et al., 2012).

Although in our cross-sectional study no information about pregnancy outcome was collected, we highlighted important inadequacies in both the folate status and the Hcy status in women of childbearing potential in Sicily, a Mediterranean area. In fact, considering a cutoff value of 305 nmol/l, about half of the women (49.0%) were deficient in RBC folate and all women had an RBC folate level <906 nmol/l, even though 51% of the subjects reported use of folic acid supplements. Furthermore, considering women who did not take supplements, only 12.0% had an adequate RBC folate level (>305 nmol/l). Our results confirmed those recently reported in a sample of healthy subjects living in a Southern province of Italy (Zappacosta et al., 2011), where the prevalence of an adequate RBC folate level was 14.5% in women of childbearing age and none showed adequate levels considering the higher cut-off value of 906 nmol/l. Furthermore, in our population, considering diet and supplement usage, the prevalence of inadequate folate intake was outstandingly higher in non-pregnant women (87.4%), suggesting a possible deleterious shift from the traditional MD in the young. In our population, vegetables and fruit were the principal folate-contributing food followed by cereals and by a large number of other food groups.

Furthermore, pregnant women consumed more vegetables, fruit and cereals than the non-pregnant women, although no significant difference was found (data not shown). Those food groups are typical of the MD, a complex dietary pattern that is generally characterized by frequent consumption of fruit, vegetables, legumes, cereals and fish; low consumption of meat and cheese, and, as a major common characteristic, use of olive oil. Further research is needed in order to describe –in pregnant and nonpregnant women – to what extent dietary intake is shifting from the MD also in terms of food groups consumed in order to design targeted public health interventions in our population.

An ideal Hcy concentration ($<10 \mu\text{mol/l}$) was observed only in 8.5% of women and as much as 79.1% had tHcy concentration $>13 \mu\text{mol/l}$. However, with respect to pregnancy, our results confirm that in pregnant women the mean plasma concentration of tHcy is lower than in non pregnant women, a finding that, according to some authors, is only in part explained by the hemodilution and the reduced plasma albumin level during pregnancy (Refsum et al., 2004). Although in previous studies a tHcy concentration $>10 \mu\text{mol/l}$ is uncommon in pregnant women (Hogeveen et al., 2012; Refsum et al., 2004; Hogg et al., 2000), in our study a substantial proportion of pregnant women (90.5%) showed elevated tHcy levels, which indicates unfavourable cardiovascular disease conditions and a higher risk of birth defects and pregnancy complications. Besides, in our study, subjects who reported consuming folic acid supplements had a significantly higher mean RBC folate level and a significantly lower plasma concentration

of tHcy than those who did not take supplements, showing that folic acid supplementation increases blood folate and decreases Hcy concentrations (Ubeda et al., 2011). Despite supplementation, the optimal level of folate required to prevent NTD was never reached, as this would depend on the elimination kinetics of folate in RBCs and on the various forms, doses and duration of daily supplementation (Pietrzik et al., 2007).

Public health strategies aiming at improving supplementation with folic acid in pregnancy, lactation and infancy are required as well as human intervention trials to obtain a stronger evidence base for the amount of folic acid that has optimal effects on health (Hermoso et al., 2011).

Furthermore, RBC folate levels and folate intake were negatively correlated with tHcy concentrations, thus confirming that a reduction in tHcy concentration can be achieved by an increase in dietary folate intake, as suggested by other authors (Yoshino et al., 2010; Baker et al., 2009; Abdollahi et al., 2008). The FFQ used in our study was previously validated against a 4-day weighted dietary record (Agodi et al., 2011) and includes alcoholic

beverages that are likely to affect folate levels. However, women reported a very low current alcohol intake that contributes minimally to the total folate intake (data not shown). Furthermore, a study limitation is that we did not record the consumption of medication that is also likely to affect folate levels, even though we have enrolled healthy women.

In the present study, folate intake, which was assessed by FFQ, was negatively correlated with tHcy concentrations ($r_s = -0.17$; $p = 0.015$). On the contrary, no correlation between folate intake and RBC folate levels was shown, also controlling for supplement use. This can be explained by the defined reference period of the FFQ, which, in our study, was the previous month, a period shorter than the normal lifespan of erythrocytes, which is 3 months (Colić Barić et al., 2009).

Various lifestyle factors, such as smoking, have been associated with an increased level of tHcy (Haj Mouhamed et al., 2011; Das et al., 2010) and, thus, identification of such factors could help to prevent diseases related to hyperhomocysteinemia. In our study, an impressive high proportion of smoking pregnant women was found (25.6%). Furthermore, the mean tHcy concentration was higher in smokers than in nonsmokers, and almost all smokers (96.7%) had tHcy concentration $>10 \mu\text{mol/l}$, although differences were not statistically significant. Furthermore, a deficiency in the folate status in smokers, independent of dietary intake, has been reported previously, and cigarette smoking has been associated with lower concentrations of RBC folate (Yoshino et al., 2010), although it has yet to be determined whether components of cigarette smoke directly cause folate depletion.

In our study, although smokers showed a higher average folate intake, their mean RBC folate level was lower than in nonsmokers, and, among smokers, a higher proportion had RBC folate levels below the recommended reference value. Some authors (Haj Mouhamed et al., 2011; Okumura et al., 2011) propose a number of mechanisms that may cause this deficiency, including the interaction of some components of tobacco smoke with folic acid coenzymes, which transforms them into biologically inactive compounds that lead to decreased circulating folic acid concentrations in smokers. Besides, a limitation of our study is that we did not record the daily number of cigarettes smoked and the duration of smoking, which are important elements related to Hcy concentration (Haj Mouhamed et al., 2011). Finally, since a relationship between folate, Hcy concentration and second-hand exposure to cigarette smoke has been reported (Okumura et al., 2011), it would have been appropriate to assess exposure by means of serum cotinine. Plasma tHcy concentrations are influenced by genetic factors such as polymorphisms in folate and Hcy metabolic enzymes. The allele variant 677T of the *MTHFR* gene is the most prevalent known genetic cause of elevated

plasma Hcy levels in the general population (McNulty et al., 2008), since individuals with the TT genotype have reduced MTHFR activity (up to 60–70%), which results in impaired folate metabolism and then elevated tHcy levels (McNulty et al., 2008). Individuals with the TT genotype have increased dietary folate requirements because they have lower RBC folate levels compared with those without this genetic variant, and the increase in tHcy is found mostly in patients with folate deficiency. However, in our study, mean RBC folate level and mean plasma concentration of Hcy did not vary significantly by genotype, and no correlation between folate intake and circulating levels stratified by genotype were found, also considering pregnancy and smoking status, confirming previous results reported in the general population (Johansson et al., 2010), in women of childbearing age (Barbosa et al., 2008) and in folic acid-supplemented pregnant women (Ubeda et al., 2011). Nevertheless, our results should be interpreted with caution due to the small number of women studied. Therefore, larger population-based studies may be necessary to evaluate the interaction of polymorphisms and Hcy and folate concentrations in women of childbearing age. Interestingly, in our survey, younger women were shown to be at higher risk of inadequate RBC folate levels. Additionally, younger women were also at higher risk of carrying the TT genotype, which is particularly unfavourable in the setting of a low folate status (Agodi et al., 2010; Klerk et al., 2002). Notably, lower education was associated with a greater inadequacy for folate intake in a recent report in a Spanish population (Rodríguez-Bernal et al., 2012).

The scenario depicted in this study confirms results of a recent review of European studies (Dhonushe-Rutten et al., 2009) showing that in some populations both the intake and the folate status are inadequate and that Hcy concentration is higher than ideal, thus emphasizing that in these groups an improvement in the folate status is needed via a food-based approach, food fortification or supplements. Furthermore, Mediterranean populations, such as those examined in this study, are known to have a higher folate level than non-Mediterranean populations (Guéant-Rodríguez et al., 2006), thus, it is plausible that the herein reported problem of an inadequate folate status in women of childbearing age is even worse in non-Mediterranean geographical areas. The current lifestyle of today's young women, if unchanged, will impact on their future health status as well as on their children's well-being. Our study discloses the possibility for future targeted chemopreventive interventions, especially among younger and less educated women, in order to prevent NTDs and other adverse pregnancy outcomes.

5.3 Levels of mtDNA 4977-bp deletion in lymphocytes of healthy young women, folate intake, RBC folate levels, and distribution of the *MTHFR* C677T polymorphism

Folate is also necessary to nucleotide synthesis and DNA methylation and folate insufficiency is thought to influence DNA stability involved in cancer carcinogenesis (Fenech M, 2001a; Choi and Mason, 2000). Those relationships are modified by the *MTHFR* gene C677T polymorphisms (Kim, Y.I. 2007; Piyathilake et al., 2009; Agodi et al., 2010). Several recent studies have demonstrated that folate status could also modulate mitochondrial DNA (mtDNA) stability (Branda et al., 2002; Crott et al., 2005; Chou et al., 2007). Particularly, mtDNA deletions and low folate status, proposed characteristics of carcinogenesis, have been suggested in relation to human liver cancer (HCC) susceptibility in a recent cross-sectional study (Wu et al., 2009).

In order to obtain new insights regarding the association between folate intake, RBC folate level and any variation of the levels of the mtDNA 4977-bp deletion, we studied a cohort of healthy women of childbearing age showing a high prevalence of inadequate folate intake and of folate deficiency. Notably, our study was conducted in a country, Italy, where no folic acid fortification has been introduced but only supplementation in the periconceptional period is recommended. It is always assumed that pregnant women consume and/or take more folate either through the choices of foods containing folate or as supplements. In fact, in our study, pregnant women have a significantly higher mean folate intake and mean RBC folate level than non-pregnant women.

Somatic mtDNA deletions obtained from samples of venous blood cells may serve as useful biomarkers for the early detection of global genomic instability and cancer risk, as it has been suggested elsewhere (Wu et al., 2009). We hypothesised that nutritional factors and specifically folate intake and folate status may have an impact on lymphocytic mtDNA 4977-bp deletion levels in humans, particularly in young healthy women, and thus on mitochondrial genomic instability, as suggested from experimental studies in rodents (Chou, Y.F. and Huang, R.F.S., 2009).

In our observational study no association between characteristics of women and the mtDNA 4977-bp deletion levels, as biomarker selected to measure mitochondrial genomic instability, were found. Additionally, no correlation was found when folate intake and RBC folate levels were explored. Furthermore, our data report lower mean levels of the mtDNA 4977-bp deletion (mean $\Delta C_t = 1.24$) in lymphocytes of young healthy women when compared with data from the literature, and particularly with those obtained - with the same methodology -

in a healthy group of older males and females (mean $\Delta C_t = 3.5$, in the control group) (Wu et al., 2009). Younger age and gender may explain, at least in part, such observed differences. However, the findings regarding an age-dependent increase of mtDNA 4977-bp deletion are still controversial. Very low mtDNA 4977-bp deletion levels were previously reported in peripheral blood lymphocytes of young and elderly subjects and no accumulation of the 4977-bp deletion with increasing age was shown (Meissner et al., 2008, Ross et al., 2002). A recent study has shown that the proportion of mtDNA 4977-bp deletion carriers was similar in elderly and in centenarians individuals, but the individual mutational load was on average much lower in the centenarians than in the elderly, probably reflecting a healthier lifestyle that can be attributed to their very advanced ages, that may result in a lower ROS production and thus less overall DNA damage (healthy survivor effect) (von Wurmb-Schwark et al., 2010).

Moreover, the low level of mtDNA deletion observed in our study may be due in part to the high turnover of blood cells, which may inhibit the accumulation of genetically abnormal mtDNA (Ross et al., 2002, Mohamed et al., 2004), leading to a dilution effect, due to rapid cytoplasmic division, and a selective effect, by which cells harboring mtDNA with large-scale deletion are eliminated during growth (Ye et al., 2008). In addition, a decreased proportion of mtDNA 4977-bp deletion in tumor tissues as compared with corresponding non-tumorous tissues has been observed in different cancers (Ye et al., 2008, Pavicic, W.H. and Richard, S.M. 2009) and a recent meta-analysis confirmed that an higher frequency of common deletion is detected in cancer patients - cancerous tissues and adjacent non cancerous tissues - compared with tissues from the healthy controls (Nie et al., 2013).

In the present study 35.1% of women were overweight or obese, confirming the recently reported prevalence among women representative of the Italian adult population (33.8%) (Gallus et al., 2013). Although obesity has been associated with mitochondrial dysfunction in adipose tissue (Vernochet et al., 2012), nutritional status was not an independent factor associated to mtDNA deletion levels in our regression analysis.

Numerous exogenous factors including lifestyle habits and in particular tobacco smoke are known to result in an increase in mtDNA deletion in different tissues (Fahn et al., 1998). In our study, pregnant *versus* non-pregnant status, obese *versus* non-obese (either overweight, normal weight or under weight), smokers *versus* non-smokers, *MTHFR* 677TT mutated genotype *versus* *MTHFR* 677CT/CC genotypes, also when present in several combinations in one individual, might have resulted in an effect on mtDNA deletion levels. After

multivariate logistic regression analysis in order to take all the above mentioned variables into account, no statistically significant influence of any of them on the mtDNA deletion was observed, possibly due to the vast renewal capacity of blood cells and the capability of mtDNA for quick restoration (von Wurmb-Schwark et al., 2010).

Very little is known about mtDNA deletions in lymphocytes associated with nutritional factors such as folate especially in healthy individuals. Results from the study of Chou and Huang (Chou, Y.F. and Huang, R.F.S., 2009) in lymphocytes of rodents after dietary folate deprivation suggest that accumulated lymphocytic mtDNA deletions may serve as a biomarker of mitochondrial genomic instability responding to dietary folate deprivation as well as depleted cellular folate storage. On the contrary in our study, no effect of folate deficiency on mitochondrial genomic stability was found. As previously suggested mtDNA deletion levels in lymphocytes could represent a snapshot of the mutational events that happened in a relatively short period of time before blood sample collection, and inadequate folate intake and folate deficiency represent risk factors that lead to fast but transient short-term changes in leukocyte mtDNA deletion accumulation (von Wurmb-Schwark et al., 2010). Furthermore, if a long exposure to folate deprivation is necessary to genomic instability, young age of our population (median age 27 years) may have played a role. In addition, dose of folate intake and blood levels of folate are important issues to be considered. An effect of folate deprivation on genomic instability (i.e. micronuclei frequency) has been clearly demonstrated only in subjects with very low folate intake ($\leq 206.64 \mu\text{g/d}$) (Fenech et al., 2005). Blood levels of folate required to prevent anaemia (Institute of Medicine, 2001) are accurately defined, nevertheless, it is becoming evident that such accepted levels may be much lower than the averaged concentration levels at which DNA damage is minimized (Fenech, M. 2012). Thus, there is an increasing need to establish requirements for preventing damage to both nuclear and mitochondrial DNA (Fenech, M. 2001b). Lack of association found in the present study was obtained defining inadequate folate intake considering estimated average dietary requirements (520 $\mu\text{g/d}$ for pregnant and 320 $\mu\text{g/d}$ for non pregnant women) and folate deficiency as RBC folate $< 305 \text{ nmol/L}$ (Institute of Medicine, 2001). Although it has been reported, from intervention studies in humans, that different biomarkers of DNA damage are minimised when RBC folate concentration is greater than 700 nmol/l (concentration achievable at intake levels more than 400 $\mu\text{g/d}$ folic acid per day) (Fenech, M. 2001b), in our study, after multivariate logistic regression analysis, negative results were obtained also considering the last proposed cut-off values (data not shown).

The issue of statistical power is critical in negative studies. Given the lack of reference data regarding the association between folate deficiency and mitochondrial genomic instability in human, we have evaluated a posteriori the power of our study (using EpiInfo version 6) founding a low power. Thus, the sample size of our study may be too small to detect a significant association. Furthermore, other factors should be discussed to explain our results. Although different characteristics have been included in the regression model to control and adjust simultaneously for various confounders, other possible uncontrolled factors should be considered, such as preconceptional, peri or post partum or infertile status. Furthermore, the mean folate intake was not weighted by season of the FFQ collection in order to control for difference across seasons, even though a recent large study (Park et al., 2005) reports no systematic variations for folate intake according to the season of dietary intake collection also in our country. Besides, although the FFQ administered have been previously validated (Agodi et al., 2011), generally dietary questionnaire data may suffer from inaccuracies of volunteer recall. Additionally, studies are difficult to compare since they differ considerably in terms of the sensitivity of the methodologies applied and the demographic characteristics of the participants, highlighting the need to promote a “gold standard method” to investigate the role of the mtDNA 4977-bp deletion in different observational contexts.

Our results indicate that mtDNA 4977-bp deletions are maintained at low levels in lymphocytes of young healthy women despite the wide range of variation of folate intakes and folate status, suggesting that the mtDNA deletion levels related with low folate dietary intake, were not so extensive to lead to mitochondrial genomic instability. We cannot exclude the absence of mtDNA damage in other tissues. To our knowledge this is the first study conducted in order to explore the association between folate deficiency and mitochondrial genomic instability in young healthy women. Future studies, carefully designed to address limits and methodological issues related to variation of this biomarker as an effect of different dietary patterns and of folate status, could provide further insight on the specific mechanisms that are acting in lymphocytes of healthy subjects under usual folate intake (Agodi et al., 2013).

5.4 Interactions between *TNFA* -308 G>A polymorphism and adherence to MD pattern or FA intakes, on overweight/obesity risk

Dietary FAs impact the expression of the cytokine genes *TNFA* and alter TNF α production. In addition, sequence variants in this gene has also been shown to alter his gene expression and plasma levels, and are associated with obesity (Joffe et al., 2013).

In 2010 UNESCO has recognized the MD as an Intangible Cultural Heritage of Humanity (UNESCO, 2010). A number of epidemiological studies have shown that greater adherence to the traditional MD, is associated with a significant reduction in total mortality, and particularly of death due to coronary heart disease and to cancer (Trichopoulou et al., 2005), could reduce overall cancer risk (Couto et al., 2011), and provide a consistent protection for the occurrence of major chronic degenerative diseases (Sofi et al., 2010). Even though most of these chronic conditions are also associated with obesity, the link between MD and obesity is not clear. In recent years, in Southern European countries a decrease in the adherence to the MD as well as an increasing prevalence of overweight/obesity have been reported (Buckland et al., 2008). The present study was conducted in a representative population of a southern European Mediterranean region with a poor adherence to MD (median value of MDS equal to 4), and a high prevalence of overweight and obesity (32.6%). Notably, in this population, the risk of being overweight/obese due to poor adherence to MD was 3.5 fold increased. Furthermore, in our research, accordingly to a recent study (Olmedo-Requena R, 2013), younger age was associated with poor adherence to MD. However, although exploring the relationship between the MD and overweight/obesity is complex, and important methodological differences (such as the methodology used to construct MD indices) and limitations in the studies make it difficult to compare results, the evidence points towards a possible role of the MD in preventing overweight/obesity and in protecting against weight gain, and additionally, physiological mechanisms can explain this protective effect (Buckland et al., 2008).

In recent years, southern European countries, which used to eat a traditional MD, have also been adopting a more Western-style diet and a dramatic change in the sources of fat intake in the general population have been observed. This change mainly consists in replacing polyunsaturated or monounsaturated FA, that have been considered as healthy lipids because they reduce the incidence of cardiovascular disease with saturated FA, known risk factors for cardiovascular disease (Hu FB and Willett WC, 2002; Ruano et al., 2011). In order to support this change, in our study, women with greater adherence to MD consume significantly (g/day) more unsaturated FA than women with poor adherence and notably, considering the mean FA intakes as daily %E, the mean saturated FA intake was statistically significant higher in women with poor adherence to MD than in the other.

Furthermore, unsaturated and saturated FA intakes (g/day) were higher in underweight/normal weight than in overweight/obese women, but, considering the mean FA

intakes as daily %E, differences between the two groups were not statistically significant (data not shown).

Some studies reports that the MD pattern may be protective against the development of obesity through its high-fiber content and low energy density (Trichopoulos D, 2002; Buckland et al., 2008), but others studies (Ferro-Luzzi 2002) have speculated that the high-fat content, particularly from olive oil, of the MD may promote excess energy intake and weight gain and for this may explain the high prevalence of overweight and obesity in Mediterranean countries. In our population, mean energy intake was higher in women with greater adherence to MD than in women with poor adherence and a similar association was previously reported (Romaguera et al., 2009; Martinez-González et al., 2012). Nevertheless, it may be possible that this association is methodologically driven given that energy intake was not corrected for when constructing the MDS. Furthermore, mean energy intake was higher in underweight/normal weight women than in overweight/obese, confirming that the root physiological cause of obesity is energy imbalance as a consequence of low physical activity and/or high energy intake, and several lifestyle factors may influence whether or not a person can maintain energy balance over the long term (Romaguera 2009; Mozaffarian et al., 2011; Lassale et al., 2012). In fact, some studies supports the theory that the problem of obesity in Mediterranean countries is likely to be related to limited physical activity in conjunction with excessive positive energy balance brought about by the westernization of their diet (Garcia-Closas et al., 2006; Willett WC, 2006).

Lifestyle changes are the most important determinants of the rapid rise in the prevalence of obesity worldwide, and genetic factors likely to modify the susceptibility to these changes (Romaguera et al., 2009). The current lack of understanding of the numerous gene–gene and gene–environment interactions in obesity, poses one of the major obstacles for the development of effective preventive and therapeutic intervention strategies (Stryjecki and Mutch, 2011). It has been described that the A allele of the *TNFA* -308 G>A polymorphism produces a two-fold increase of *TNFA* transcription and subsequent increase in TNF α production (Wilson et al., 1997).

Despite the many studies showing independent association between the *TNFA* SNPs and obesity, only few studies have investigated diet–gene interactions. Nieters et al. found that German Caucasian men and women with the –308 A allele, who were in the highest tertile for intake of the *n*-6 PUFAs LA and AA (%E), had an increased obesity risk (Nieters et al. 2002).

Conflicting results have been reported on the relationship between A allele and obesity risk and this may be explained by the interaction between genotype and dietary FAs intake, that potentially modulate the risk of overweight/obesity, similarly to what demonstrated in German-Caucasian men and women (Nieters et al. 2002) and in two studies of different ethnic populations (Joffe et al., 2010 and Joffe et al., 2011). In the present study, no evidence for an independent effect of the *TNFA* -308 G>A polymorphism on overweight/obesity risk was found. However, significant interactions between *TNFA* -308 G>A genotypes and FA intakes were reported. Particularly, there were significant interactions between A allele and linoleic acid, γ -linolenic acid, α -linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, poly-unsaturated FA, mono-unsaturated FA and total unsaturated FA intake on overweight/obesity risk. In fact, carriers of *TNFA* -308 A allele and with unsaturated FA intake below or equal to the 75th percentile of the FA intake distribution (g/day) had an increased risk of overweight/obesity. Similarly to that reported in previous studies (Joffe et al., 2010; Sookoian et al., 2005; Nieters et al. 2002), the results suggest that the presence of the A allele by itself does not confer risk but rather may be indicative of a greater responsiveness or sensitivity of an individual to changes in FA intake.

Furthermore, the risk of overweight/obesity was higher in women carriers of the A allele and with poor adherence to MD than the reference group.

In conclusion, in our population, the risk of being overweight/obese due to poor adherence to MD was 3.5 fold increased and younger women with the lower adherence to MD are at increased risk to become obese. Therefore, women carriers of A allele with low unsaturated FA intake or with poor adherence to MD are a cause for concern and a target group for possible nutritional intervention.

In a recent review (Joffe et al., 2013) when interactions between dietary fatty acids with *TNFA* SNPs on obesity and serum lipid were analyzed, it became evident that both the quantity and quality of dietary fatty acids modulate the relationship between *TNFA* SNPS on obesity and serum lipid profiles, thereby impacting the association between phenotype and genotype, however the nutrigenetic interactions are complex and it is difficult to assess the magnitude of their impact in managing an individual's diet.

Nevertheless, the present research has certain limitations that need to be taken into account when considering the study and its contributions. Its cross-sectional design, could limit the inference on the time sequence of the association between MD and nutritional status. In addition, selection bias, recall bias, and confounding might be present, for example, misreporting of diet and of energy intake especially in the obese women may be an

important factor that was not included in our analyses as a confounder factor. Furthermore, although our FFQ is validated, it may contain measurement errors. Additionally, our research was limited to the assessment of only one SNP from a single gene and it is known that a number of other candidate genes have been implicated in the pathogenesis of obesity. Furthermore, the inter-ethnic variability reported should caution us with regards singular dietary recommendations, as it cannot be assumed that fatty acids and other nutrient metabolism is uniform for all populations.

In addition to diet–single polymorphism interactions, it is also important to understand the combined effect of a number of polymorphisms on the same or different genes interacting with the environment. For this reason, the identification and analysis of haplotype-nutrient interactions may provide additional insights in future research.

The results of this study should therefore be confirmed in future prospective studies on a larger cohort, taking into account the above mentioned limitations, in order to further knowledge on gene–diet interactions in the obesity risk that might constitute the basis of new kinds of dietary recommendations. The future study of nutrigenomics offers the opportunity to clarify the underlying molecular mechanisms governing the interactions between dietary fatty acids and the inflammatory phenotype, potentially elucidating the observed differences between different ethnic groups and genders in developing population-specific dietary recommendations.

5.5 Association between MD pattern, folate intake and LINE-1 methylation level

DNA methylation is a well-defined epigenetic mechanism involved in the regulation of a wide variety of biological processes, including gene expression, genomic stability and parental imprinting (Bird A, 2002). Abnormal DNA methylation patterns have been associated with various human diseases, including cancer, cardiovascular disease and autoimmune disease (Wilson et al., 2007). Because of his high representation throughout the genome, LINE-1 has been used as surrogate marker for estimating global DNA methylation levels (Weisenberger et al., 2005). More recently, global DNAmethylation has been examined in surrogate tissues such as leukocyte DNA. A lower level of leukocyte DNA methylation has been associated with an increased risk of several cancers (Hsiung et al., 2007; Moore et al., 2008; Lim et al., 2008; Choi et al., 2009; Hou et al., 2010) after adjusting for known risk factors. This suggests that leukocyte DNA methylation may serve as a surrogate biomarker for systemic genomic methylation and provide an independent risk factor for cancer development (Zhang et al., 2011a).

The possible impact of the environment on epigenetic regulation has attracted considerable interest, with environmentally induced changes in gene expression being associated with altered DNA methylation patterns or with altered histone modifications. The interest in DNA methylation relates to its involvement in key developmental and mechanistic pathways as well as its possible association with phenotypical changes due to the interplay between environmental exposures and epigenetics (Borgel et al., 2010; Feil and Fraga, 2012; Law and Jacobsen, 2010; Sincic and Herceg, 2011). Epigenetic marks could be useful to personalize nutrition, to early detect those individuals with more risk to develop metabolic disorders or to better respond to a treatment. However, a wider approach should be adopted, and epigenetic marks have to be studied at the same time that SNPs, miRNA expression and mRNAs in order to decipher the interactions among DNA sequence, epigenetics and gene expression, always taken into account the diet and other environmental factors (Milagro et al., 2013). A number of bioactive dietary components that appear to have potential to prevent disease and promote overall health have been identified (Howells et al., 2007; De Kok et al., 2008). This may be used from a chemopreventive standpoint to incorporate anticancer nutrients into one's daily routine to impede disease mechanisms. From a therapeutic perspective many nutrients have been and are being studied for their ability to prevent and reduce the risk or severity of certain diseases and for their anticarcinogenic properties (Hardy and Tollefsbol, 2011).

Furthermore, obesity is a multifactorial disorder, reflecting complex interactions of genes, environment and lifestyle (Newel et al., 2007), associated with a high risk of chronic diseases such as diabetes, cardiovascular disease and certain cancers (Couto et al., 2011), thus, assessment of overall dietary patterns and their link to chronic disease risk and diet-related alterations in the epigenome are becoming increasingly recognized as important (Piyathilake et al., 2012). The Mediterranean Diet (MD) has long been reported to be the optimal diet for preventing NCDs and preserving good health. A meta-analysis confirms the significant and consistent protection provided by adherence to the MD in relation to the occurrence and mortality of major chronic degenerative diseases (Sofi et al., 2010). Even though it is logical to assume that dietary recommendations focused on promoting a healthier overall dietary pattern rather than encouraging consumption of certain foods or food categories should be the first line of intervention for prevention of many different types of cancers, use of biomarkers to monitor the effectiveness of these interventions should be an integral part of such efforts.

The main purpose of the current study was to determine the relationship between MD, folate intake and the degree of LINE-1 leukocyte methylation in a cancer-free population, in order to define the scientific evidences useful for an integrated strategy of primary prevention against cervical cancer, in terms of translational research from basic molecular epidemiology to cancer prevention.

The present study was conducted in a representative population of 177 southern European Mediterranean non pregnant women with a poor adherence to MD (median value of MDS equal to 4.1), and a high prevalence of overweight and obesity (32.8%).

The comparison of mean crude dietary folate intake (245.8 $\mu\text{g}/\text{day}$) with recommended intake of this vitamin (400 $\mu\text{g}/\text{day}$, Institute of Medicine, 2001), reported a significant lack of folate in our population ($p=0,000$). Notably, our data were similar to a recent study, in the framework of the EPIC cohort (European Prospective Investigation into Cancer and Nutrition), where adjusted mean dietary folate intake, assessed by a 24 h recall in most European centres ranged from 200 to 300 $\mu\text{g}/\text{d}$ in women (Park et al., 2012).

The folate intake (both crude and nutrient density estimation) tended to increase with increasing MD adherence. Accordant results, indeed, were confirmed in the Spanish population, where a significant relationship between a higher MDS and higher folate intake was observed (Monteagudo et al., 2013; Serra-Majem et al., 2009).

Further, some authors reported significant associations between dietary folate intake and higher educational level (Planells et al., 2003) and increased age (Monteagudo et al., 2013) and, conversely, decreased folate intake among current smokers (Park et al., 2012). However, others found no relationship with socio-economic status (Rouhani et al., 2007). In our study, no association was detected between dietary folate, educational level, age, employment and smoking status.

With regard to the MDS, mean adherence tended to increase in older women, as reported by Patino-Alonso et al. (2013), where logistic regression analysis revealed that increase physical activity, older age, and moderate alcohol consumption were associated with improved MD adherence. In our population, following the age percentile distribution, a positive trend was reported ($p=0.001$).

Epidemiological evidences point towards a possible role of the MD in preventing overweight/obesity whilst physiological mechanisms can explain why key components of MD might protect against weight gain. Indeed, the MD is rich in plant-based foods that provide a large quantity of dietary fibre, which has been shown to increase satiety through mechanisms, such as prolonged mastication, increased gastric detention and enhanced

release of cholecystokinin. On the contrary, energy density has an important role in weight gain, as palatable energy-dense food leads to poor appetite control and consequently to over-consumption. The MD has a low energy density and a low glycaemic load compared with many other dietary patterns. These characteristics, together with its high water content, lead to increased satiation and a lower calorie intake (Buckland et al., 2008).

In our study, mean MDS was, lower in obese women than non obese ($p= 0.007$), despite, trend within BMI classification resulted in a borderline difference ($p=0.05$).

Furthermore, mean MDS was significant lower in women with low-mean education level than in women with mean-high education level ($p=0.042$) and women who consumed folate or vitamin supplements reported higher mean MDS, compared with others ($p=0.000$).

It is rather reasonable to assert that people with higher degree of education could be keener to be aware of the nutritional quality of food and thus to choose high-quality products. These data may support the necessity to implement people's "nutrition knowledge" on health-related issues in order to promote healthier choices in terms of dietary habits, independently from other less-modifiable risk factors such as socioeconomic position (Bonaccio et al., 2012)

Taking into account the use of supplements, further classifying each woman as consumer or not consumer of supplements, the prevalence of inadequate folate intake was 83.1%. Notably, the risk of being overweight/obese due to folate deficiency was 3.8 folds increased and 4.1 folds increased considering both folate deficiency and poor MD adherence, on the contrary, no association was shown in the group of women with greater adherence to MD.

Global DNA hypomethylation is associated with genomic instability and chromosomal aberrations (Hoffmann and Schulz, 2005). Because of, methylation of repetitive elements such as LINE-1 has been shown to be a major contributor to total genomic DNA methylation in the human genome, we measured LINE-1 methylation, by pyrosequencing, as a surrogate biomarker of changes in genome wide methylation.

Hypomethylation-mediated reactivation of active forms of LINE-1 in relation to human cancer risk has been under investigation for several years. LINE-1 retrotransposons have been shown to be hypomethylated in many cancers (Suter et al., 2004; Cho et al., 2007; Pattamadilok et al., 2008), suggesting potential activation of LINE-1 in these cancers. Based on these observations, it is biologically plausible that reactivation of LINE-1 elements through hypomethylation could induce genomic instability, transforming cells into a precancerous or cancerous state (Piyathilake et al., 2011).

LINE-1 methylation analysis was carried out in the subgroup of 177 non pregnant women (on a total of 380 women), in order to control any confounding effect of pregnancy on epigenetic changes.

In this cancer-free population, mean leukocytes LINE-1 methylation level was: 65.3% (SD \pm 3.3; median: 65.3%). This result was similar to mean methylation level (64% \pm 7) reported among 273 controls from a CIN case-control study (Piyathilake et al., 2011), from which we adapted the pyrosequencing method (i.e. the primer set). However, our values were lower than other cancer-free population, where median levels ranged between 73.5% and 74.7% (Zhang et al., 2011a; Hsiung et al., 2007; Choi et al., 2009).

The comparison of mean global methylation level according to the study characteristics did not report any significant difference.

Because normal mammalian development is dependent on DNA methylation, there is enormous interest in assessing the potential for changes in folate intake to modulate DNA methylation both as a biomarker for folate status and as a mechanistic link to developmental disorders and chronic diseases including cancer (Crider et al., 2012). Dietary factors may exert their effects on carcinogenesis through DNA methylation. Studies have shown that insufficient amounts of methyl-group donors such as folate, vitamin B-12, vitamin B-6, and methionine may cause DNA hypomethylation, leading to an increased risk of cancer (Davis CD, Uthus EO. 2004). A reduced methylation in leukocytes may indicate systematic genomic hypomethylation and reflect the cumulative environmental impact on carcinogenesis (Jirtle RL, Skinner MK 2007).

Intervention studies in humans show that DNA damages are minimized when red cell folate concentration is greater than 700 nmol/L. Such concentration is achievable at intake levels at or above current recommended dietary intakes of folate (i.e. >400 $\mu\text{g}/\text{day}$) depending on an individual's capacity to absorb and metabolize this vitamin which may vary due to genetic and epigenetic differences (Fenech M, 2012).

In the present study, in univariate analysis, we detected a strong association between folate deficiency and global hypomethylation, in fact women with folate deficiency had a 4 folds increased risk to be in the lowest tertile of global methylation level (T1, hypomethylation) compared with women with no folate deficiency (T3 vs. T1: OR = 4; 95% CI: 1.3–11.8) and the association showed a dose-response relationship (p -trend = 0.032).

However in multivariate analysis, this association decreased, with a borderline confidence interval ($p=0.053$, OR = 3; 95% CI: 1 – 3.3).

The human studies of DNA methylation and folate/folic acid intake vary widely in their study design, timing of exposure, tissue tested, assays, and, not surprisingly, the findings. At this time, the evidence suggests that low folate status is associated with decreases in global DNA methylation, which in some studies has been related to an increased risk of cancer (Davis CD, Uthus EO 2004; Crieder et al., 2012). A reduced methylation in leukocytes may indicate systematic genomic hypomethylation and reflect the cumulative environmental impact on carcinogenesis (Jirtle RL, Skinner 2007).

Although controversial findings are widely reported, several studies reported that dietary folate intake and global DNA methylation were not associated among cancer-free controls (Hsiung et al., 2007; Moore et al., 2008, Zhang et al., 2011a). In addition, intervention studies found that dietary folate restriction or folic acid supplementation resulted in changes in DNA methylation (Jacob et al., 1998; Pufulete et al., 2005), whereas others did not observe methylation changes in response to folate depletion or treatment (Axume et al., 2007), even if results vary stratifying by age and MTHFR polymorphisms.

In a recent report, Figueiredo et al. (2009) showed that folate supplementation did not alter LINE-1 methylation levels in normal colorectal mucosa, but in a large prospective cohort study, it has been found that low folate and, to a lesser degree, vitamin B6 intake and excess alcohol consumption were associated with increased risk of colon cancers with LINE-1 hypomethylation. Together together, these data could suggest that folate levels may not be relevant in terms of LINE-1 methylation in normal mucosa, with relatively normal cellular kinetics, but once neoplasia develops some factor, possibly the increase in cellular proliferation, may reveal the relationship between folate and LINE-1 methylation (Schernhammer et al., 2010).

Recently, LINE-1 methylation in peripheral blood mononuclear cells was investigated in CIN risk, in US population where folic acid fortification was begin in 1996-1998. Interestingly, women with supraphysiologic concentrations of plasma folate and sufficient vitamin B12 were significantly more likely to have highly methylated leukocytes compared with women with lower folate and lower vitamin B12 (OR 3.92, 95% CI: 1.06–14.52, p=0.041). Therefore, said observations strengthen previous finding that supraphysiologic plasma folate concentrations seen in the era after US folic acid fortification do not increase the risk of CIN in premenopausal women of child-bearing age. Moreover, even in the presence of sufficient vitamin B12 status, the same micronutrient combination is able to exert a positive influence on leukocytes methylation, which in turn decreases the risk of

CIN2+. However, these results do not exclude the possibility of adverse effects of exposure to higher folate on other health conditions in this population (Piyathilake et al., 2011).

Taken together, these findings suggest that there may be a differential global DNA methylation response to folate depletion and repletion dependent on age, genotypes, duration, and magnitude of exposure (Crieder et al., 2012). This data is not unexpected taking into account that DNA methylation is part of a complex and highly regulated system, so that additional researches are needed to elucidate the relationship between folate and DNA methylation.

MD has been considered an healthy dietary life style, which is associated with a reduced overall cancer risk and in total mortality, (Trichopoulou et al., 2005; Couto et al., 2011) and although recent studies evaluated the beneficial effects of a healthier dietary pattern on LINE-1 methylation, to the best of our knowledge, the present study is the first to investigate the hypothesis of a possible association between MD and global DNA methylation in a cancer-free population.

A recent study (Zhang et al., 2011a), which identified two dietary patterns, the prudent dietary pattern, characterized by a high intake of vegetables (dark green vegetables, orange vegetables, and other vegetables including tomatoes but not including legumes and potatoes) and fruits and the Western dietary pattern, characterized by a high intake of energy dense foods such as grains, meats, potatoes, oils, and dairy, has shown that the prudent dietary pattern, was associated with a lower prevalence of leukocytes LINE-1 hypomethylation in a dose-dependent manner, furthermore, there was no apparent association between the Western dietary pattern and global leukocyte DNA methylation.

When the 13 food items were individually examined by levels of DNA methylation, only the consumption of dark green vegetables was positively associated with global DNA methylation. Participants with low DNA methylation or DNA hypomethylation (i.e. < median) consumed fewer dark green vegetables compared with those with high DNA methylation (i.e. > median) who consumed more ($p = 0.04$). Furthermore, participants, with low DNA methylation consumed more saturated fat than participants with high DNA methylation ($p = 0.02$), however, participants with high and low levels of DNA methylation did not differ in their daily intake of those nutrients involved in 1-carbon metabolism, such as dietary folate, vitamin B-6, riboflavin, vitamin B-12, and methionine.

Piyathilake and colleagues, in 2012, evaluated the association between dietary patterns and peripheral blood mononuclear cells LINE-1 methylation in women free of cervical pre-cancer, but at higher risk for developing cervical pre-cancer or cancer because they were

diagnosed with abnormal pap and tested positive for HR-HPVs. The study observed that the women with the healthiest dietary pattern (seafood, beans and lentils, tofu and meat substitutes, whole grains, fresh fruits, canned fruits, vegetables, peanut butter, low fat dairy, chicken and turkey, cereals, water, yogurt, dressings and gravy, and phytochemical rich foods) were significantly more likely to have higher peripheral blood mononuclear cells LINE-1 methylation than those with the unhealthiest dietary pattern (high sugar beverages, pasta and starchy foods, margarine, butter, refined grains, desserts and sweets, snacks, high fat dairy, fatty meat, sausages and bacon, condiments, pizza, macaroni, and cheese). Additionally, the study reported that the intakes of several methyl donor micronutrients (folate, vitamins B12, B2, and B6) were significantly higher in the healthiest dietary pattern than the unhealthiest dietary pattern, confirming the biologic plausibility for higher LINE-1 methylation observed in women with the healthiest dietary pattern. Results did also show that pre-cancer-free women with the healthiest dietary pattern were 3.3 times more likely to have higher leukocytes LINE-1 methylation than women with the unhealthiest one ($p=0.04$). In our study we did not find any association between poor adherence to MD and global methylation level, with regards to the three CpG sites, however, when all the 9 Mediterranean food components were individually examined by DNA methylation level, women whose consumption of fruit and nuts was below the median (201 gr/day) had a 2.8 fold increased risk to be in the lowest tertile of methylation in comparison with those women whose consumption was above the median value (T3 vs. T1: OR = 2.8; 95% CI: 1.3–5.8) and the association showed a dose-response relationship ($p\text{-trend} = 0.019$).

Even in multivariate logistic regression analysis, results, adjusted for age, nutritional status, smoking habits and dietary caloric intake, showed a significant relationship between low fruits and nuts intake and low methylation levels ($p = 0.006$; OR = 3.8; 95% CI: 1.5–9.7).

As recently reported, among all the food groups, vegetables, cereals, fruit and nuts are the main contributors of dietary folate intake in the European population (Park et al., 2012). We could consequently assume that high consumption of fruit and nuts, provides multiple nutrients, such as 1-carbon nutrients, antioxidants, and others which may interact in the prevention of DNA hypomethylation. As a result, intervention on the overall dietary pattern rather than on single nutrients or food groups may be a more effective way to protect against cancer risk through epigenetic regulation (Zhang et al., 2011a).

Taking into account only methylation level at CpG locus 1, hypermethylation (> 75 th percentile) was significantly higher in women with the healthiest dietary pattern ($MDS \geq 5$) than women with the unhealthiest dietary pattern ($MDS \leq 6$) ($p = 0.001$; OR = 3.5; 95% CI:

1.6–7.8), furthermore, consumption of fruit and nuts and consumption of vegetables was associated with global DNA methylation. After multivariate analysis for locus 1 results did not show any significant relationship between the above listed factors and methylation level at locus 1. These results, may lead to the interpretation that a single locus is not indicative of global methylation level, and that a broader analysis of LINE-1 methylation (three or more CpG site) may serve as a unique epigenetic marker for monitoring the effectiveness of such dietary interventions.

Our study has some limitations.

The cross-sectional design of the present study in which diet and DNA methylation were simultaneously assessed limits the study's ability to draw strong conclusions about a causal relationship between dietary patterns and genomic DNA methylation.

Statistical power calculation and sample size estimation were performed at the end of the study, due to the explorative nature of our research in order to evaluate the possible association between MD and global methylation level, thus our data suggest the need to enroll a larger population to clarify the protective effect of MD adherence on DNA hypomethylation.

However, we used a standard approach to identify dietary patterns in our study, such as the MDS, which has been widely adopted in the European cohort, the association between MD patterns and DNA methylation may be confounded by other lifestyle factors such as physical activity, use of oral/hormonal contraceptives and genetic polymorphisms. Although we adjusted for some of these variables in the analyses, residual confounding may still exist. Moreover, women's diet can be especially difficult to assess, as women tend to underreport their intakes more often than men and are more likely to do so if they are overweight or obese (Olafsdottir et al., 2006) and this phenomenon will bias diet-disease relationships. Additionally, despite our FFQ has been previously validated in order to estimate folate intake, we could not compare our data with micronutrient analysis of blood.

Due the lack data, we did not investigate the role of physical activity on LINE-1 methylation, despite it is well acknowledged that physical activity confers many health benefits and some of them may occur by alterations in epigenetic landscapes. Thus, Zhang et al. (2011b) have reported that, although the differences are small, individuals with high physical activity (30 min/day) have a significantly higher level of global genomic DNA methylation in LINE-1 in peripheral blood than those with low physical activity (10 min/day).

Furthermore, we did not investigate the distribution of the *MTHFR* polymorphisms in relation to folate intake and folic acid supplementation and methylation level. Recent studies reported that healthy young women may have higher folate needs due to increasing prevalence of the T allele and reduced folate intake compared with older groups (Agodi et al 2011). Moreover, Friso et al. 2002, found an *MTHFR* genotype-dependent association between lower global DNA methylation and lower plasma folate concentration.

Besides, we did not assess prenatal and early postnatal dietary exposure, which may represent a critical window for environmental influences on DNA methylation (Waterland RA, Michels KB, 2007). Nevertheless, in support of our findings, studies of monozygotic twins found remarkable differences in global DNA methylation in the peripheral blood of older than of younger twins, likely to reflect the cumulative impact of the environmental exposure on DNA methylation over the lifetime (Fraga et al., 2005). Significant changes in global DNA methylation have also been identified in the peripheral blood of the same adult population followed for >10 y (Bjornsson et al., 2008).

We measured LINE-1 methylation as a surrogate for global DNA methylation by pyrosequencing, because methylation of repetitive elements such as LINE-1 has been shown to be a major contributor to total genomic DNA methylation in the human genome. Pyrosequencing has been extensively used to measure global DNA methylation. Although variation in measuring global methylation can come from the accuracy of measuring the area under the curve for different peaks, this variation is generally very small. Previous studies suggest that pyrosequencing is a reproducible assay with a $SD \leq 2\%$ (Yang et al., 2004). In addition, we evaluated global DNA methylation in peripheral blood. Christensen et al. (2009) analyzed normal human tissues from 10 anatomic sites and found that age-related changes in global DNA methylation had similar patterns irrespective of tissue types including peripheral blood, suggesting common mechanisms may underlie methylation changes and the use of blood may be relevant for epidemiologic studies.

Finally, a subtle source of bias may result from the diet modifying the proportions of leukocyte cell types that have been shown to be differentially methylated (Wu et al., 2011). We cannot rule out the existence of such a mechanism that would affect leukocyte DNA methylation not by absolute loss or gain but by a change in the density of leukocyte populations.

In conclusion, we found an inverse association between high consumption of fruit, which represents a main source of folate, and DNA hypomethylation, suggesting that dietary pattern

characterized by a high intake of fruits and, in a broader view, great adherence to MD may protect against DNA hypomethylation.

These findings highlight the need for future large-scale studies to evaluate whether the association between dietary pattern and DNA methylation holds longitudinally.

Therefore, intervening to change the unhealthiest dietary patterns in favour of the healthiest one may reduce the risk of hypomethylation, and consequently, to related diseases (Wentzensen et al., 2009; Lim et al., 2008). Leukocytes LINE-1 methylation may serve as a unique epigenetic marker for monitoring the effectiveness of dietary interventions. Taken together, LINE-1 methylation may serve as a biomarker for dietary pattern interventions that are targeted to reduce the risk of cancerous and pre-cancerous conditions.

6. FURTHER PERSPECTIVES

Cervical cancer is the second leading cause of cancer deaths in women worldwide (Ferlay et al., 2010). A persistent HPV infection is a prerequisite for the development of precursor lesions, CIN and invasive cervical cancer. Epidemiologic and molecular studies have shown a causal relationship between infection with high-risk (HR) HPV and cervical cancer. Nutritional status and food consumption may be important HPV cofactors that increase risk of persistence and progression to CIN; however, there is insufficient evidence to support the association between nutritional status and cervical carcinogenesis (Garcia-Closas R, 2005).

The protracted course from HPV infection to CIN and invasive disease make CIN an ideal candidate for chemoprevention. The use of chemical agents, to prevent the initiating and/or promoting events in the carcinogenic process completes a triad of preventive strategy for cancer control, which also includes the elimination or avoidance of carcinogens in the environment (e.g. smoking cessation, low fat diets, referred to as primary prevention) and participation in screening programs and early detection of cancer (referred to as secondary prevention) (Vlastos et al., 2003).

A recent study documented that a higher degree of DNA methylation in LINE-1 of peripheral blood mononuclear cells was associated with 56% lower risk of being diagnosed with higher grades of CIN (CIN 2+), a common precancerous lesion found among sexually active women exposed to carcinogenic or high risk (HR) types of HPVs (Piyathilake et al., 2011). Furthermore, several studies have shown that healthy and balanced diet leading to provide high serum levels of antioxidants may reduce cervical neoplasia risk (Ghosh et al., 2008; Hwang et al., 2010; Siegel et al., 2010; Tomita et al., 2010; Tomita et al., 2011; Piyathilake et al., 2012). Particularly, i) serum lycopene concentration was associated with reduced risk of cervical dysplasia and invasive cancer (ii) serum tocopherols were inversely associated with CIN2 and CIN3 (Tomita et al., 2010) and iii) a higher consumption of dark green and deep yellow vegetables and fruits rich in carotene (including β -, α - and γ -carotene) and tocopherols was associated with lower risk of having CIN, especially among smokers (Tomita et al., 2011). Thus, it has been suggested that HPV associated risk of developing CIN 2+ may be reduced by improving dietary patterns and the degree of peripheral blood mononuclear cells LINE-1 methylation may serve as a biomarker for monitoring the effectiveness of dietary modifications needed for reducing the risk of CIN 2+ (Piyathilake et al., 2012).

Studies conducted in the 1950s and 1960s led to the recognition of the role of folic acid to prevent megaloblastic anemia in pregnancy. The second major achievement with the use of folic acid occurred in the 1990s, when researchers suspected an association between maternal folate status and fetal malformations, particularly neural tube defects (Tamura and Picciano, 2006). Folic acid had an important role in the aminoacids metabolism, in the nucleid acid synthesis and in the DNA methilation. Previous research has shown that higher circulating concentrations of folate were associated with a lower likelihood of becoming HR-HPV positive and of having a persistent HR-HPV infection, and when infected, a greater likelihood of clearing HR-HPV and lower likelihood of developing HR-HPV–associated CIN 2+ (Piyathilake et al., 2004; 2009).

The apparent role of folate in carcinogenesis in several tissues has stimulated investigations on polymorphisms in genes coding for folate-metabolizing enzymes. Methylen-tetrahydrofolate reductase (MTHFR) regulates the metabolism of folate and methionine, which are important factors in DNA methylation and synthesis. The *MTHFR* C677T polymorphism can lead to abnormal DNA methylation and DNA synthesis, possibly resulting in an increased risk of cancer. However, the effect of *MTHFR* polymorphisms on cancer susceptibility remains controversial.

In a population of women in Catania, Italy, with high prevalence of HR-HPV infection (Agodi et al 2009), a decreased risk for CIN of individuals homozygous for the *MTHFR* T allele has been previously reported (Agodi et al., 2010). However, some studies have supported the existence of gene-folate status interactions in the etiology of cervical cancer. Specifically it has been reported that the *MTHFR* T allele and reduced dietary folate may increase the risk for cervical squamous intraepithelial lesions (Goodman et al, 2001), and on the contrary, a study conducted after folate fortification reported that *MTHFR* polymorphism is associated with reduced risk of CIN 2 or 3 (Henao et al, 2005). The above *scenario* suggests a possible role of folate as a modulating factor on the risk of cervical cancer.

Even though it is logical to assume that dietary recommendations focused on promoting a healthier overall dietary pattern rather than encouraging consumption of certain foods or food categories should be the first line of intervention for prevention of many different types of cancers, use of biomarkers to monitor the effectiveness of these interventions should be an integral part of such efforts. To our knowledge, there have been no systematic studies conducted to derive biomarkers of dietary patterns which are also associated with higher risk of developing precancerous lesions (Piyathilake et al., 2012).

Our preliminary results on a small cancer free-population provide useful informations for an integrated strategy of primary prevention against multifactorial diseases, such as cancer, in terms of translational research from basic molecular epidemiology to effective prevention. Our future research will investigate the association between nutritional profiles, in terms of MD adherence of a population at risk of cervical cancer and, particularly, of the protective effects of an adequate folate intake, as well as of other macro and micronutrients, controlling for specific genetic polymorphisms, and cancer specific DNA methylation profile of three tumor suppressor genes (TGS, *CDHI*, *DAPK*, and *HIC1*, as reported by Flatley et al. 2009), as well as LINE-1 methylation, which is a validated biomarker of intermediate effect of changes in genome wide methylation, in order to propose corrective and preventive measures to be taken in a more effective chemoprevention strategy of cervical cancer associated with intake of food groups of micronutrients, and healthier dietary patterns.

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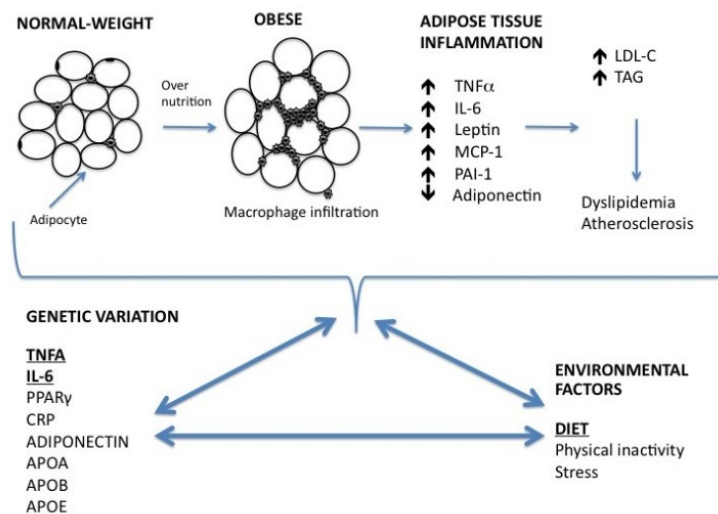
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8. TABLES AND FIGURES

Figure 1. Diagram for obesity-associated low-grade inflammation, and the relationship of diet–gene interactions on obesity and dyslipidemia.

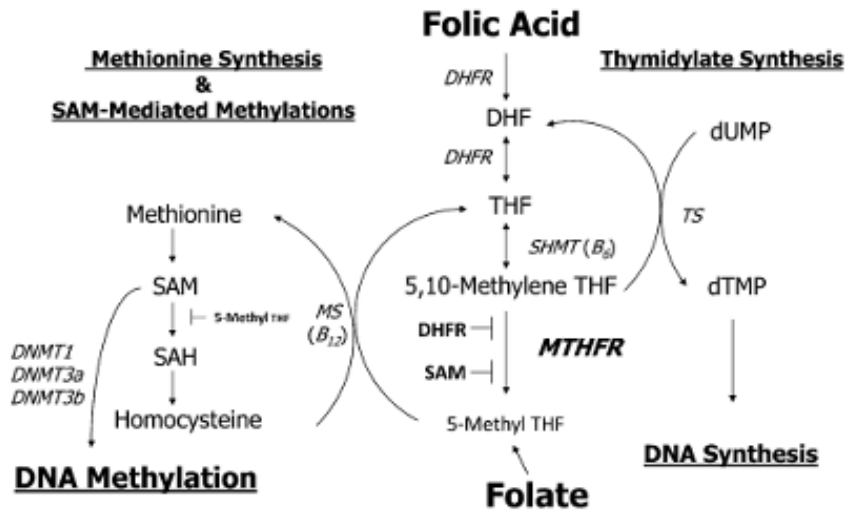
Adipocytes become hypertrophic through over-nutrition. Expansion of adipose tissue in obesity leads to a subsequent increase in the production of chemokines by the adipocytes, resulting in increasing macrophage infiltration and enhanced production of pro-inflammatory cytokines, such as TNF α and IL-6. Obesity-associated low-grade inflammation results in an increase in serum triglycerides, and LDL-C concentrations and is associated with dyslipidemia. Environmental factors and DNA sequence variations in inflammatory genes, interact to impact molecular processes of the inflammatory pathway, serum lipids and the obese phenotype (Joffe et al., 2013).



APOA, Apolipoprotein A; *APOB*, Apolipoprotein B; *APOE*, Apolipoprotein E; *CRP*, C-reactive protein; IL-6, interleukin-6; LDL-C, low-density lipoprotein cholesterol; MCP-1, monocyte chemotactic protein-1; PAI, plasminogen activated inhibitor; *PPAR* γ , peroxisome proliferator-activated receptor gamma; TAG, triglycerides; TNF α , tumor necrosis factor.

Figure 2. Folic acid metabolism. This schematic shows the process by which folate/folic acid is used for DNA methylation.

The *MTHFR* 677C/T variant reduces enzyme activity (175) and may help to divert the available methyl groups from the DNA methylation pathway toward the DNA synthesis pathway. The pathway is complex and highly regulated, with feedback loops and interactions not shown in the schematic. Gene names for enzymes are in italics and cofactors are in parentheses (Crieder et al., 2012).



DHF, dihydrofolate; DHFR, dihydrofolate reductase; DNMT, DNA methyltransferase; dTMP, thymidylate; dUMP, deoxyuridine monophosphate; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase

Figure 3. Gene—environment vs epigen—environment interplay: a model of possible genetic and epigenetic paths linking environmental exposures to health effects (Bollati et al., 2011).

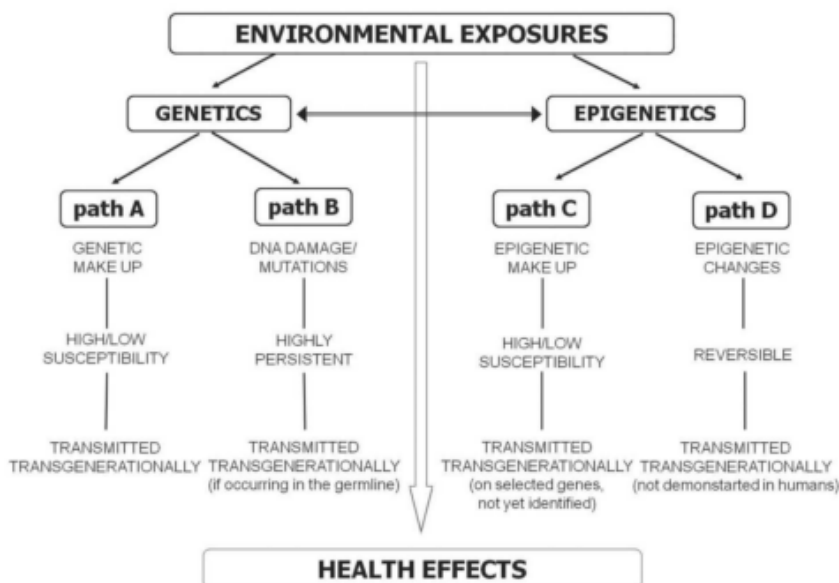


Figure 4. Epigenetic mechanisms provide the link between environmental factors and phenotypical changes during the whole lifetime (Tammen et al., 2013).

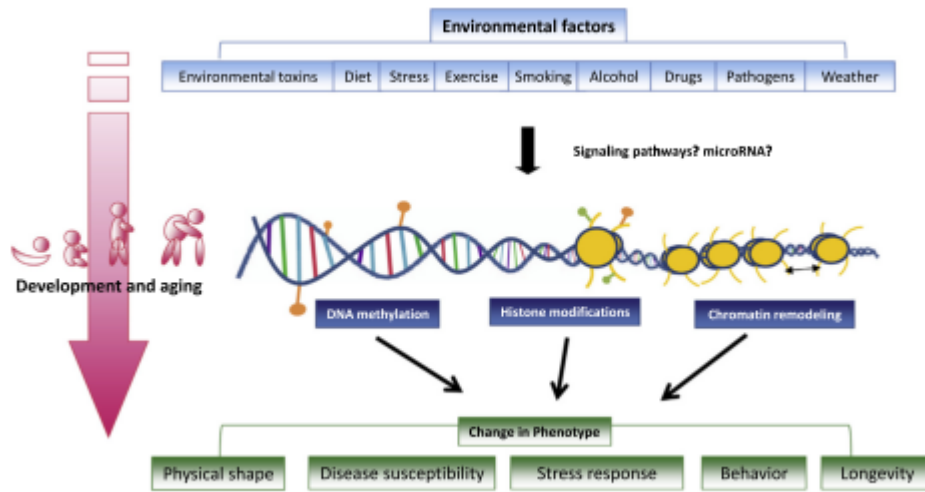
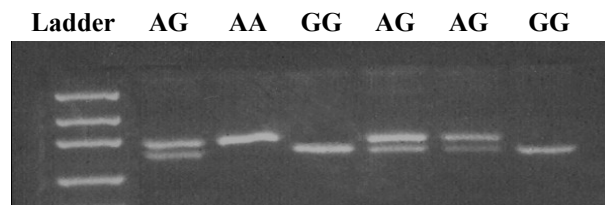


Figure 5. Electrophoresis of the digested PCR products of TNFA -308 G>A polymorphism, after digestion with NcoI.



- Line 1:** Ultra Low Range DNA ladder;
- Lines 2, 5 and 6:** heterozygous individual (AG)
- Line 3:** homozygous mutated individual (AA);
- Line 4 and 7:** homozygous wild type individual (GG).

Figure 6. Allelic Discrimination of *MTHFR* C677T polymorphism

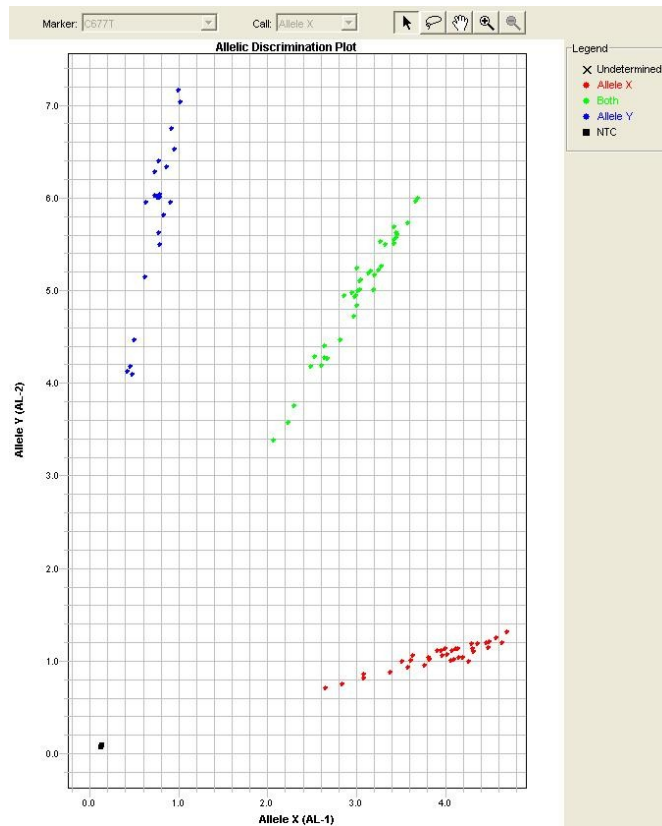


Figure 7. Real-time PCR analysis of mtDNA deletions and results calculation by the Sequence Detection System software (SDS)

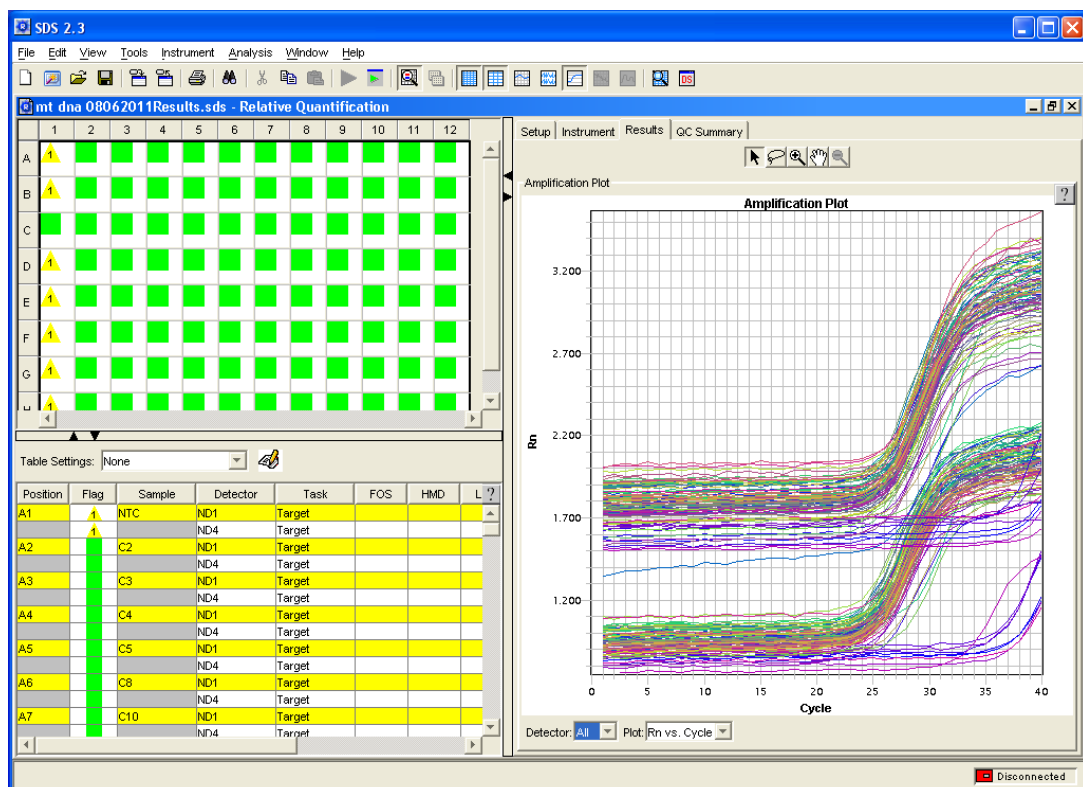
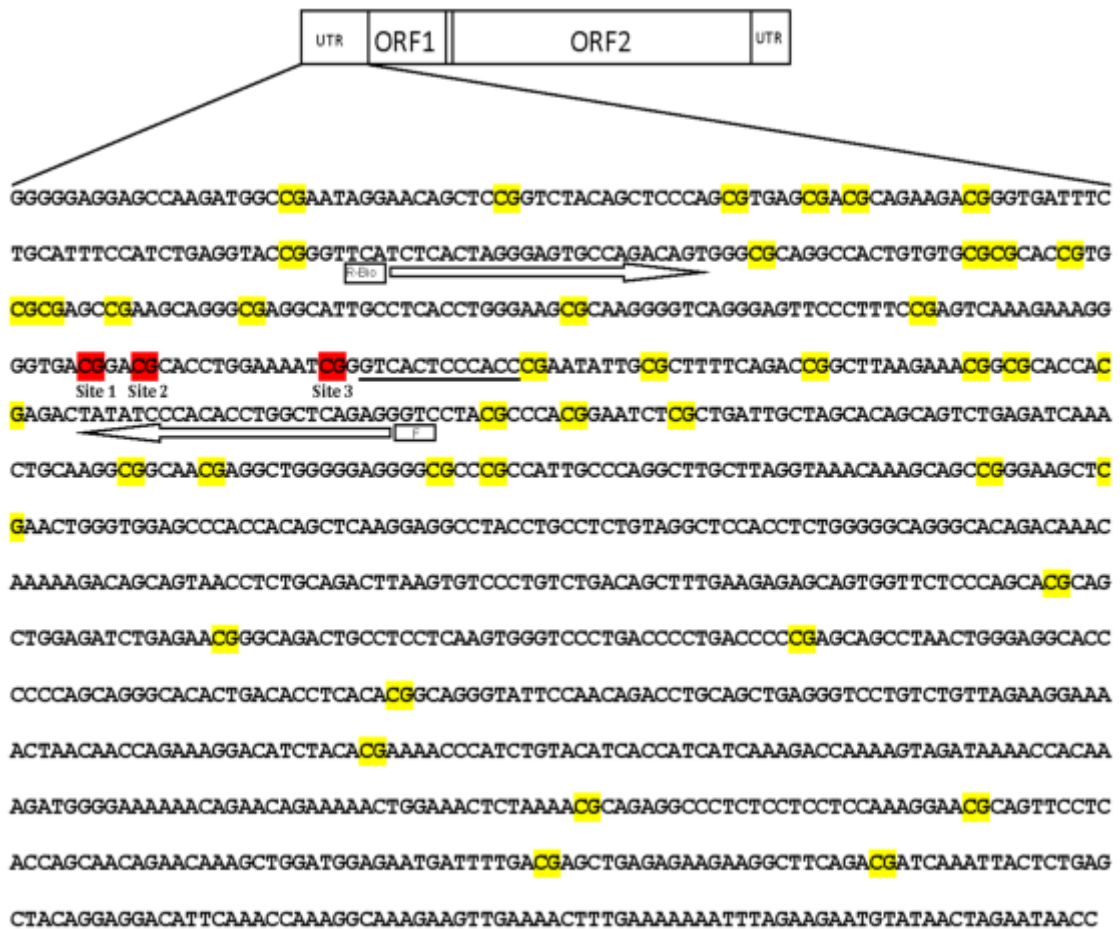


Figure 8. Location of LINE-1 pyrosequencing sites 1-3. CpG island region of the human LINE-1 transposon (GenBank accession no. X58075, nucleotide position 1 to 1147). Yellow highlights represent single CpG sites, and red highlights (Site1, Site 2 and Site 3) represent the CpG sites analyzed by pyrosequencing. Horizontal arrows indicate the location of primers (F, forward primer; R, reverse primer; -bio, biotinylated primer). The sequencing primer is underlined. The complementary strand was analyzed (Piyathilake et al., 2012).



Dispensing Order: GTCGATTAGTAGTCAGTCGCT
Sequence to analyze: TYGATTTTTTAGGTGYGTTYGTTA (Y=C/T)

Forward primer (5'-TTTTTTGAGTTAGGTGTGGG-3')
Reverse biotinylated primer (5'-biotin-TCTACTAAAAAATACCAAACAA-3')
pyrosequencing primer (5'-GGGTGGGAGTGAT-3')

Figure 9. PyroMark Q24 Vacuum Workstation.



Solution and volumes:

Trough 1: Ethanol (70%), 50 ml

Trough 2: Denaturation Solution. 40 ml

Trough 3: Wash Buffer, 50 ml

Trough 4: High-purity water, 50 ml

Trough 5: High-purity water, 70 ml

Figure 10. Illustration of the PyroMark Q24 Cartridge seen from above. Annotations correspond to the label on the reagent vials.

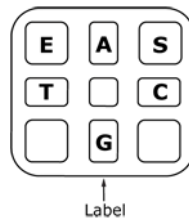
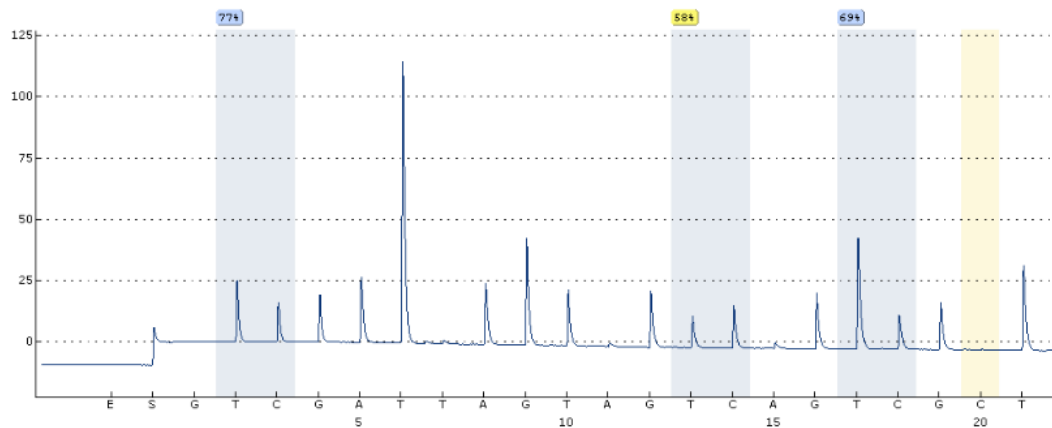


Figure 11. Pyrogram trace obtained after analysis of samples.



Blu areas: three CpG sites with corresponding quantification of methylation level, yellow area: Bisulfite control.

Table 1. Characteristics of study participants (N= 307)

	n (%)	Mean	s.d.	Range
<i>Nationality</i>				
Italian	289 (94.2)			
European countries	9 (2.9)			
Non-European countries	9 (2.9)			
Age (years)		28.9	7.6	14–49
<i>Education (years of schooling)</i>				
<5	1 (0.3)			
5	24 (7.8)			
8	134 (43.6)			
13	122 (39.7)			
>13	26 (8.5)			
<i>Employment status</i>				
Employed	89 (29.0)			
Unemployed	10 (3.2)			
Student	34 (11.1)			
Housewife	174 (56.7)			
<i>Smoking^a</i>				
Current smokers	94 (30.9)			
Non-smokers	171 (56.3)			
Former smokers	39 (12.8)			
Body mass index ^b		24.9	5.5	15.6–47.7
Underweight	22 (7.2)			
Normal weight	160 (52.1)			
Overweight	76 (24.8)			
Obese	49 (16.0)			
<i>Pregnancy</i>				
No	169 (55.0)			
Yes	138 (45.0)			
<i>Trimester of pregnancy^c</i>				
First	35 (25.5)			
Second	42 (30.7)			
Third	60 (43.8)			

^aData missing for three women

^bBased on criteria from the World Health Organization (1995)

^cData missing for one woman

Table 2. Folic acid supplements and folate intakes

<i>Folic acid supplements use</i>	<i>n (%)</i>	<i>P-value</i>		
Overall	133 (43.3)			
Non-pregnant women	13 (7.7)	0.000 ^a		
<i>Pregnant women</i>	120 (87.0)			
First trimester	25 (71.4)	0.001 ^a		
Second trimester	37 (88.1)			
Third trimester	57 (95.0)			
<i>Folate intake (µg per day)</i>	<i>Mean</i>	<i>P-value</i>		
Overall population	208.5			
Non-pregnant women	196.9	0.026 ^b		
<i>Pregnant women</i>	222.8			
First trimester	197.3	> 0.05 ^b		
Second trimester	223.9			
Third trimester	235.6			
<i>Prevalence of inadequate folate intake^c</i>	<i>n (%)</i>	<i>P-value</i>		
Overall population	158 (51.5)			
Non-pregnant women	141 (83.4)	0.000 ^a		
<i>Pregnant women</i>	17 (12.3)			
First trimester	9 (25.7)	0.013 ^a		
Second trimester	5 (11.9)			
Third trimester	3 (5.0)			
<i>Tertiles of folate intake</i>	<i>First tertile</i>	<i>Second tertile</i>	<i>Third tertile</i>	<i>P-value</i>
Overall population (limit, µg per day)	155.7	234.1	939.7	
<i>n (%)</i>	102 (33.2)	103 (33.6)	102 (33.2)	
Folic acid supplements (yes)	40 (39.2)	42 (40.8)	51 (50.0)	0.244 ^a
Low-medium educational level	49 (48.0)	59 (57.3)	48 (47.1)	0.271 ^a
Medium-high educational level	53 (52.0)	44 (42.7)	54 (52.9)	
Working women	41 (40.2)	49 (47.6)	43 (42.2)	0.543 ^a
Housewife women	61 (59.8)	54 (52.4)	59 (57.8)	
Normal weight women	49 (48.0)	50 (48.5)	61 (59.8)	0.164 ^a
Overweight or obese women	53 (52.0)	53 (51.5)	41 (40.2)	

^a χ^2 test^b Student's t-test^c Prevalence of inadequate folate intake was estimated by comparing folate intake with the estimated average requirement for folate (Food and Nutrition Board, Institute of Medicine, 2001)**Table 3.** Distribution of MTHFR C677T and A1298C genotypes and allele frequencies ^a

	<i>Genotype frequencies, n (%)</i>			<i>Allele frequencies, n (%)^b</i>	
C677T	CC 101 (32.9)	CT 143 (46.6)	TT 63 (20.5)	C 345 (56.2)	T 269 (43.8)
A1298C	AA 144 (46.9)	AC 139 (45.3)	CC 24 (7.8)	A 427 (69.5)	C 187 (30.5)

^aThe allelic distribution of both MTHFR polymorphisms follows the Hardy–Weinberg equilibrium expectations.^bTotal number of alleles for each polymorphism: 614

Table 4. Distribution of C677T and A1298C genotype and allelic frequencies in the four groups according to birth date

<i>MTHFR</i>	<i>Frequencies by date of birth, n (%)</i>			
	1959–1973	1974–1979	1980–1984	1985–1994
<i>C677T</i>				
CC	32 (41.6)	21 (30.9)	25 (32.1)	23 (27.4)
CT	34 (44.2)	36 (52.9)	31 (39.7)	42 (50.0)
TT	11 (14.3) ^a	11 (16.2)	22 (28.2) ^a	19 (22.6)
C allele ^b	98 (63.6)	78 (57.3)	81 (51.9)	88 (52.4)
T allele ^b	56 (36.4) ^{c,d}	58 (42.7)	75 (48.1) ^c	80 (47.6) ^d
<i>A1298C</i>				
AA	36 (46.8)	31 (45.6)	40 (51.3)	37 (44.0)
AC	33 (42.9)	33 (48.5)	32 (41.0)	41 (48.8)
CC	8 (10.4)	4 (5.9)	6 (7.7)	6 (7.1)
A allele ^b	105 (68.2)	95 (69.8)	112 (71.8)	115 (68.4)
C allele ^b	49 (31.8)	41 (30.2)	44 (28.2)	53 (31.6)

^a χ^2 test: p=0.03

^b Total number of alleles for each polymorphism: 614.

^c χ^2 test: p=0.04

^d χ^2 test: p=0.04

Table 5. RBC folate levels, plasma concentrations of tHcy and folate intake of the study women

	All women (n= 204)	Pregnant (n= 117)	Non-pregnant (n= 87)	p-value*
RBC folate levels (nmol/L)				
Mean (\pm SD)	331.5 (\pm 125.6)	374.5 (\pm 134.6)	273.6 (\pm 82.8)	<0.001
Median	309.3	367.7	262.6	
Interquartile range	231.8-411.4	256.7-464.7	221.6-315.3	
Range	111.9-622.1	111.9-622.1	114.4-540.0	
Plasma concentrations of tHcy (μ mol/L)				
Mean (\pm SD)	14.5 (\pm 2.7)	14.0 (\pm 2.6)	15.2 (\pm 2.7)	0.002
Median	15.2	14.5	15.7	
Interquartile range	13.6-16.2	13.0-15.7	14.4-16.4	
Range	4.3-26.5	4.3-21.9	7.7-26.5	
Folate intakes (μ g/d)				
Mean (\pm SD)	212.0 (\pm 109.1)	222.5 (\pm 119.0)	197.9 (\pm 92.8)	0.112
Median	196.5	204.1	188.7	
Interquartile range	138.9-257.9	145.8-272.8	133.1-238.9	
Range	47.4-939.7	67.2-839.7	47.4-579.3	

Student's t test for comparisons of means between pregnant and nonpregnant women (two-sided p values).

Table 6. RBC folate level, plasma concentration of tHcy and dietary folate intake (means \pm SD) stratified by MTHFR C677T genotypes

	<i>MTHFR C677T genotypes</i>				
	CC	CT	TT	CT or TT*	CC or CT**
Mean RBC folate level (nmol/L) (\pm SD)	321.0 (\pm 116.9)	329.1 (\pm 116.5)	348.1 (\pm 150.0)	335.8 (\pm 129.0)	326.0 (\pm 116.4)
Mean plasma concentration of tHcy (μ mol/L) (\pm SD)	14.8 (\pm 1.8)	14.2 (\pm 3.0)	14.7 (\pm 3.1)	14.4 (\pm 3.0)	14.5 (\pm 2.6)
Mean folate intake (μ g/d) (\pm SD) [§]	215.8 (\pm 97.0)	211.2 (\pm 121.6)	209.2 (\pm 99.2)	210.5 (\pm 113.9)	213.0 (\pm 112.4)

No significant differences were observed between genotypes (Student's t test for the comparison of means).

^a Carriers of the T allele

^b Carriers of the C allele

Table 7. Mean levels (mean Δ Ct) of the mtDNA4977-bp deletion in the study population by age, nutritional and lifestyle factors, and *MTHFR* C677T polymorphism

Variables	Number of women	Mean Δ Ct (\pm SD)	<i>p</i> -value
Age 1 st quartile (14-23 years)	124	1.24 \pm 0.51	0.67
Age 2 nd quartile (24-28 years)	125	1.28 \pm 0.54	
Age 3 rd quartile (29-34 years)	111	1.20 \pm 0.51	
Age 4 th quartile (35-49 years)	105	1.23 \pm 0.52	
Age \leq 27 years (median value)	233	1.28 \pm 0.54	0.09
Age > 27 years	232	1.20 \pm 0.51	
Smokers	196	1.21 \pm 0.540	0.29
Non-smokers	266	1.26 \pm 0.54	
Inadequate folate intake ^a	213	1.21 \pm 0.49	0.20
Adequate folate intake	252	1.27 \pm 0.55	
Folate deficiency ^b	243	1.04 \pm 0.26	0.22
No folate deficiency	222	0.99 \pm 0.26	
Pregnant	229	1.24 \pm 0.52	0.96
Non-pregnant	236	1.24 \pm 0.53	
Underweight and normal weight ^c	301	1.29 \pm 0.55	<0.01
Overweight and obese	164	1.14 \pm 0.44	
Supplement users	212	1.24 \pm 0.53	0.97
Supplement non-users	253	1.24 \pm 0.51	
Homozygous 677TT mutated genotype	95	1.00 \pm 0.24	0.19
Other genotypes ^d	370	1.05 \pm 0.26	

^a Prevalence of inadequate folate intake was determined by comparing folate intake with the estimated average dietary requirements: 520 μ g/d for pregnant and 320 μ g/d for non pregnant women (Food and Nutrition Board, 2001).

^b Folate deficiency was defined as RBC folate <305 nmol/L (Food and Nutrition Board, 2001).

^c Based on criteria from the World Health Organization (1995).

^d *MTHFR* 677CC and *MTHFR* 677CT.

Table 8. Multivariate analysis for mtDNA 4977-bp deletion levels (Δ Ct)

Population characteristics and variables	Δ Ct below the median value ^a (N ^b)	Δ Ct above the median value ^a (N ^b)	Multivariate analysis	
			<i>p</i>	OR (CI 95%) ^c
Age (below the median value: 27 years)	46.8% (109)	53.4% (124)	0.77	1.09 (0.62-1.89)
Smokers	46.1% (107)	38.7% (89)	0.92	0.97 (0.57-1.65)
Pregnant	48.9% (114)	49.6% (115)	0.25	1.68 (0.70-4.03)
Overweight and obese	38.6% (90)	31.9% (74)	0.32	0.75 (0.43-1.32)
Inadequate folate intake	46.8% (109)	44.8% (104)	0.55	1.52 (0.39-6.00)
Folate deficiency	52.1% (155)	52.7% (88)	0.18	1.52 (0.83-2.80)
Supplement non-users	55.8% (130)	53.0% (123)	0.33	0.46 (0.10-2.18)
Homozygous 677TT mutated genotype	22.4% (59)	18.0% (36)	0.43	1.27 (0.71-2.27)

^a Δ Ct median value was 1.09

^b N: Number of women

^c OR for Δ Ct above the median value

Table 9. Characteristics of study participants (N= 380)

	n (%)	Mean	SD	Range
Age at recruitment (years)		28.7	9.5	13-85
Nationality				
- Italian	362 (95.3)			
- European countries	11 (2.9)			
- Non-European countries	7 (1.8)			
Education (years of schooling)				
- ≤8 (low)	202 (53.3)			
- ≤13 (medium)	126 (33.2)			
- >13 (high)	51 (13.5)			
Employment status				
- Employed	120 (31.9)			
- Unemployed	33 (8.8)			
- Student	46 (12.2)			
- Housewife	177 (47.1)			
BMI		24	5.3	14.9-45.9
- Underweight	33 (8.7)			
- Normal weight	223 (58.7)			
- Overweight	78 (20.5)			
- Obese	46 (12.1)			
Smoking				
- Current smokers	90 (23.7)			
- Non smokers	244 (64.4)			
- Former-smokers	45 (11.9)			
Pregnancy status (yes)	203 (53.4)			
MDS		4.1	1.65	0-9
- 0-3 (≤25 th percentile)	140 (36.8)			
- 4-6 (>25 th percentile - ≤90 th percentile)	209 (55.0)			
- 7-9 (>90 th percentile)	31 (8.2)			
TNFA -308 G>A genotypes				
- GG	306 (80.5)			
- GA	69 (18.2)			
- AA	5 (1.3)			

Abbreviations: SD, Standard Deviation; BMI, Body Mass Index; MDS, Mediterranean Diet Score.

Table 10. Fatty acids and energy intakes in underweight/normal weight and overweight/obese women[#]

Nutrient intake (g/day)	Underweight/ normal weight	Overweight/ obese	p-value*
	Mean ± S.D.	Mean ± S.D.	
Linoleic acid	17.07 ± 12.09	12.66 ± 8.05	0.000
Arachidonic acid	0.18 ± 0.28	0.13 ± 0.06	0.012
γ-linolenic acid	1.60 ± 1.10	1.20 ± 0.54	0.000
α- linolenic acid	1.60 ± 1.11	1.21 ± 0.53	0.000
Eicosapentaenoic acid	0.13 ± 0.23	0.08 ± 0.07	0.011
Docosahexaenoic acid	0.30 ± 0.67	0.20 ± 0.14	0.031
Polyunsaturated fatty acids	18.43 ± 10.10	14.50 ± 9.10	0.000
Monounsaturated fatty acid	55.25 ± 25.24	47.41 ± 23.88	0.004
Total unsaturated fatty acids	73.68 ± 33.27	61.91 ± 31.62	0.001
<i>Unsaturated/saturated fatty acids ratio</i>	2.34 ± 0.69	2.34 ± 0.66	0.990
<i>Saturated fatty acids</i>	33.65 ± 18.13	27.33 ± 13.65	0.000
<i>Palmitic acid</i>	19.35 ± 13.18	15.01 ± 7.12	0.000
<i>Energy intake (Kcal/day)</i>	2272.0	1811.1	0.000

[#] Statistically significant p-values (p<0.01) are indicated in bold font

*t-Student test for the comparison of means between underweight/normal weight and overweight/obese women (two-sides p-values)

Table 11. Fatty acids and energy intakes between women with greater adherence to MD and women with lower adherence to MD[#]

Nutrient intake (g/day)	MDS≤6	MDS>6	p-value*
	Mean ± S.D.	Mean ± S.D.	
Linoleic acid	15.24 ± 10.94	20.05 ± 12.39	0.021
Arachidonic acid	0.16 ± 0.24	0.17 ± 0.08	0.830
γ-linolenic acid	1.44 ± 0.98	1.80 ± 0.75	0.017
α- linolenic acid	1.44 ± 0.99	1.80 ± 0.67	0.008
Eicosapentaenoic acid	0.11 ± 0.20	0.16 ± 0.10	0.021
Docosahexaenoic acid	0.26 ± 0.58	0.34 ± 0.16	0.081
Polyunsaturated fatty acids	16.69 ± 9.53	22.35 ± 12.85	0.002
Monounsaturated fatty acid	51.36 ± 24.27	67.64 ± 28.95	0.000
Total unsaturated fatty acids	68.05 ± 31.95	89.99 ± 39.90	0.000
<i>Unsaturated/saturated fatty acids ratio</i>	2.32 ± 0.68	2.67 ± 0.62	0.006
<i>Saturated fatty acids</i>	31.26 ± 16.93	35.26 ± 18.18	0.210
<i>Palmitic acid</i>	17.74 ± 11.88	20.16 ± 9.65	0.197
<i>Energy intake (Kcal/day)</i>	2065.7 ± 897.5	2750 ± 1006.7	0.000

[#] Statistically significant p-values (p<0.01) are indicated in bold font

*t-Student test for the comparison of means between MDS≤6 and MDS>6 (two-sides p-values)

Table 12. *TNFA* -308 G>A polymorphism and unsaturated fatty acid intake interactions on overweight/obesity risk#

Nutrient intake	A* OR (95%CI)	B° OR (95%CI)	A+B§ OR (95%CI)
Linoleic acid	2.291 (0.730-7.185)	3.312 (1.655-6.628)	4.065 (1.751-9.432)
Arachidonic acid	4.327 (1.549-12.082)	2.11 (1.126-3.953)	1.857 (0.827-4.170)
γ-linolenic acid	2.708 (0.851-8.617)	3.138 (1.603-6.144)	3.505 (1.549-7.931)
α- linolenic acid	3.5 (1.125-10.886)	3.855 (1.882-7.898)	4.136 (1.747-9.792)
Eicosapentaenoic acid	4.292 (1.456-12.651)	2.675 (1.389-5.152)	2.517 (1.113-5.694)
Docosahexaenoic acid	2.408 (0.846-6.854)	2.289 (1.205-4.348)	2.634 (1.175-5.904)
Polyunsaturated fatty acids	2.462 (0.782-7.752)	3.068 (1.566-6.011)	3.556 (1.567-8.067)
Monounsaturated fatty acid	2.372 (0.798-7.055)	2.211 (1.182-4.136)	2.512 (1.147-5.502)
Total unsaturated fatty acids	2.188 (0.741-6.461)	2.0 (1.082-3.698)	2.316 (1.067-5.026)
<i>Unsaturated/saturated fatty acids ratio</i>	0.838 (0.286-2.455)	0.778 (0.45-1.345)	1.21 (0.592-2.474)

Statistically significant p-values ($p < 0.01$) from χ^2 -test are indicated in bold font

*A: Risk of developing overweight/obesity in carriers of *TNFA* -308 A allele

°B: Risk of developing overweight/obesity in women with FA intake below or equal to the 75th percentile of the unsaturated FA intake distribution (g/day)

§A+B: Risk of developing overweight/obesity in women exposed to both A and B

Table 13. *TNFA* -308 G>A polymorphism and saturated fatty acid intake interactions on overweight/obesity risk

Nutrient intake	A* OR (95%CI)	B° OR (95%CI)	A+B§ OR (95%CI)
Palmitic acid	1.002 (0.536-1.872)	0.217 (0.098-0.478)	1.447 (0.504-4.151)
Saturated FA	1.158 (0.622-2.156)	0.335 (0.165-0.678)	1.176 (0.402-3.438)

*A: Risk of developing overweight/obesity in carriers of *TNFA* -308 A allele

°B: Risk of developing overweight/obesity in women with FA intake above the 75th percentile of the saturated FA intake distribution (g/day)

§A+B: Risk of developing overweight/obesity in women exposed to both A and B

Table 14. Characteristics of the study population (N= 177)

Characteristics	% (N.)
Mean age (years) – median	30.7 – 28
Age percentile distribution	
13-23 years	24.3% (43)
24-28 years	22.6% (40)
39-37 years	19.8% (35)
38-85 years	33.3% (59)
Nutritional status	
- Underweight	6.8% (12)
- Normalweight	60.4% (107)
- Overweight	18.1% (32)
- Obese	14.7% (26)
Mean MDS - median	4,1 - 4
-0/3 ($\leq 25^{\text{th}}$ percentile)	36.7% (65)
-4/6	53.7% (95)
-7/9 ($\geq 90^{\text{th}}$ percentile)	9.6% (17)
Mean food folate intake – median ($\mu\text{g}/\text{die}$)	245.8 – 228.8
Dietary folate deficiency (cut-off: $400\mu\text{g}/\text{day}^{\circ}$)	
-Yes	88.1% (156)
-No	11.9% (21)
Supplement users	
-Yes	6.8% (12)
-No	93.2% (165)
Overall folate deficiency	
-Yes	83.1% (147)
-No	16.9% (30)
Smoking status	
-Current	25% (44)
-Non current	75% (132)
Employment status	
-Employed (part/full-time)	35.6% (63)
-Unemployed	64.4% (114)
Education level	
-Low-medium (Less than high school education)	43.5% (77)
-Medium-high (High school education or greater)	56.5% (100)
Smoking status	
-Current	29.5% (53)
-Non current	64.4% (114)
-Former	5.6% (10)

$^{\circ}$ Recommended daily intake by Food and Nutrition Board, 2001

Table 15. Mean levels of dietary folate intake in the study population by age, nutritional and lifestyle factors

Characteristics		Folate intake (µg/day)	p	Adjusted folate intake (µg/1,000 kcal)	
Age (median)	≤28	244.6	0.894	125.1	0.204
	>28	247.0		133.9	
Age percentile distribution	13-23	225.8	0.237*	117.8	0.248*
	24-28	266.0		134.6	
	39-37	222.4		136.8	
	38-85	260.4		130.0	
BMI	≤30	254.9	0.017	130.9	0.290
	>30	192.5		120.6	
Nutritional status	Underweight	318,9	0.002 *	123.4	0.664*
	Normalweight	262.8		132.3	
	Overweight	204.6		129.2	
	Obese	192.5		120.4	
Education level	Low-medium	244.0	0.868	129.0	0.921
	Medium-high	247.1		129.7	
Employment status	Employed	250.8	0.689	128.4	0.820
	Unemployed	243.0		130.0	
Smoking status	Current	263.5	0.254	131.2	0.717
	Non current	238.8		128.3	
Supplement users	Yes	309.9	0.063	128.4	0.934
	No	241.1		129.9	
MDS	0-3	176.9	0.000 *	110.9	0.000*
	4-6	273.7		138.5	
	7-9	353.0		149.3	

* Analysis of variance

Table 16. Mean levels of MDS in the study population by age, nutritional and lifestyle factors

Characteristics		MDS	p
Age (median)	≤ 28	3.7	0.002
	>28	4.5	
Age percentile distribution	13-23	3.3	0.001 *
	24-28	4.3	
	39-37	4	
	38-85	4.7	
BMI	≤ 30	4.3	0.007
	> 30	3.3	
Nutritional status	Underweight	4.0	0.05 *
	Normalweight	4.3	
	Overweight	4.2	
	Obese	3.3	
Education level	Low-medium	3.8	0.042
	Medium-high	4.4	
Employment status	Employed	4.3	0.366
	Unemployed	4.0	
Smoking status	Current	4.3	0.430
	Non current	4.1	
Supplement users	Yes	5.7	0.001
	No	4.0	
Dietary folate deficiency	Yes	4.00	0.010
	No	5.05	
Overall Folate deficiency	Yes	3.89	0.000
	No	5.27	

* Analysis of variance

Table 17. Folate deficiency and risk of overweight/obese

	Folate deficiency		p	OR (95% CI)
	Yes	No		
Overweight/obese	54 36.7%	4 13.3%	0.000	3.8 (1.3 – 11.4)
Underweight/nbormalweight	93 63.3%	26 86.7%		

Table 18. Degree of LINE-1 methylation in Leukocytes

	Locus 1	Locus 2	Locus 3	Mean
% Methylation	80.1 ± 2.9	57.8 ± 4.4	62.9 ± 4.6	65.3 ± 3.3

Table 19. Global methylation level of leukocyte DNA according to study characteristics

Characteristics	N	Mean (%)	SD (%)	p
Age (median)				
≤ 28	89	65.3	3	0.133
>28	86	65.2	3.5	
Age percentile distribution				
13-23	43	65.5	3.46	0.740*
24-28	39	65.2	2.6	
39-37	34	64.7	2.8	
38-85	59	65.4	3.8	
BMI				
<30	149	65.2	3.1	0.668
≥30	26	65.5	4.1	
Nutritional status				
Unverweight	12	65.3	3.6	0.958*
Normalweight	105	65.1	3.2	
Overweight	32	65.4	2.7	
Obese	26	65.5	4.1	
Education level				
Low-medium	77	65.5	3.4	0.419
Medium-high	98	65.0	3.1	
Employment				
Employed	62	65.5	3.5	0.415
Unemployed	113	65.1	3.1	
Smoking status				
Current	44	66.2	3.8	0.06
Non current	132	65	3	
Dietary folate deficiency				
Yes	154	65.2	3.2	0.347
No	21	65.9	3.4	
Supplement users				
Yes	11	65.4	4.4	0.882
No	164	65.2	3.2	
Overall Folate deficiency				
Yes	146	65.0	3.2	0.05
No	29	66.3	3.3	

* Analysis of variance

Table 20. Folate deficiency and risk of hypomethylation

	Folate deficiency		OR	95%CI
	Yes	No		
Methylation level				
First tertile	39% (57)	17.2% (5)	4	1.3-11.8
Second tertile	31.5% (46)	31% (9)	2.3	0.7-7.1
Third tertile	29.5% (43)	51.7% (15)	<i>rif</i>	-

Table 21. Fruit and nuts consumption and risk of hypomethylation

Methylation level	Fruit and nuts MDS		OR	95%CI
	0	1		
First tertile	63.9% (39)	39% (23)	2.8	1.3-5.8
Third tertile	36.1% (22)	61% (36)	<i>rif</i>	-

Table 22. First multivariate analysis for hypomethylation level

Population characteristics and variables ^o	Methylation level		Multivariate analysis		
	First tertile	Third tertile	p	OR	95% CI
Folate deficiency (yes)	39% (57)	29.5% (43)	0.053	3	1- 3.3
Fruit and nuts MDS (= 0)	63.9% (39)	36.1% (22)	0.033	2.39	1.1 - 4.9

^o considering only those variables associated in univariate analysis

Table 23. Second multivariate analysis for hypomethylation level*

* Adjusted for smoking status, daily energy intake and nutritional status

Population characteristics and variables	Methylation level		Multivariate analysis		
	First tertile	Third tertile	p	OR	95% CI
Smoking status (yes)	23.0% (14)	36.2% (21)	0.089	0.442	0.173 - 1.131
Daily energy intake (> median)	40.3% (25)	53.4% (31)	.0766	1.167	0.422 - 3.228
Folate deficiency (yes)	91.9% (57)	74.1% (43)	0.102	3.065	0.799 - 11.756
Age (> median)	46.8% (29)	48.3% (28)	0.751	1.162	0.460 - 2.933
Overweight/obese (yes)	35.5% (22)	31.0% (18)	0.862	1.089	0.415 - 2.858
MDS cereals (=0)	51.6% (32)	60.3% (35)	0.187	0.529	0.206 - 1.361
MDS vegetables (=0)	51.6% (32)	48.3% (28)	0.516	0.744	0.306 - 1.813
MDS fruit and nuts (=0)	62.9% (39)	37.9% (22)	0.006	3.768	1.460 - 9.721
MDS meat (=0)	46.8% (29)	58.6% (34)	0.285	0.607	0.243 - 1.515
MDS dairy (=0)	53.2% (33)	46.6% (27)	0.285	1.579	0.683 - 3.651
MDS fish (=0)	59.7% (37)	46.6% (27)	0.092	2.153	0.881 - 5.258
MDS legums (=0)	48.4% (30)	55.2% (32)	0.234	0.580	0.237 - 1.421
MDS unsaturated/saturated FA (=0)	43.5% (27)	51.7% (30)	0.314	0.646	0.275 - 1.513
MDS alcol (=0)	87.1% (54)	79.3% (46)	0.568	1.442	0.411 - 5.057