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AN EPIDEMIOLOGICAL APPROACH TO STUDY THE INTERPLAY BETWEEN GENOME, EPIGENOME AND EXPOSOME IN WOMEN

PhD Thesis

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List of publications

This PhD thesis is based on the following original publications:

1. Barchitta M, Maugeri A, La Rosa MC, Magnano San Lio R, Favara G, Panella M, Cianci A, Agodi A. Single Nucleotide Polymorphisms in Vitamin D Receptor Gene Affect Birth Weight and the Risk of Preterm Birth: Results From the "Mamma & Bambino" Cohort and A Meta-Analysis. Nutrients. 2018 Aug 27;10(9). pii: E1172. doi: 10.3390/nu10091172. PubMed PMID: 30150529; PubMed Central PMCID: PMC6164379.

2. Maugeri A, Barchitta M, Agrifoglio O, Favara G, La Mastra C, La Rosa MC, Magnano San Lio R, Panella M, Cianci A, Agodi A. The impact of social determinants and lifestyles on dietary patterns during pregnancy: evidence from the "Mamma & Bambino" study. Ann Ig. 2019 Mar-Apr;31(2 Supple 1):81-89. doi: 10.7416/ai.2019.2280. PubMed PMID: 30994167.

3. Maugeri A, Barchitta M, Favara G, La Rosa MC, La Mastra C, Magnano San Lio R, Agodi A. Maternal Dietary Patterns Are Associated with Pre-Pregnancy Body Mass Index and Gestational Weight Gain: Results from the "Mamma & Bambino" Cohort. Nutrients. 2019 Jun 10;11(6). pii: E1308. doi: 10.3390/nu11061308. PubMed PMID: 31185656; PubMed Central PMCID: PMC6627583.

4. Barchitta M, Maugeri A, Magnano San Lio R, Favara G, La Rosa MC, La Mastra C, Quattrocchi A, Agodi A. Dietary Patterns are Associated with Leukocyte LINE-1 Methylation in Women: A Cross-Sectional Study in Southern Italy. Nutrients. 2019 Aug 9;11(8). pii: E1843. doi: 10.3390/nu11081843. PubMed PMID: 31395820; PubMed Central PMCID: PMC6722720

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6. Barchitta M, Maugeri A, Magnano San Lio R, Favara G, La Mastra C, La Rosa MC, Agodi A. Dietary Folate Intake and Folic Acid Supplements among Pregnant Women from Southern Italy: Evidence from the "Mamma & Bambino" Cohort. Int J Environ Res Public Health. 2020 Jan 19;17(2):638. doi: 10.3390/ijerph17020638. PMID: 31963813; PMCID: PMC7013905.

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16. Maugeri A, Magnano San Lio R, La Rosa MC, Giunta G, Panella M, Cianci C, Caruso M, Agodi A, Barchitta M. The effect of nutrient intake on telomere length of cell-free circulating DNA from amniotic fluid: findings from the Mamma & Bambino cohort. *Submitted.*

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Acknowledgments

Abbreviations

5mC	5-Methyl-cytosines
ACS	American Cancer Society
AFAR	American Federation for Aging Research
AGA	Adequate for gestational age
ANC	Antenatal care
AUC	Area Under the Curve
BiB	(The) Born in Bradford (study)
BMI	Body mass index
CDAI	Composite Dietary Antioxidant Index
cfDNA	Cell-free circulating fetal DNA
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CpG	C--phosphate—G
DII	Dietary Inflammatory Index
DNMT	DNA methyltransferase
DOHaD	Developmental Origins of Health and Disease (hypothesis)
DTaP	Diphtheria – Tetanus – and acellular Pertussis
EDEN	Etude de cohorte généraliste, menée en France sur les Déterminants pré et post natals précoces du développement psychomoteur et de la santé de l'Enfant
ELBW	Extremely low-birth weight
EORTC QLQ-C30	European Organization for the Research and Treatment of Cancer Quality-of-Life Questionnaire–Core 30
EWAS	Epigenome-wide association study
FAO	Food and Agricultural Organization
FFQ	Food Frequency Questionnaire
GDM	Gestational diabetes mellitus
GWAS	Genome Wide Association Study
GWG	Gestational weight gain
HELIX	Human Early-Life Exposome (study)
HGP	Human Genome Project
HIV	Human Immunodeficiency Virus
HLA-E	Human Leucocyte Antigen E
HPV	Human Papilloma Virus
HWE	Hardy-Weinberg Equilibrium
IARC	International Agency for Research on Cancer
INMA	Infancia y Medio Ambiente (cohort)
IOM	Institute of Medicine
IPAQ	International Physical Activity Questionnaire
IQR	Interquartile range
IUGR	Intrauterine growth restriction
KANK	Kaunas cohort

LBW	Low-birth weight
LGA	Large for gestational age
LINE	Long Interspersed Nuclear Element
MD	Mediterranean Diet
MDS	Mediterranean Diet Score
mlDNA	Maternal leucocyte DNA
MMR	Measles – Mumps - Rubella
MoBa	(The) Norwegian Mother and Child Cohort Study
MUFA	Monounsaturated fatty acid
NIH	National Institute of Health
NK	Natural killer (cells)
OR	Odds ratio
ORF	Open reading frame
PCA	Principal component analysis
PCR	Polymerase chain reaction
PE	Preeclampsia
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PTB	Pre-term birth
PUFA	Polyinsatured fatty acid
QLQ-BR23	Quality of Life Questionnaire Breast Cancer Module 23
QoL	Quality of life
qPCR	Quantitative polymerase chain reaction
RCT	Randomized controlled trial
ROC	Receiver Operating Characteristic (curve)
ROS	Reactive oxygen species
SD	Standard deviation
SDG	Sustainable Development Goals
SE	Standard error
SES	Socioeconomic status
SGA	Small for gestational age
SINE	Short Interspersed Nuclear Element
SNP	Single Nucleotide Polymorphism
USDA	U.S. Department of Agriculture
UTR	Untranslated Region
VDR	Vitamin D receptor
VDRE	Vitamin D receptor elements
VLBW	Very-low birth weight
VPD	Vaccine-preventable disease
WHO	World Health Organization

1. Introduction

1.1 The relationship between genome and exposome

From Mendel's discovery of trait inheritance models to the identification of DNA as the genetic material [1], genetics grew as a field of biology in the 1900s. In the mid to late 1970s [2], the development of sequencing technologies allowed to sequence the first genes [3] and genomes [4]. In the early 2000s, the identification of the first human genome by the Human Genome Project (HGP) represented a great scientific achievement, a turning point for human genetics and a starting point for human genomics [5]. Following the first human genome sequencing [6, 7], several projects - such as the Personal Genome Project - were proposed to take genomics into the medical field for personalized healthcare [8], with the aim to identify genetic factors involved in several disease etiology. In 2005, the development of genome-wide association studies (GWAS) started solidifying genomics into an understanding of disease at genome level [9], including larger cohorts for evaluating neurological [10], cardiovascular [11], metabolic [12], renal [13], hepatic [14], and reproductive factors [15] over time.

In parallel with the concept of the genome, the terms "exposome" has been coined to address chronic human health issues in a broader perspective [16, 17]. In 2005, Christopher Wild firstly introduced the concept of the exposome, which comprises all environmental exposures across the lifetime. Thus, the exposome is defined as the totality of exposure that individuals experience from conception until death and its impact on chronic diseases [18]. Exposure is a generic term that includes toxicants in the environment, diet, other lifestyles and socio-economic characteristics [19]. In general, the exposome includes: i) internal factors, ii) specific external factors, and iii) general external factors, which are overlapping and intertwining with each other. Internal factors include all internal biological factors such as age, metabolic factors, gut microflora, inflammation, oxidative stress. Specific external factors regard contaminants such as diet and tobacco, as well as physical, biological, and physiological exposures. The third domain includes social factors such as education level and socioeconomic status, as well as urban environment and climate [20] (**Figure 1**).

The definition of the exposome was further expanded by Miller and Jones as "the cumulative measure of environmental influences and associated biological responses throughout the lifespan, including exposures from the environment, diet, behavior, and endogenous processes" [16].

As such, in epidemiology the exposome approach is considered and presented as highly innovative [21]. Since the research suggests that environmental exposures have a greater impact on health and disease than genetic factors alone, an integrated approach is necessary to understand the relationship between genome, exposome and human health [22]. In fact, the low penetrance of genetic variants

— as opposed to their high prevalence — implies that their contribution to disease burden is crucially linked to the presence of some exposures. Conceptually, the exposome lies in a two-fold relation with the genome. On the one hand, the innovations of genome-sequencing are continually mentioned in discussions on the exposome. On the other hand, the idea of the exposome as the necessary complement of the genome puts it into a critical position, underlying the need for new and different approaches [23]. However, tools that could integrate the genome and exposome for complex trait analyses are currently scarce.

Technologies such as omics, sensors, and geographic information are allowing for a more comprehensive understanding of the exposome, which is dynamic, as opposed to the static genome [16]. To a large extent, the exposome aims to move the developments of the omic techniques to epidemiological research, trying to get a similar level of precision in measuring the exposures and their effects on human health [22].

1.2 Biomarkers

In 1998, the lack of agreement on the term "biomarker" led the National Institutes of Health (NIH) to define biomarkers as those "characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [24]. Next, the word "genotype" was added to the definition, indicating how the focus of the research has changed since 2001. Furthermore, the *World Health Organization* (WHO) has stated that an accurate definition of biomarkers includes "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" [25]. In fact, a common objective of studies on biomarkers is to investigate the association between an exposure (e.g., a hypothesized risk factor for a disease) and the risk of disease or, more generally, of any health outcome [26]. However, an even broader definition takes into account not just incidence and outcome of disease, but also the effects of treatments. For these reasons, an ideal biomarker would be highly sensitive and specific, with biologic plausibility and capability of being easily measured and rapidly reported. According to the Dictionary of Epidemiology, a biomarker - or biological marker - is "a cellular, biochemical, or molecular indicator of exposure, biological, subclinical, or clinical effects, or of possible susceptibility". Specifically, a biomarker of exposure is an exogenous substance or its metabolite that is measured in a compartment within an organism to confirm and assess the exposure of individuals or populations to a particular substance [27]. Differently from biomarkers of exposure, a biomarker of effect is a biochemical, physiological, behavioral, or other measurable

alteration within an organism that can be recognized as associated with an established or possible disease [27]. Thus, the linkage of biomarkers between the exposure and its effects contributes to the definition the so-called dose-response relationships. In addition to the above-mentioned, a biomarker of susceptibility is an indicator of pre-existing genetic factors regardless of exposure, but which may affect the probability of disease development as a result of exposure [27]. In the context of the gene-environment interaction, genetic variants assume the role of effect modulators determining an increase (susceptibility) or a decrease (protection) of the strength of the interaction. Particularly, several genetic variants are potentially considered to be responsible for disease susceptibility [28].

In addition to these biomarkers, epigenetic biomarkers are considered as emerging tools for the early detection of various diseases, for prognostic and treatment monitoring, and for predicting the risk of future diseases in a personalized medicine perspective [29]. Epigenetic modifications involve the interplay between DNA methylation, histone modification and micro-RNA expression in modulating gene expression and, in turn, the risk of several diseases (e.g., cardiovascular diseases, obesity, type-2 diabetes and cancer) [30-32]. More recently, several lines of evidence proposed that biological aging is reflected by highly reproducible DNA methylation changes at specific sites in the genome, suggesting a panel of DNA methylation-based biomarkers of aging that characterize the so-called "epigenetic age". Additionally, telomere length represents a promising biomarker for biological aging and age-related diseases, suggesting how shorter telomeres are associated with diabetes [33], cancer [34] and cardiovascular disease [35] in adults. In this scenario, quantitative biomarkers of aging can be considered as valuable tools to measure physiological age and to potentially improve human health, preventing age-associated diseases [36].

1.2.1 Single Nucleotide Polymorphisms

In 2006, it has been introduced the concept of "Genomic Biomarker", which is defined as a DNA or RNA characteristic that is an indicator of normal biological processes, pathogenic processes, and/or response to therapeutic or other intervention. Robust and reproducible accessible genomic biomarkers are of diagnostic value and may lead to the identification of causal factors. They could be used for the diagnosis of a disease, as well as to guide molecularly targeted therapy and to personalise regimens [37]. Therefore, a genomic biomarker should reflect the expression, the function, and the regulation of gene activity. With respect to DNA characteristics, the following should certainly be considered: single nucleotide polymorphisms (SNPs), variability of short sequence repeats, DNA modification, insertions/deletions, copy number variation and cytogenetic rearrangements. Nowadays, SNPs are largely the easiest to ascertain, and the most applied markers

in genetic studies because of their high prevalence in the genome and their amenability to automated analyses [38]. Particularly, SNPs are gene variations occurring approximately every 500–1000 base pairs. They can occur in noncoding regions of the genome, as well as in genes (both exons and introns), with a frequency of at least 1%. Depending on the potential application, SNP markers might be selected outside of exons or the direct cause of a genetic mutation [39].

Recently, it has been suggested that SNPs can be used as biological markers to identify and map common diseases such as high blood pressure, diabetes, and cardiovascular disease, which in turn are caused by the interplay of genetic and non-genetic factors [40]. Accordingly, genetic variants could determine a different answer to an environmental exposure, resulting in the development of several diseases. For this reason, new technologies and computational approaches are being studied to tease out the gene and environment interactions that underpin disease. Thus, studies on gene–environment interaction are required to improve accuracy and precision in the assessment of both genetic and environmental influences [41].

1.2.2 Epigenetic biomarkers

Epigenetics mechanisms - including DNA methylation, histone modifications, histone variants, chromatin remodelling and non-coding RNAs - can be defined as hereditary modifications that influence gene expression and the phenotype without changing the genotype. These molecular processes characterize the epigenome, which is dynamic in response to environmental signs, modifiable during cell differentiation and heritable in daughter cells [42].

Nowadays, an epigenetic biomarker could be redefined as "any epigenetic mark or altered epigenetic mechanism which is stable and reproducible during sample processing and can be measured in the body fluids or primary types of tissue preparations (i) that predicts risk of future disease development; (ii) defines a disease; (iii) reveals information about natural history of disease (iv) predicts the outcome of disease; (v) responds to therapy; and (vi) monitors responses to therapy or medication, and and/or (vii) allows simultaneously conduction of diagnosis and targeted therapy (theragnosis)"[29]. Moreover, compared to a genetic biomarker, an epigenetic biomarker can provide relevant information about the gene function in individual cell types, as well as on the effects of the environment and lifestyle [43].

DNA methylation- known as the covalent addition of a methyl group to the C-5 position of cytosine to form 5-methylcytosine (5mC) - is one of the most investigated epigenetic mechanisms. DNA methylation almost exclusively occurs within CpG islands – short sequences that typically contain about 5-10 CpG dinucleotides per 100 bp [44]. In humans, approximately 60% promoters include CpG islands and ~90% of them is unmethylated [45]. However, up to 80% CpG dinucleotides occur

in repetitive sequences scattered throughout the genome [46], such as Long Interspersed Nuclear Elements (LINEs), disrupting gene expression and eventually leading to genomic instability. In mammals, DNA methylation is regulated by the activity of three DNA methyltransferases (DNMTs): while DNMT1 has a maintenance role, DNMT3a and 3b are *de novo* methylases. To protect from this potential deleterious effect, DNMT1 works to maintain these sequences highly methylated [47, 48]. DNMTs functions are associated with several key physiological processes, including genomic imprinting, X-chromosome inactivation, regulation of gene expression, maintenance of chromosome integrity through chromatin modulation, DNA stabilization and DNA-protein interactions [49]. Dysregulation of DNA methylation is implicated in the pathogenesis of numerous diseases, such as cardiovascular diseases, obesity, type-2 diabetes, and cancer [30-32]. Nowadays, methylation levels can be assessed through global and gene-specific DNA methylation approaches. Gene-specific DNA methylation refers to methylation level of specific gene, whereas global DNA methylation refers to the average methylation status that occurs across the whole genome [50]. As Long Interspersed Nuclear Element-1 (LINE-1) constitutes approximately 17% of the human genome, LINE-1 methylation is considered as a surrogate marker of global DNA methylation [51]. Specifically, LINE-1 elements are retrotransposons capable of independent and autonomous retro-transposition via RNA intermediate. LINE-1 sequence consists of a 5' untranslated region (UTR), a 3'UTR containing a polyadenylation signal and 2 Open Reading Frames (ORF1 and ORF2) encoding an endonuclease and a protein machinery for retrotransposition [52]. Interestingly, LINE-1 is amplified to more than 500,000 copies and occurs in a truncated form on both homologous chromosomes in the human genome [46]. Even if only a restricted proportion of LINE-1 sequences exists in a potentially active form, accumulating evidence suggests their important role in various processes. In fact, LINE-1 can continuously rearrange the genome and influence gene expression, inducing genetic variations and polymorphisms through the recombination and rearrangement [53]. Although LINE-1 methylation is not universally accepted as a solid marker of global methylation, aberrant methylation of these sequences was associated with cancer, cardiovascular and neurodegenerative diseases [54-56].

1.2.3 Biomarkers of aging

The aging phenotype can be described as a complex mosaic resulting from the interplay of environmental, genetic, and epigenetic events during lifetime [57, 58]. Human aging is currently defined as a dynamic process involving the recurrent adaptation to internal and external stressors [59]. Despite its enormous complexity, a slight number of molecular mechanisms underpin the aging process, which represents an important risk factor for many non-communicable diseases [36].

Given its complex nature, biomarkers of aging are multifaceted. Following the guidelines of The American Federation for Aging Research (AFAR), a biomarker of aging should predict the rate of aging and monitor the process that underlies the aging progression, not the effects of disease [60, 61]. Moreover, a biomarker of aging should be minimally invasive and reproducible without harm to human subjects and testable in both laboratory animals and humans.

In general, measurable biomarkers could be classified into the following hallmarks of aging: genomic instability [62-65], telomere attrition [66, 67], epigenetic alterations [68-74], loss of proteostasis [75, 76], mitochondrial dysfunction [77, 78], cellular senescence [79-82], deregulated nutrient-sensing [83, 84], steam cell exhaustion [85] and altered intercellular communication [86, 87].

Telomeres of vertebrates are repetitive (TTAGGG)n sequences at the ends of chromosomes that progressively shorten with cell division [88]. Shorter telomere length in leukocytes has been associated with aging [89] and age-related diseases, such as cardiovascular diseases [35], cancer [34], and neurological disorders [90]. The advantages of using telomere length as a biomarker of aging include its correlation with chronological age throughout lifetime, the predictive power for disease condition and mortality, and the huge responsiveness to exposures [91]. However, its compliance with the first two criteria is rather questionable [92]. Nevertheless, despite this uncertainty, telomere length currently remains one of the most widely used biomarker of aging in epidemiological studies, and a potential biomarker in personalized medicine [93].

With respect to epigenetic alterations, several lines of evidence have demonstrated substantial DNA methylation changes that occur with aging [94-96]. In normal tissues, CpG islands methylation within gene promoters rises with aging, while global DNA methylation levels decrease [60, 96, 97]. Interestingly, some of these changes might be associated with higher risk for various diseases, including cancer [55, 98-103].

During the last decades, several biomarkers have been proposed to improve the measure of biological aging compared to conventional chronological age [104, 105]. Accordingly, the analysis of methylation profiles outlined a panel of DNA methylation-based biomarkers of aging that characterize the so-called "epigenetic age" of an individual. Epigenetic age is a strong biomarker for aging, even if it is unclear as to what age-related biological process it is measuring [106]. Various age-predictors used methylation measures from CpG sites across the genome to predict chronological age in humans [68, 69]. For instance, Horvath and colleagues developed an age predictor index - named "epigenetic clock" - based on the DNA methylation levels of 353 CpG sites, summarizing the most important DNA methylation-based age estimators [69]. Horvath and

colleagues presented the first multi-tissue epigenetic age estimator, trained on 8000 microarray profiles of different tissue and cell types from children and adults, to overcome the Hannum's algorithm based on whole blood from adults [68]. Next, Levine and colleagues developed the DNAm PhenoAge based on 513 CpG sites, with the aim to predict mortality, risk for cardiovascular disease, and numerous indexes of multimorbidity [71]. Finally, Weidner and colleagues presented an epigenetic age estimator, based only on three CpG sites, that might predict chronological age with an absolute deviation of less than 5 years [107]. Hence, a positive difference between epigenetic age and chronological age has been identified as a measure of accelerated "biological" aging that is associated with the risk of obesity, age-related diseases, several cancers and all- cause of mortality in later life[108].

1.3 The Predictive, Preventive, Personalized and Participatory Medicine

As stated by the physician Caleb Parry in the 18th century: "It is much more important to know what kind of patient has a disease than to know what kind of disease a patient has". The concept of Personalized Medicine, embracing the concept of "right medicine, right patient, right time," has been around at least since the late 1990s. Then, results reached by the Human Genome Project have changed and improved the biomedical research towards the development of treatments tailored to specific genomic profiles and the proposal of targeted therapies for specific individual genetic variations [109]. In recent years, huge advances have been made in developing a wide range of biomedical technologies, which have enhanced the description of several diseases. In this scenario, the idea of medicine as Predictive, Preventive, Personalized and Participatory ('P4') has long been encouraged by Leroy Hood and other pioneers of systems medicine, that is the application of systems biology to the challenge of human disease [110-112]. P4 medicine proposes the integration of numerous aspects of biological data, including molecular, cellular, and phenotypical measurements, as well as genome sequences of individuals [113, 114]. In fact, with the implementation of high-performance technology, data are gathered on multiple levels and regard genomic, transcriptomic, proteomic, metabonomic and interactomic information (−omics). An integrative analysis of these multiple omics data may lead to a systematic evaluation of the patient to find a personalized and an applicable health approach. In this way, from a clinical point of view, the implementation of the P4 medicine aims to create predictive and personalized models for better defining the wellbeing of every patient, predicting transitions to illness, and orienting medical interventions [115, 116].

Particularly, predictive diagnoses for Predictive Medicine are conventionally represented by tests based on the genome and on imaging markers. For Preventive Medicine, measures against complex

illnesses are to identify people at risk before the development of symptoms. Thus, preventive biomarkers should be able to stratify the individuals with higher risk by measuring the association between their molecular profile and disease phenotype [117]. Additionally, Personalized Medicine promotes the wellbeing, increasing the opportunity of a successful prevention, detection and treatment of diseases [118]. In line, personalized medicine is being developed taking into account patient-oriented research [119]. Thus, with Participatory Medicine approach patients seeking medical care take a more active role is encouraged to participate actively in every step of the medical process. By adding the 'participatory' component, P4 medicine exploits the effectiveness of medical systems by expanding its application out from hospital settings. With the addition of self-assessments in the participatory component, new data will be aggregated and used to develop health strategies for public health [120].

Thus, P4 medicine implies a deep understanding of inter-individual differences due to genetic and environmental factors. In this context, both biological and socio-cultural aspects can affect risk factors, prevalence, age of onset, clinical manifestation, prognosis, biomarkers and treatment effectiveness [121]. Evidence of sex and gender differences has been reported in chronic diseases, such as diabetes, cardiovascular disorders, neurological diseases[122], cancer [123], and aging [124]. Additionally, differences in lifestyle (i.e., diet, physical activity, tobacco and alcohol consumption) have been also associated with the epidemiology of diseases [125].

Therefore, the challenge is to find strategies for translating the potential research excellence into clinical reality, which should be tailored on the basis of individual genome profile, lifestyle and environment.

1.4 The determinants of health

The "health sciences" are grounded in the belief that the phenomena which we label "health" display more or less associations with other events. Those events bearing a relationship to health are labelled "determinants of health." The health sciences deal with the detection, characterization, and the effects of such determinants on health. The combination of many factors affects the health of individuals and populations. To a large extent, environmental and genetic factors, socio-economic characteristics and lifestyles have considerable impacts on health, whereas the more commonly considered factors (i.e., access and use of health care services) often have less of an impact [126]. In this scenario, the WHO Department of Equity, Poverty and Social Determinants of Health defines health equity as "the absence of unfair and avoidable or remediable differences in health among population groups defined socially, economically, demographically or geographically". Since 2015, the Agenda 2030 for Sustainable Development has tackle the emerging problem of health

inequalities, proposing the 17 Sustainable Development Goals (SDGs) as a global action plan which all states are committed to achieving by 2030. In this perspective, SDGs have been designed for achieving sustainable development in its three dimensions – economic, social and environmental [127] (**Figure 2**).

In general, health inequalities can arise already in the foetus, delaying the maturation of tissues and having unfavourable effects in adulthood [128]. In this scenario, Barker was the first who proposed the Developmental Origins of Health and Disease (DOHaD) hypothesis, which states that uterine environment programs the fetus for environmental challenges that is likely to experience after birth [129]. Additionally, during childhood, biological programming is associated with social and environmental factors that constitute the main etiopathogenetic mediators of health inequalities in adulthood [130]. In recent years, health inequalities have represented an important issue in both European and global public health, whose mission is to reduce human suffering and enhance quality of life. These efforts are guided by public health principles, which include a scientific basis for action, an orientation towards the prevention of diseases and promotion of wellness, a population-wide perspective, community-based participation and problem solving, and a respect for diversity. Thus, public health strategies should address all the determinants of health – social, economic, and environmental - to ensure health equity and to promote wellness for both individuals and communities.

1.4.1 The social determinants of health

The social determinants of health – the non-medical factors that influence health outcomes – are conditions in which people are born and coexist, and the wider set of forces and systems shaping the context of daily life. According to the definition of the Centers for Disease Control and Prevention, the social determinants of health are "conditions in which people are born, grow up, live, work and age, which affect the health of an individual, or more generally of a community or population" [131].

Specifically, the main social determinants of health are represented by:

- Social gradient - at each level of the social position, those in an advantaged social position have a better health profile than those in a less one. This disadvantage has been documented for a wide range of health indicators, in both sexes, for all age groups, and for different social indicators [132, 133];
- Early life social determinants – with significant association of low social position with adverse pregnancy outcomes (i.e., pre-term birth, low birth weight, stillbirth) [134, 135], as well as with childhood obesity [136];

- Social exclusion - which concerns, in addition to the homeless and immigrants, especially children and adolescents from poor families [131];

- Employment and working conditions [137];

- Unemployment – which affects health with poverty, stress and behavior modification [138]

- Lifestyles – including smoking status, alcohol consumption, diet, physical activity, and breastfeeding [139].

Therefore, the social determinants of health have an important effect on health inequities found within and between countries. This inequity is seen in the conditions of early childhood, employment status, lifestyles and, in general, quality of life. Social stratification, thus, determines differential access to health care, with consequences for well-being, disease prevention, and survival [140]. With respect to the social dimension of sustainable development, the following SDGs aim to combat social inequalities through critical global action:

- Goal 4 - Ensure inclusive and equitable quality education and promote lifelong learning opportunities for all;

- Goal 5 - Achieve gender equality and empower all women and girls;

- Goal 10 - Reduce inequality within and among countries;

- Goal 11 - Make cities and human settlements inclusive, safe, resilient and sustainable;

- Goal 16 - Promote peaceful and inclusive societies for sustainable development, provide access to justice for all and build effective, accountable and inclusive institutions at all levels;

- Goal 17 - Strengthen the means of implementation and revitalize the global partnership for sustainable development [127].

With respect to the economic dimension of sustainable development, the following SDGs aim to combat inequalities through critical global action:

- Goal 1 - End poverty in all its forms everywhere;

- Goal 2 - End hunger, achieve food security and improved nutrition and promote sustainable agriculture;

- Goal 3 - Ensure healthy lives and promote well-being for all at all ages;

- Goal 8 - Promote sustained, inclusive and sustainable economic growth, full and productive employment and decent work for all;

- Goal 9 - Build resilient infrastructure, promote inclusive and sustainable industrialization and foster innovation [127].

1.4.2 The environmental determinants of health

Human health is influenced by economic factors, employment, education status, access to green spaces, walkability, water and air quality and individual behaviors [141-145]. From a health perspective, therefore, the definition of environmental determinants of health may be wide. However, not considering this broad perspective of environment, environmental determinants are characterized by all the physical, chemical, and biological factors external to the individual that affect health. Nowadays, local health hazards (i.e., air and water pollution) have been added to by other global risk, such as stratospheric ozone depletion and climate change. In general, the Global Burden of Disease Study estimated that environmental factors are responsible for 13–20% of the burden of disease in Europe [146]. However, it is difficult to provide definitive estimates of the environmental burden of disease because of issues of definition, incomplete evidence about aetiology, and the complexities of assessing exposures and their longer-term effects [147].

With respect to the environmental dimension of sustainable development, the following SDGs aim to combat inequalities through critical global action:

- Goal 6 - Ensure availability and sustainable management of water and sanitation for all;
- Goal 7 - Ensure access to affordable, reliable, sustainable and modern energy for all;
- Goal 12 - Ensure sustainable consumption and production patterns
- Goal 13 - Take urgent action to combat climate change and its impacts;
- Goal 14 - Conserve and sustainably use the oceans, seas and marine resources for sustainable development;
- Goal 15 - Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and halt biodiversity loss [127].

1.4.3 The determinants of health for women during the reproductive age

As stated above, several factors are considered as determinants of health and their complex interactions may have a profound impact on human health [148]. In general, men and women – especially during the reproductive age - are exposed to determinants of health in different ways. In 2008, the Social Determinants of Health - promoted by WHO – have been developed to identify and determine health inequalities according to groups of people [149], also considering that women's health during the reproductive age affect long-term health of theirs and their family. Even though women account for more than half of the population and drive the majority of health care decisions, in 2018 the Centers for Disease Control and Prevention found that most avoidable deaths among women in reproductive age were attributable to "broader failures of social support". Thus, there are

some ways by which social determinants of health impact women in reproductive age, including economic security, caregiving, violence, health care access (especially for black women) and maternal health. During the reproductive age, women could have high and varying determinants of health-related needs, with disparities across ethnicities and other socioeconomic dimensions. These needs regard food, medical care, housing, and heating, as well as employment assistance and childcare support [150]. Reproductive outcomes confirm that social determinants of health play an important role also during pregnancy. Women with low socioeconomic status experience adverse pregnancy outcomes, such as spontaneous abortions, infant mortality, and low birth weight. This is a critical issue since infant health, particularly for metabolic and respiratory pathologies, affects health in adult life. Similarly, differences in lifestyles, such as smoking, use of alcohol, unhealthy diet, obesity, and physical inactivity, have a similar effect [151].

For all these reasons, prioritizing health of women helps the achievement of the 4th – "Reduce child mortality" - and 5th – "Improve maternal health" - goals of Millennium Development Program, later replaced by several SDGs. Particularly, ensure healthy lives and promote well-being for all at all ages (Goal 3) also implies to reduce the number of women deaths due to preventable causes related to pregnancy and childbirth, for aiming an average global ratio of less than 70 deaths per 100 000 births by 2030. In fact, according to the Barker's theory, any insult at a critical period of embryonic and foetal development can have effects both at birth and during adulthood, thereby predisposing an individual to several diseases [152]. Moreover, guarantee education equality (Goal 4), gender equality (Goal 5) and decent work (Goal 8) will help to reduce health inequalities (Goal 10) [127].

1.5 Pregnancy

Pregnancy is a physiological condition in women which leads to rapid and continuous changes in body composition and metabolism. Socio-demographic and lifestyle characteristics of pregnant women are changing over the years. Worldwide, about 140 million births take place every year. Deaths from complications during pregnancy, childbirth, and the postnatal period have declined by 38% in the last two decades, but at an average reduction of just 3% per year [153]. Particularly, every day in 2017, approximately 810 women died from preventable causes related to adverse pregnancy outcomes, with 94% of all maternal deaths occur in low and lower middle-income countries. For this reasons, SDGs offer an opportunity to accelerate progress for improving maternal health for all women, in all countries and circumstances [154].

In Italy, deliveries represent one of the most common interventions for healthcare. The most detailed information on assistance during pregnancy is those collected at the time of birth and taken from certificates of childbirth assistance, for which the most recent national survey has been

published in 2007. These data partly describe changes occurred in the profiles of pregnant women, showing, for instance, that age is increasing over the years. In fact, the mean age of Italian pregnant women was 32.3 years, while it was 28.8 years for foreigners that represented 15.9% of pregnancies [155].

In 2016, the WHO guidelines have provided a set of updated recommendations on *antenatal care* (ANC), which provides a platform for important health-care functions, consisting in health promotion, screening and diagnosis, and disease prevention. Particularly, these recommendations are about:

- Nutritional interventions, including calcium, zinc, iron and folic acid supplements, as well as Vitamin A, B, C, D and E supplements and restricted caffeine intake;
- Maternal and fetal assessment;
- Preventive measures;
- Interventions for common physiological symptoms, such as nausea and vomiting;
- Health system interventions to improve utilization and quality of ANC.

1.5.1 Pregnancy and adverse outcomes

During the 282 days from the first day of the last menstruation to delivery, which represent the average duration of a term pregnancy, the fetus goes through different stages of development [155]. Since growth is due to many factors, such as maternal nutrition and *in utero* environment, prenatal monitoring plays a key role for the prevention of adverse pregnancy outcomes [156]. Although some of these outcomes do not represent an issue for health-care interventions, others, including pre-term birth (PTB), low-birth weight (LBW), small for gestational age (SGA) and intrauterine growth restriction (IUGR), as well as gestational diabetes mellitus (GDM) and preeclampsia (PE), continue to be major Public Health problems [157]. For this reason, preventing deaths and complications start with a healthy pregnancy and the quality of care - before, between and during pregnancies - ensures all women have a positive pregnancy experience.

PTB, the major cause of death among newborns and the second leading cause of death under five years, is defined as the condition in which mother gives birth before 37 weeks of gestation [158]. Every year, approximately 15 million babies are born pre-term, representing more than one in ten babies. Moreover, nearly one million children die each year due to complications of preterm birth [159], while survivors face a lifetime of disability. Recent data show that PTB and its complications are responsible for the highest rates of mortality and morbidity. Indeed, 70% of neonatal deaths and 75% of cases of neurological, respiratory and gastrointestinal disorders could

be attributed to PTB [160]. Although PTB is common, its etiology is still unclear. In addition to well-established genetic susceptibility [161], more recently it has been also demonstrated the effect of novel fetal and maternal genomic variants which in turn can affect intrauterine environment and pregnancy duration [162]. It is worth mentioning that the increased risk of PTB is associated with maternal age (both in younger and older), short inter-pregnancy interval, multiple gestation, drug abuse, smoking, low maternal pre-pregnancy weight or inadequate gestational weight gain [163].

If fetal growth does not occur regularly during pregnancy, growth restriction can manifest as LBW, SGA and ,in general, as IUGR [156]. LBW refers to newborns weight under 2.5 Kg, which in turn can be classified as very-low birth weight (VLBW, birth weight less than 1.5 kg) and extremely low-birth weight (ELBW, birth weight less than 1.0 kg). As reported by the WHO, LBW contributes from 60% to 80% of all newborn's deaths. While LBW is independent of gestational age, SGA indicates a birth weight below the 10th percentile for gestational age, often caused by placental insufficiency and mal-perfusion [164-166]. IUGR is a prenatal outcome of growth restriction in which fetal growth velocity, defined as the change in fetal size between two time points during pregnancy, is reduced [167]. About one third of the risk of SGA is due to genetic susceptibility, while two thirds to maternal behaviors, such as diet, smoking and alcohol consumption [168-170]. The burden of SGA is also motivate by the fact that children born SGA are at higher risk of developing obesity and metabolic diseases as diabetes mellitus [171].

Inadequate gestational weight gain (GWG) affects a growing number of pregnancies. To face the increasing burden of several adverse pregnancy outcomes, the Institute of Medicine (IOM) published a set of guidelines on recommended GWG. In spite of these recommendations, weight gain outside of the suggested values is still widespread. As shown in **Figure 3**, the adequate GWG depends on pre-pregnancy nutritional status, with particular attention on women who start pregnancy with a higher body mass index (BMI).

GWG influences the intrauterine environment and, in general, the long-term health. With regard to mother health, excessive GWG is associated with an increased risk of high blood pressure [172], diabetes [173], cesarean section [174], postpartum weight retention [175] and obesity [176]. For newborns, an excessive GWG causes a lower chance of survival.

1.5.2 Lifestyles and pregnancy

Women's health throughout life represents an important target for Public Health strategies, due to several biological and socio-cultural reasons. During the reproductive period - especially in pregnancy - environmental factors and lifestyles can affect physiological and pathological conditions both in women and in future generations. For instance, several intrauterine exposures

and factors (i.e., famine, folate intake, pre-pregnancy BMI and hyperglycaemia) are associated with fetal growth and hence with birth weight [177-179].

With respect to women nutrition, maternal diet plays a key role in ensuring the correct development and growth of newborns, suggesting an interplay between newborn and mother metabolism, which in turn involves nutrient stores and intakes [180-182]. For this reason, maternal diet should be as varied as possible [155], including: proteins, fats, carbohydrates, mineral salts, vitamins and water, fruit and vegetables, flour products (i.e. bread, pasta, rice, potatoes), proteins derived from fish, meat, legumes, plenty of fiber derived from whole-meal bread, and dairy products like milk, cheese, yogurt [183]. Moreover, it is also recommended to take 4-5 meals a day and to drink at least 2 liters of mineral water a day.

Since micronutrients – vitamins and mineral supplied to organism in small amounts – support maternal health and fetal development, it is necessary to determine their intake by assessing the physiological requirements of nutrients for a healthy pregnancy [184]. Examples of micronutrient interventions include supplementation of (i) folic acid to prevent neural tube defects, that can cause lifelong problems affecting health, growth and learning [185]; (ii) iodine to prevent cretinism; (iii) zinc to reduce the risk of PTB and (iv) iron to reduce the risk of LBW. Moreover, during pregnancy, the fetus is entirely dependent on maternal sources of vitamin D, which regulates placental function [186] and it is associated with several adverse outcomes [187, 188] [189] [190, 191] [192]. For these reasons, maternal micronutrient deficiencies are a global public health concern, compromising length of gestation and fetal development, which can lead to pregnancy loss, preterm delivery, small birth size, birth defects and long-term metabolic disturbances. Thus, both preconception and periconception interventions are needed to further assess the full effect of micronutrient adequacy on pregnancy outcomes [184]. However, since diet is characterized by a mixture of foods and nutrients, the best approach to investigate the relationship between maternal nutrition and adverse pregnancy outcomes requests the study of dietary patterns. Thus, several lines of evidence suggest an association of Mediterranean diet (MD) with reduced risk of PTB [193] [194] and shorter gestational age [195]. By contrast, women who do not adhere to MD are at higher risk of having neonates born LBW or SGA [196]. By contrast, those who adhere to a traditional diet have a lower risk of SGA [197]. Beyond diet, physical activity during pregnancy may reduce the risk of adverse outcomes for both mothers and their children. Current recommendations suggest pregnant women to perform at least 30 minutes of physical activity on most days of the week [198]. However, at the dawn of the 21st century, the larger part of humanity faces two major epidemics: the sedentary lifestyle and the obesity epidemic [199]. Obese women – those who are less likely

physically active in pregnancy [200] – have an increased risk for adverse outcomes [201, 202]. Of note, the health risks dramatically increase as mothers gain excessive weight during pregnancy [203]. Similarly, women who smoke are at higher risk for LBW, placental abruption, and sudden infant death syndrome [204, 205].

In conclusion, healthy lifestyles during pregnancy play an important role for maternal and child health during lifetime [18].

1.5.3 The relationship between exposome, genome and epigenome during pregnancy

Individual exposome is dynamic, making its assessment more challenging. Several critical life stages – such as fetus development - have been identified as those in which some exposures may have a greater impact on future diseases [206]. Embryos rapidly mature and may not have all the protective mechanisms in place to repair damage experienced from an exposure. For example, in the 1950s and 1960s, offspring exposed *in utero* to diethylstilboestrol had at risk of reproductive tract cancers, decreased fertility, and difficult pregnancies [207, 208].

Beyond maternal diet – considered as major determinant of fetal growth – it has been well established that also genetic risk factors are associated with adverse neonatal outcomes. For instance, polymorphisms in genes involved in the one-carbon metabolism affect the normal metabolic functions and several SNPs can create metabolic deficiencies, influencing dietary requirements for pregnant women. Similarly, genetic variants in genes involved in fatty acids, proteins and micronutrients metabolism can affect the course of pregnancy. Therefore, deficiencies or imbalance of nutrients, as well as polymorphisms in both maternal and fetal genes, may influence fetal growth [209, 210].

However, predicting adverse outcomes or risk of diseases from genetic background is complicated by its interactions with environmental risk factors. The so-called gene-environment interactions are ubiquitous and may account for the greater part of disease risk seen across genotypes [211]. Despite the difficulties, with the recent revolutionary advances in high-throughput genotyping technology, a large body of GWAS have been conducted and located hundreds of genomic variations related to several diseases, such as risk of obesity, type 2 diabetes, and cardiovascular disease [212, 213].

Because the genetic background of a certain population has been relatively constant for many generations, modifications of lifestyles could represent a main-stream prevention approach in public health practice. Thus, genetic variation plays a key role both for determining individual susceptibility to diseases and for influencing the response to the diet modifications. The novel knowledge of gene-diet interaction will be useful for tailoring lifestyle modifications to a personalized manner [214].

In this scenario, the term "Nutrigenomics" - also defined as "nutritional genomics"- refers to the study of the relationship between human genome, nutrition, and health. While Epigenetics can be defined as hereditary changes that modify gene expression and influence the phenotype, without altering the genotype, Epigenomics is the study of genome-wide epigenetic modifications [215, 216]. In line with these broader definitions, Nutrigenomics investigates the influence of dietary components on the Genome, Transcriptome, Proteome and Metabolome [217-219]. Nowadays, evidence that nutrient-induced changes in gene expression could determine modifications in metabolism and disease susceptibility has become more than a hypothesis [220, 221]. Environmental and lifestyle factors can potentially influence epigenetic mechanism, which in turn are involved in adult obesity, diabetes mellitus, aging process and cancer development [222]. For instance, several classes of nutrients - folate, polyphenols, selenium, retinoids, fatty acids, isothiocyanates and allyl compounds – modulate DNA methylation process via different mechanisms [223], leading to genome reprogramming in exposed individuals and in future generations [224]. The strong relationship between the intrauterine environment and the long-term health of the growing fetus can explain fetal origin of adult diseases [225-227]. A well-balanced supply of maternal nutrients before conception, as well as during pregnancy and breastfeeding, promotes the optimal development of the fetus and the offspring [228]. The significant role of maternal diet in epigenetic mechanisms has been confirmed by several studies, showing how DNA methylation [229], microRNAs [230] and histone modifications [231] are involved in IUGR and other adverse outcomes.

1.6 The female cancers

Worldwide, cancer is a leading cause of death among women in both high and middle-income countries. In 2012, there were an estimated 6.7 million new cancer cases and 3.5 million deaths among females [232]. However, cancer burden is expected to increase by 2030, due to increasing prevalence of risk factors (i.e., smoking, excess body weight, physical inactivity, and changes in reproductive patterns), as well as to the increasing average life expectancy derived from the improvement of the control of infectious diseases and from the reduction in maternal and childhood mortality [233]. Addressing the cancer burden in women is important for its health impact and for tackling gender inequalities. In this context, breast cancer is the most diagnosed cancer among women in 140 of 184 countries, whereas cervical cancer is the most common in 39 countries.

With respect to breast cancer, it represents the most frequently diagnosed cancer and the leading cause of cancer-related death among women worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012 [234]. Established risk factors for breast cancer include both genetic – as

family history of the disease, BRCA1 or 2 mutations [235] – and non-genetic factors [236]. Among these, it has been established the role of reproductive factors [237], which influence endogenous estrogenic exposure, as well as lifestyles such as obesity [238], physical inactivity, smoking [239] and alcohol drinking [240]. On the other hand, breastfeeding has been reported to slightly reduce breast cancer risk [241]. Since about 20% of breast cancers are due to modifiable risk factors, a potential reduction in the disease burden could be possible by increasing healthy behaviors [242].

In 2012, an estimated 6.2 million women who had survived after 5 years from the diagnosis of breast cancer. Among these survivors, many experience lasting physical effects [243] and treatment-related side effects (i.e., impaired fertility or premature menopause) [244], as well as several long-term effects (i.e., increased risk of osteoporosis, cognitive impairment, chronic fatigue, and vaginal dryness) [245]. Thus, breast cancer survivors represent an important target population for promoting prevention strategies [246].

As regard cervical cancer, it is the fourth most frequently cancer and the fourth leading cause of cancer-related death. In 2012, there were an estimated 527,600 cases and 265,700 deaths worldwide.

Established risk factors for cervical cancer include the chronic infection with human papillomavirus, HPV. According to the IARC classification, among the 12 HPV types that are carcinogenic for humans, [247] HPV 16 and 18 are responsible for 70% of cervical cancers worldwide [248]. However, only women with persistent infections are at risk of cervical cancer, while 80-90% of these infections are cleared within a few years [249]. HPV infection interacts with multiple cofactors, which influence the risk of virus persistence and progression, such as having multiple sexual partners, higher parity, oral contraceptive use, HIV infection, smoking [250, 251] and diet [252]. Nowadays, cervical cancer is considered nearly completely preventable thorough screening and HPV vaccine. Prevention plays a key role in reducing the burden of cervical cancer, which is responsible for short and long-term effects experienced by cervical cancer survivors, such as treatment-related impaired sexual function and worse quality of life [253].

1.6.1 Breast cancer

In addition to age, a variety of non- modifiable risk factors for breast cancer (e.g., race, ethnicity, and genetics) and modifiable exposures (e.g., diet, physical inactivity, exogenous hormones and reproductive factor) have been identified [232].

To date, several GWAS studies have been conducted to discover the potential effects of genetic risk factors for breast cancer, accounting for more than 200 common susceptibility loci [254] .However, even if all the common genetic factors are taken together, they could explain only about 30% of the

familiar risk. For this reason, it is necessary to understand how genetic factors combine with environmental factors to influence the risk of breast cancer [255]. By accounting for the gene-environment interactions, better estimates of the population-attributable risk in specific subgroups can be obtained [256]. However, inconclusive results have been reported by several studies conducting in European populations [254]. Beyond genetic risk factors, epigenetic aberrations are known to play an important role in the development and progression of breast cancer. Hypermethylation of promoters for tumor suppressor genes and/or oncogenes hypomethylation can affect cancer development [257, 258], explaining how epigenetic can translate the same genotype into different phenotype. Recent developments in high-throughput technologies have led to the identification of distinctive genetic and epigenetic modifications in different breast cancer molecular subtype [259]. Nowadays, gene expression signatures in peripheral blood cells have shown a good performance to early detect breast cancer [260]. Similarly, DNA methylation changes might be useful markers for early detection of breast cancer since DNA can be easily obtained from different samples [261]. In this scenario, the identification and characterization of exposures, as well as their potential interactions with genetic and epigenetic factors, could be useful for the detection of individual breast cancer risk, which would enable personalized breast cancer prevention.

1.6.2 Cervical cancer

As already stated for breast cancer, numerous genetic and non-genetic risk factors are involved in the development and the progression of cervical cancer [262]. Among modifiable risk factors, several lines of evidence have suggested a relationship between healthy diet – rich in fruit and vegetables, antioxidants, folates, and minerals – and decreased risk of HPV infection, precursor lesions, and cervical cancer [263-268]. Additionally, inherited genetic predisposition may contribute to the risk of cervical cancer, suggesting how genetic polymorphisms in tumor suppressor genes might be related to HPV persistence and cancer progression [269]. A persistent high-risk HPV infection is not sufficient to immortalize and transform host epithelial cells, thus genetic and epigenetic alterations are needed for the development of carcinogenesis [270]. Since in cancer-free cells there is a well-controlled balance between DNA methylation and demethylation, aberrant DNA methylation might be an important event in cervical carcinogenesis [271, 272]. Specifically, DNA methylation is essential for the progression of cervical cancer, as reflected by its sensitivity for prognosis and therapeutic scopes in clinical practice. More than 100 human host genes have been reported as candidate methylation biomarkers of cervical cancer [273]. Furthermore, several studies have shown that methylation has a good performance to detect lesions

of cervical intraepithelial neoplasia (CIN 2) or more severe lesions (CIN2+ or high-grade intraepithelial lesions), and that can be used as a triage method in women with positive hrHPV status [274]. Since the occurrence of cancer is dependent on the interplay between the genome, the epigenome, and the exposome, the epigenotype could be useful also for assessing the effectiveness of dietary modifications to reduce such risk. For these reasons, studies that address the relationship between exposome, epigenome and genome are needed for developing appropriate public health strategies and interventions [275]. Nowadays, there is an increasing focus on epigenetics, because genotype alone does not account for all cancer risk, and that many of them could be avoided through changes in lifestyle, which in turn affects epigenetic mechanisms.

1.7 Rationale and specific aims

In line with the current state of the art, investigating the relationship between exposome, genome and epigenome is becoming an attractive field of research with perspectives for personalized medicine. Indeed, uncovering this relationship would be important to understand the molecular mechanisms underpinning the effect of lifestyles – and particularly of diet – on women health during lifetime. Thus, this thesis describes several studies applying an integrated approach of nutritional and molecular epidemiology to answer the following questions:

What are the main determinants of the adherence to the MD in healthy women?

Several studies have already demonstrated how the determinants of health might act on some behaviors such as the adherence to healthy dietary patterns. To confirm this evidence in Sicilian women, we conducted a cross-sectional analysis to assess the overall degree of adherence to MD and its main determinants, among healthy women from Catania, Italy.

How do lifestyles affect DNA methylation in healthy women?

Although lifestyles, such as dietary habits and weight status, affect the global methylation level, further studies are necessary to investigate the influence of obesity and complex dietary patterns among Sicilian women. To do that, we first derived different dietary patterns among healthy women from Catania, and then we assessed their relationship with global DNA methylation levels, using LINE-1 sequences as surrogate marker. Moreover, we evaluated the association of BMI and obesity with LINE-1 methylation levels.

Could differences in lifestyle explain the social effects on DNA methylation?

Previous studies suggested some social disparities in epigenetic markers, including DNA methylation levels. However, the social status alone cannot explain these inequalities that might be

mediated by behavioral factors. For this reason, we tested the mediating effect of behaviors (i.e., adherence to MD, smoking status, physical activity, and weight status) in the relationship between socioeconomic status and LINE-1 methylation level.

What are the main determinants of women choices before and during pregnancy?

The determinants of health often influence our choices about recommendations on healthy behaviors. Since pregnancy is a crucial stage of life for the health of women and their children, we investigated the main determinants of some women choices before and during pregnancy. In particular, we focused on the intake of specific nutrients and supplements (i.e., folate and folic acid supplements), on the adherence to dietary patterns of women, and on their vaccination status. To do that, we used data from the Mamma & Bambino cohort, a prospective study enrolling pregnant women from Catania, Italy.

What is the effect of dietary habits on pregnancy outcomes?

Maternal dietary habits before and during pregnancy could affect weight trajectories, which in turn are associated with pregnancy outcomes. However, studies on the association between maternal dietary patterns and GWG during pregnancy are still lacking. To fill this gap, we used data from the Mamma & Bambino cohort to derive maternal dietary patterns and examine their relationship with GWG.

What are the effects of genetic variants on pregnancy outcomes?

As discussed above, genetic variants might affect enzymatic activity and hence individual dietary requirements. However, the study on this field of research is complex because it is necessary to simultaneously assess the genetic background, the intake of specific nutrients, and how they interact on our health. In this scenario, as for an example, we investigated the effect of some polymorphisms in Vitamin D receptor (VDR) gene on pregnancy outcomes (i.e., PTB and neonatal anthropometric measures), taking into account the intake of vitamin D through the diet and supplements. To corroborate our findings obtained on the Mamma & Bambino cohort, we also carried out a systematic review with meta-analysis of previously published epidemiological studies.

How do nutrients and foods affect telomere length in women and their children?

Biological aging is a dynamic process that starts in the early phase of our life as a response to internal and external stressors. However, further studies are needed to understand what maternal factors affect the aging process of their children. Here, we first tested the relationship between

maternal nutrient intake and telomere length in blood and amniotic fluid samples obtained from the Mamma & Bambino cohort. In this context, we also carried out a systematic review of epidemiological studies investigating the effect of alcohol consumption on telomere length. The lack of evidence about this effect during pregnancy has pushed us to conduct a pilot study using data and sample from the Mamma & Bambino cohort.

Is there a relationship between telomere length and adverse outcomes of pregnancy?

Beyond maternal dietary habits, other factors might modulate the risk of adverse pregnancy outcomes, also affecting the biological aging process. However, studies on this field of research are scarce and further efforts are needed to understand the relationship between aging and risks for pregnant women. For these reasons, we also assessed if inadequate GWG – a risk factor of adverse pregnancy outcomes – might produce an early effect on telomere length of DNA extracted from amniotic fluid.

What factors influence breastfeeding practice and how the adherence to recommendations affect DNA methylation signatures?

As defined by the WHO, breastfeeding is an important practice to sustain healthy development and growth of newborns. For this reason, the understanding of factors that might be associated with the adherence to the WHO recommendations is crucial. Thus, we evaluated the main determinants of breastfeeding status among women from the Mamma & Bambino cohort. Moreover, since molecular mechanisms associated with breastfeeding are currently unclear, we investigated its effect on the epigenome-wide methylation status of DNA extracted from blood samples of children. To do that, we used data and samples from the HELIX project, which involves six existing prospective birth cohort studies in Europe.

How do antioxidant and inflammatory potential of diet affect the risk of HPV infection and cervical cancer?

Bioactive food components, as well as the adherence to specific dietary patterns, might be associated with the risk of female cancer. However, there is still the need for a full understanding of the effects of antioxidant and inflammatory properties of foods and nutrients against the risk of cervical cancer. Thus, we conducted a cross-section analysis to evaluate the antioxidant and inflammatory potential of diet among women at risk for cervical cancer from Catania, Italy.

Are dietary interventions effective for improving the quality of life of breast cancer survivors?

The promotion of healthy dietary habits might be crucial for sustaining the survival and the quality of life of women diagnosed with cancer. However, the evidence about the success of dietary interventions among breast cancer survivors is still inconclusive. For this reason, we carried out a systematic review of epidemiological studies evaluating the effects of dietary interventions on the quality of life of women, after a breast cancer diagnosis.

Figure 1. The exposome concept
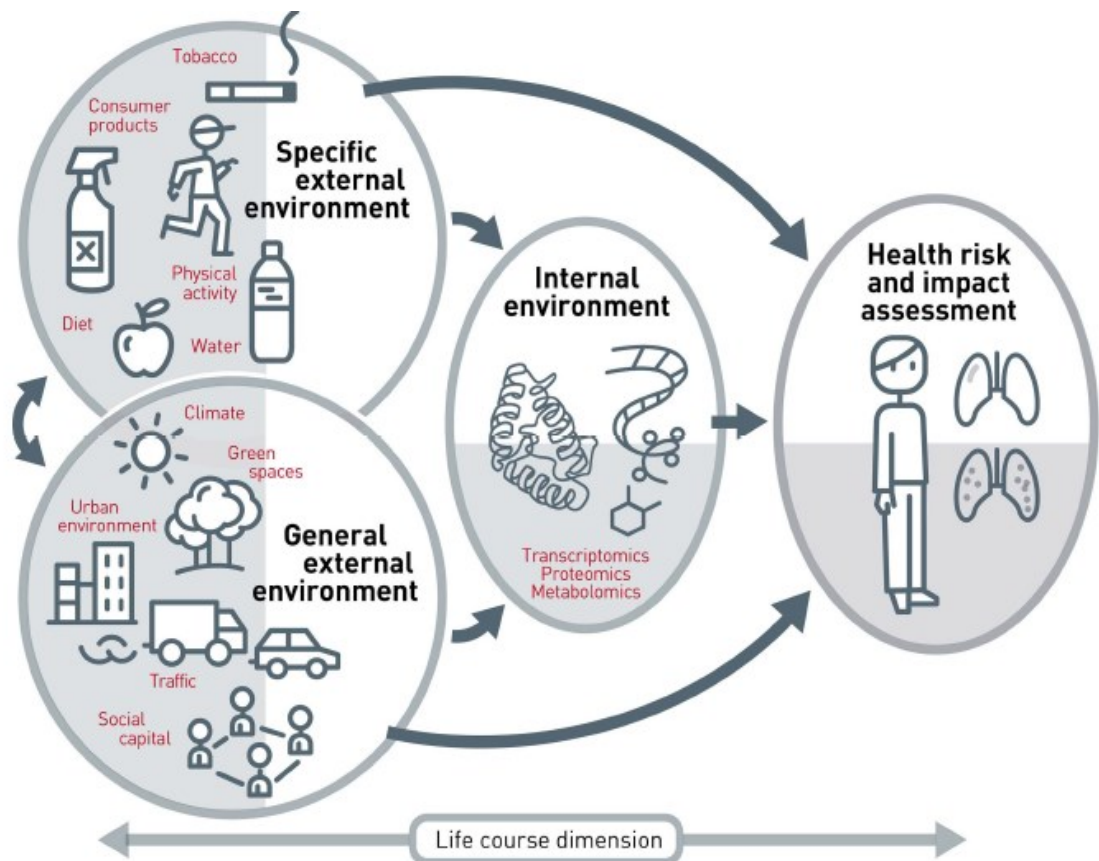
Three overlapping domains within the exposome have been described as follows: (1) a general external environment; (2) a specific external environment and (3) an internal environment.

Figure 2. The Sustainable Development Goals (SDGs)

The 17 SDGs arranged into three pillars: social, environmental, and economic.

Olivera Kostoska and Ljupco Kocarev. Sustainability 2019, 11(7), 1961;

https://doi.org/10.3390/su11071961.

Figure 3. Gestational weight gain according to pre-pregnancy BMI

Maternal Weight: Before Pregnancy	Maternal Body Mass Index (BMI)
Underweight	<18.5
Average/normal weight	18.5 - 24.9
Overweight	25 - 29.9
Obese	30+

Institute of Medicine (US). Weight gain during pregnancy: re-examining the guidelines.

Washington, DC. National Academies Press, 2009.

2. The main determinants of adherence to the Mediterranean diet: a cross-sectional study in healthy women

2.1 Background

In the last decades, several studies have demonstrated that the adherence to a "healthy" dietary pattern - rich in fruit, vegetables, whole grains, and fish - can reduce the risk of many non-communicable diseases [276-278]. However, since people consume a complex mixture of foods and nutrients that may interact synergistically or antagonistically [279], consider single nutrients or food group is not the best approach [280-283]. For this reason, nutritional research is shifting to the analysis of dietary pattern as a whole [284]. In this scenario, the MD is the common dietary pattern among the populations near to the Mediterranean Sea, and it has been associated to an encouraging health status and a better quality of life [276]. The comparison of disease rates between Mediterranean and other European countries demonstrated the beneficial effects of the MD [285]. Indeed, greater adherence to MD has been associated with a reduced risk of overall and cardiovascular mortality, cancer incidence and mortality, and incidence of Parkinson's and Alzheimer's diseases [286, 287]. Despite this evidence, a nutrition transition from the MD to a Western diet has emerged for Mediterranean populations [282]. Therefore, identifying social and behavioral factors associated with adherence to MD might help tailor nutritional strategies and interventions in a more efficient manner. In general, diet quality follows a socioeconomic gradient in terms of education, employment and income. People with higher socio-economic status eat more vegetables, fruit, whole grains, fish, and low-fat products, while those with lower socio-economic status consume more refined grains and added fats [288]. Moreover, people with unhealthy lifestyles, such as smoking and a sedentary life, tend to consume more fast-food products and fewer fruits and vegetables [289-292]. Hence, we performed a cross-sectional study on adult healthy women recruited in Catania, Southern Italy. We focused on women since their crucial role in food choice by providing meals for their families and making up most of the workforce in food-related jobs, health care and education. Our aim was to assess major social and behavioral determinants of adherence to MD in general, and the consumption of its specific food components.

2.2 Methods

2.2.1 Study Design

The current cross-sectional study was conducted on women who were referred for routine medical examination to three clinical laboratories in Catania (Italy) from 2010 to 2017. In particular, we included non-pregnant women, aged 25–64 years, with a complete assessment of social and behavioral characteristics and anthropometric measures, and no history of diseases (e.g., cancer, cardiovascular disease, diabetes, neurodegenerative and autoimmune diseases). The study protocol was approved by the Ethics Committees of the involved institutions, and the study was conducted in agreement with the Helsinki declaration. All women gave their signed informed consent to participate in the study.

2.2.2 Data Collection

Data were collected by trained interviewers using structured questionnaires. Age was considered according to tertile distribution as follows: 1st tertile (25–33 years; n = 276), 2nd tertile (34–46 years; n = 289) and 3rd tertile (47–65 years; n = 276). Educational level was categorized as low (i.e., primary education or apprenticeship), medium (i.e. secondary education), or high (i.e. tertiary education). Marital status was categorized into living alone (including single, divorced, or widowed) or living in couple (including married and other relationships). Employment status was categorized into employed (including full-time or part-time employment) or unemployed (including retired). Smoking status was categorized as current, former, or never smoking. Body weight and height were measured using standard procedures [18,19]. BMI, defined as weight in kilograms divided by height in meters squared, was classified as underweight (BMI < 18.5 kg/m2), normal weight (18.5 kg/m2 ≤ BMI < 25 kg/m2), overweight (25 ≤ BMI < 30 kg/m2) or obese (BMI ≥ 30 kg/m2), according to the WHO criteria [293]. Physical activity was assessed using the long form of the International Physical Activity Questionnaire (IPAQ-L) [294], and categorized as poor (no moderate or vigorous activity), intermediate (1–149 min/week moderate, 1–74 min/week vigorous or 1–149 min/week moderate + vigorous), or ideal (≥150 min/week moderate, ≥75 min/week vigorous or ≥150 min/week moderate + vigorous), according to the American Heart Association criteria [295].

2.2.3 Dietary Assessment

Dietary information was collected using a 95-item semi-quantitative Food Frequency Questionnaire (FFQ), referring to the previous month [97, 268, 296-300]. FFQ was adapted from a 46-item FFQ validated for the assessment of folate intake in Italian women of child-bearing age [296]. For each

food item, participants indicated: frequency of consumption - classified into specific categories, ranging from "almost never" to "six or more times a day" - and serving size (low, medium, or large). The medium serving size was defined by standard weight or volume measures commonly consumed in the Italian population, while small and large serving sizes were half a medium serving size or 1.5 times or larger than a medium serving size, respectively. A photograph atlas was used to calculate the amount of each food item and to minimize inaccuracies. Food intakes were obtained by multiplying the frequency of consumption by the daily portion size of each food group and adjusted for total energy intake using the residual method [301].

2.2.4 Mediterranean Diet Score

The adherence to MD was assessed by the Mediterranean Diet Score (MDS) [302, 303], based on the ideal/poor consumption of nine food categories: fruits and nuts, vegetables, legumes, cereals, lipids, fish, dairy products, meat products, alcohol and the ratio of unsaturated to saturated lipids. For vegetables, legumes, fruits and nuts, cereals, fish, and the ratio of unsaturated to saturated lipids, women whose consumption was below or equal to the median value of the population were assigned a value of 0, and a value of 1 was assigned otherwise. For dairy and meat products, women whose consumption was below the median were assigned a value of 1, and a value of 0 was assigned otherwise. With respect to alcohol, a value of 1 was given to women consuming 5 to 25 g per day. Thus, MDS ranged from 0 (non-adherence) to 9 (perfect adherence), and the adherence was categorized as follows: low adherence (MDS range: 0–3), medium adherence (MDS range: 4–6), or high adherence (MDS range: 7–9) [304].

2.2.5 Statistical Analysis

Statistical analyses were performed using the SPSS software (version 21.0, SPSS, Chicago, IL, USA). Continuous variables - expressed as median (interquartile range, IQR) - were tested for normality using the Kolmogorov–Smirnov test and compared using the Mann–Whitney U test or Kruskal–Wallis test. Categorical variables were expressed as frequency (percentage) and compared using the Chi-square test. Logistic regression models were applied to find independent determinants of the ideal consumption of each food category and of medium-to-high adherence to MD. The models included both social (i.e., age groups, educational level, employment status, and having children) and behavioral (i.e., smoking status, use of supplements, physical activity level, and BMI) characteristics. Results were expressed as odds ratios (ORs) and 95% confidence intervals (CIs). All statistical tests were two-sided, and p-values < 0.05 were considered statistically significant.

2.3 Results

2.3.1 Characteristics of Study Population

The present cross-sectional study included 841 women from 25 to 64 years, with a complete assessment of dietary data. In brief, 50.6% of women lived in couple and 69.0% had at least one child. Moreover, about a third (35.7%) reported a low educational level, while 55.3% were unemployed. With respect to lifestyles, 34.2% were current smokers and 17.3% were poorly physical active. According to BMI (mean = 23.87; SD = 4.69), nearly a third of women (32.5%) were overweight or obese. We also stated that 15.4% of women were in menopause, while 15.5% used folic acid supplements.

2.3.2 Determinants of Ideal Consumption of Cereals, Fruits and Vegetables, Legumes and Fish

We first evaluated women characteristics according to their consumption of food categories that positively characterized the MD – cereals, vegetables, fruits and nuts, legumes, fish, and the ratio of unsaturated to saturated lipids (**Table 1**). In particular, women with an ideal consumption of cereals were more likely to be unemployed (p = 0.030), to live in a couple (p = 0.002), to use supplements (p = 0.010), to have children (p = 0.010) and to have a reported higher BMI (p = 0.028) than those with poor consumption. Logistic regression analysis, which included all social and behavioral characteristics, showed that living in a couple was associated with an ideal intake of cereals (OR = 2.801 95%CI = 1.188–6.602; p = 0.018). Instead, women belonging to the category characterized by an ideal consumption of vegetables were older (p < 0.001), with more women between 34–46 and 47–65 years old (p < 0.001) in this category. Compared to women with poor consumption, they were also less likely to be unemployed (p = 0.029) and current smokers (p = 0.019) and more likely to live in a couple (p = 0.001). Interestingly, they also had a higher BMI if compared with their counterpart (p = 0.001). Logistic regression analysis suggested that more engagement in physical activity was the only determinant of an ideal consumption of vegetables (OR = 6.148; 95%CI = 1.506–25.104; p = 0.011). However, univariate analysis did not show statistical significance. With respect to women with an ideal consumption of fruits and nuts, they were older (p = 0.004) and most of them aged between 47–65 years old (p = 0.027). They were also more educated (p = 0.040), less likely to be unemployed (p = 0.039) and more likely to perform physical activity (p = 0.037) if compared with those with poor consumption. However, after applying logistic regression model, having children was the only positive determinant of the ideal consumption of fruits (OR = 3.149; 95%CI = 1.245–7.762; p = 0.015). Similarly, women with an ideal consumption of legumes were older (p < 0.001), with more women between 47–65 years old (p < 0.001). They were also more

likely to live in a couple (p = 0.001) and to have children (p = 0.011) and less likely to smoke (p = 0.038). They also had a higher BMI than those who consumed less legumes (p = 0.007). Logistic regression analysis revealed that more engagement in physical activity was the only determinant of an ideal consumption of legumes (OR = 5.832; 95%CI = 1.414–24.063; p = 0.015). However, univariate analysis did not show statistical significance. By contrast, univariate analysis also showed that women with an ideal consumption of fish were older (p < 0.001), with a higher proportion of women between 47–65 years old (p < 0.001). They were also more educated (p = 0.006), less likely to be unemployed (p = 0.006) and current smokers (p = 0.040) and to have children (p = 0.030) than those with poor consumption of fish. However, none of these characteristics was associated with the consumption of fish in multivariable analysis.

2.3.3 Determinants of Ideal Consumption of Meat, Dairy Products, Alcohol and Lipids

We next evaluated women characteristics according to their consumption of food categories that negatively characterized the MD (**Table 2**). Specifically, women belonging to the category of an ideal consumption of meat were older (p < 0.001) and less likely to smoke (p = 0.050) than those with poor consumption. Particularly, the highest proportion of those women were aged 47–65 years old (p < 0.001). By contrast, if compared to women with poor consumption of dairy products, women with an ideal consumption of dairy products were younger (p = 0.012), with a higher proportion of women between 25–33 years old (p = 0.029). Logistic regression analysis showed that smoking tobacco was negatively associated with an ideal consumption of meat (OR = 0.449; 95%CI = 0.0220–0.917; p = 0.028). Instead, none of the social or behavioral factors was associated with an ideal consumption of dairy products in multivariable analysis. We also found that women with an ideal consumption of alcohol were less likely to be obese (p = 0.006) and in menopause (p = 0.030) than those with poor consumption. However, as suggested by logistic regression analysis, being moderately or highly educated was a positive determinant of ideal consumption of alcohol (OR = 4.059; 95%CI = 1.311–12.570; p = 0.015; OR = 4.258; 95%CI = 1.068–16.976; p = 0.040; respectively). Finally, we observed that women with an ideal ratio of unsaturated to saturated fatty acids were less educated (p = 0.020), more likely to have children (p = 0.013) and with higher BMI (p = 0.037) than those with a poor ratio. However, none of these characteristics was significantly associated with the ratio of unsaturated to saturated fatty acids in multivariable analysis.

2.3.4 Determinants of Adherence to Mediterranean Diet

We next evaluated the adherence to MD using the MDS (mean = 4.2; range = 0–8), which allowed us to identify women with low adherence to MD (33.8%; MDS ≤ 3), as well as with medium (56.8%; 3 < MDS < 7), and high adherence to MD (9.4%; MDS ≥ 7). **Table 3** displays the

characteristics of the recruited women according to the adherence to MD. Particularly, we observed that adherence to MD increased with increasing age, and hence women with high adherence to MD were older (p < 0.001). In particular, women who highly adhered to MD were more likely to be 47–65 years old than those with medium or low adherence (p < 0.001). Moreover, women belonging to the category of high adherence to MD were more educated (p < 0.001), less likely to be unemployed (p = 0.017) and more likely to live in a couple (p = 0.017). With respect to lifestyles, people with high adherence to MD were less likely to smoke (p < 0.001). Moreover, they showed a lower BMI than those who adhered less to MD (p < 0.001), which resulted in a lower prevalence of overweight and obesity (p < 0.001). However, multivariable logistic regression analysis showed that only the engagement in physical activity was associated with moderate to high adherence to MD (OR = 5.500; 95%CI = 1.293–18.575; p = 0.031).

2.4 Discussion

Our analysis pointed out several social factors and behaviors associated with the ideal consumption of specific foods and the adherence to MD. However, in multivariable models, more engagement in physical activity was the only positive determinant of the ideal consumption of vegetables and legumes, as well as of high adherence to MD in general. People who perform more physical activity are more likely to be healthier than their sedentary counterparts [305], probably due to the mixture of foods consumed by physically and socially active people [306]. By contrast, sedentary individuals are more likely to eat fast food products and less likely to consume fruits and vegetables [289, 290]. Although a direct association between physical activity and food choices has not been yet elucidated, it has been well-known that a lack of exercise and an unhealthy diet tend to coexist among individuals [307].

Similarly, our analysis showed that current smoking was a negative determinant of the ideal consumption of meat products. This is in line with previous evidence that smokers had an unhealthier diet if compared with non-smokers [291, 292, 308]. As stated by a meta-analysis of 51 studies, people who smoked had a higher intake of energy, total and saturated fat and cholesterol, while a lower intake of vitamins and fibers than non-smokers [309]. Notably, the coexistence of these unhealthy habits may exacerbate their adverse effects on health, increasing the risk of non-communicable diseases. Thus, changes in one health behavior might promote changes in overall lifestyle. For instance, a study of 500 smokers demonstrated that reducing and quitting smoking were associated with an increased intake of fruits and vegetables and more engagement in physical activity [310].

Among social determinants, educational level is a widely used indicator of socio-economic status, due to its availability in a variety of national and international studies. In our study, having a medium-high educational level was the only positive determinant of an ideal consumption of alcohol. This partially was in line with the current concept that healthy dietary behaviors are more common among high-educated individuals, since they have more knowledge about the benefits and risks of their food choices [311]. Indeed, education might affect several health outcomes through its influence on lifestyles – such as physical activity, smoking habits, and diet – and on problem-solving capacity and values (e.g., awareness of preventive behaviors) [312, 313]. Other social characteristics, such as household size and composition, are rarely investigated in this field of research. In our study, living in couple was associated with an ideal consumption of cereals, while having children was the only positive determinant of an ideal consumption of fruits. Although these findings suggested that a healthier diet was more recurrent among those who were members of large families, an increasing household size also implies more mouths to feed and increased costs on food, which in turn might reduce diet quantity and variety [314]. However, our research on women and mothers confirmed their awareness about healthy food choices for themselves and their families. Thus, further research should be encouraged to better understand whether the relationships between household size, composition, and diet quality might be affected by the role of each member in their family.

To the best of our knowledge, it is the first study exploring the effect of social and behavioral determinants of adherence to MD among women from Southern Italy. Moreover, data were collected using standard and validated tools, and the socioeconomic information covers different aspects of the social status contributing individually to the relationship with MD. Finally, most of our results are robust, as they have been obtained after adjusting for total energy intake and by using logistic regression models.

However, our study also has some limitations. Its cross-sectional design does not allow us to assess the causality of observed relationships. With respect to dietary assessment, data were collected using FFQs, which did not preclude inaccuracies. However, other widely used tools for assessing dietary data (e.g., weighted records and 24-hour recalls) are prone to a degree of misreporting. Thus, the use of FFQs still remains a widely tool for dietary assessment in epidemiological studies [315]. Finally, we cannot completely exclude the effect of unmeasured residual factors, such as household income, food security and food access.

In conclusions, in our study we reported a low adherence to MD in Southern Italy, confirming that nutrition transition is also developing for Mediterranean populations. This reflects the need to

improve public health strategies, which should consider determinants of diet quality. Our study shows that more engagement in physical activity is a major positive determinant of the adherence to MD. By contrast, the coexistence of sedentary habits with unhealthy food choices exacerbates their adverse effects on health. However, the promotion of changes in one health behavior could lead to an overall improvement of lifestyle.

Table 1. Characteristics of women according to consumption of cereals, fruits, vegetables, legumes, and fish

Characteristics	Cereals			Vegetables			Fruits			Legumes			Fish		
	Poor	Ideal	*p*-Value	Poor	Ideal	*p*-Value	Poor	Ideal	*p*-Value	Poor	Ideal	*p*-Value	Poor	Ideal	*p*-Value
Age, years	43(14)	44(13)	0.876	43(14)	44(13)	**<0.001**	43(13)	44(14)	**0.004**	43(14)	44(14)	**<0.001**	43(14)	44(12)	**<0.001**
1st tertile (25–33 years)	35.1%	30.6%		39.8%	25.9%		35.4%	30.3%		38.6%	27.3%		37.8%	27.9%	
2nd tertile (34–46 years)	31.3%	37.4%	0.148	35.5%	33.3%	**<0.001**	36.1%	32.6%	**0.027**	36.7%	32.2%	**<0.001**	37.1%	31.7%	**<0.001**
3rd tertile (47–65 years)	33.7%	32.0%		24.8%	40.9%		28.5%	37.1%		24.8%	40.6%		25.1%	40.4%	
Educational level															
Low	32.9	38.4		38.6	32.8		39	32.4		37.6	33.8		40.9	30.5	
Medium	45.3	44.8	0.110	42.9	47.3	0.210	44.7	45.4	**0.040**	43.4	46.6	0.518	40.9	49.2	**0.006**
High	21.7	16.8		18.6	20		16.3	22.2		18.9	19.6		18.2	20.3	
Employment status (% unemployed)	51.6	59	**0.030**	59	51.5	**0.029**	58.9	51.8	**0.039**	53.6	56.9	0.350	60	50.6	**0.006**
Marital status (% living in couple)	42.8	56.3	**0.002**	44.6	58.9	**0.001**	49.3	51.9	0.540	44.3	59.4	**0.001**	50.3	50.9	0.359
Smoking status															
Never smokers	53.2	58.2		54.3	57.1		52.9	58.5		52.7	58.6		55.6	55.8	
Former smokers	11.2	9	0.300	7.9	12.4	**0.019**	9.3	10.9	0.085	9	11.2	**0.038**	7.7	12.5	**0.040**
Current smokers	35.6	32.8		37.9	30.5		37.8	30.6		38.3	30.1		36.7	21.7	
Use of supplements (% users)	10.9	18.8	**0.010**	15	16.1	0.730	16.5	14.2	0.460	15.6	15.2	0.890	15	16.1	0.737
Having children (% yes)	65.9	71.2	0.190	66.9	71.9	0.220	66.9	71.2	0.290	64.6	75	**0.011**	72.6	63.8	**0.030**
Body Mass Index. kg/m^2	23.4(4.5)	23.1(5.5)	**0.028**	22.9(4.5)	23.7(5.1)	**0.001**	23.4(5.6)	23.05(4.6)	0.820	23.1(4.7)	23.5(5.7)	**0.007**	23.2(4.9)	23.3(5.2)	0.973
Underweight	6.3	7.4		7.9	5.7		8.4	5.2		6.9	6.8		7.7	5.9	
Normal weight	58.7	62.7	0.280	63.5	58	0.062	57.3	64	0.130	64	57.6	0.235	58.2	63.2	0.425
Overweight	22.5	21.1		18.3	25.3		23.4	20.2		19.1	24.4		23.4	20.2	
Obese	12.6	8.8		10.3	11		10.8	10.5		10	11.2		10.6	10.7	
Physical activity															
Poor	17.6	17		19.6	15.8		15.7	18.6		16.3	17.9		13.3	19.8	
Intermediate	74.7	75	0.980	75	74.8	0.270	80.1	70.6	**0.037**	77.8	73.2	0.570	78.3	72.7	0.276
Ideal	7.7	8		5.4	9.5		4.2	10.8		5.9	8.9		8.4	7.5	

This table is adapted from Maugeri et al., Int. J. Environ. Res. Public Health 2019

Table 2. Characteristics of women according to consumption of meat, dairy products, alcohol, and lipids

Characteristics	Meat			Dairy Products			Alcohol			Unsaturated/Saturated Ratio		
	Poor	Ideal	*p*-Value	Poor	Ideal	*p*-Value	Poor	Ideal	*p*-Value	Poor	Ideal	*p*-Value
Age. Years	43(13)	44(14)	**<0.001**	45(12)	42(15)	**0.012**	43(13)	45(15)	0.481	44(14)	44(13)	0.056.
1st tertile (25–33 years)	39.0%	26.7%		29.5%	36.2%		31.9%	36.5%		34.3%	31.4%	
2nd tertile (34–46 years)	34.4%	34.3%	**<0.001**	33.7%	35.0%	**0.029**	34.9%	32.4%	0.524	35.2%	33.5%	0.343
3rd tertile (47–65 years)	26.6%	39.0%		36.8%	28.8%		33.2%	31.2%		30.5%	35.2%	
Educational level												
Low	38.2	33.1		34.2	37.1		37.1	30		31.7	39.7	
Medium	45.6	44.5	0.054	46.8	43.3	0.582	44.6	47.1	0.174	46.2	43.9	**0.021**
High	16.2	22.4		19	19.5		18.3	22.9		22.1	16.4	
Marital status (% living in couple)	49.5	51.8	0.621	52.4	49	0.426	51.8	45.2	0.223	48.5	52.5	0.352
Smoking status												
Never smokers	53.6	57.9		61	50.5		55.1	30.2		59.2	52.3	
Former smokers	8.6	11.7	0.050	9.8	10.5	**0.006**	9.7	58	0.407	8.8	11.4	0.113
Current smokers	37.9	30.5		29.3	39		35.2	11.8		32	36.3	
Having children (% yes)	66.2	72	0.155	67.3	70.3	0.453	69.6	66.3	0.523	63.8	73.7	**0.013**
Body Mass Index. kg/m^2	23.4(5.7)	22.9(4.6)	0.774	23.3(4.9)	23.2(5.1)	0.331	23.14(4.7)	23.8(6)	0.211	23.05(5.1)	23.43(5)	**0.037**
Underweight	7.7	6		5.5	8.1		5.4	12.4		8.6	5	
Normal weight	58.9	62.6	0.612	60.7	60.8	0.465	62.3	54.4	**0.006**	59.7	61.7	0.147
Overweight	22	21.6		22.5	21.1		21.2	24.3		22.3	21.3	
Obese	11.5	9.8		11.3	10		11.1	8.9		9.4	12	
Physical activity												
Poor	19.8	15.2		16.9	17.8		17.5	16.7		16.1	18.3	
Intermediate	72.7	76.8	0.501	75.8	73.6	0.866	74.7	75.6	0.986	76.8	73.3	0.741
Ideal	7.6	8.1		7.2	8.6		7.9	7.7		7.1	8.4	
Menopause (% yes)	13	18	0.143	16.1	14.8	0.715	17.3	7.9	**0.030**	12.6	18	0.112

This table is adapted from Maugeri et al., Int. J. Environ. Res. Public Health 2019

Table 3. Characteristics of women according to adherence to Mediterranean diet

Characteristics	Mediterranean Diet			
	Low	**Medium**	**High**	*p*-Value [b]
Age, years	36.0 (17.0)	41.0 (20.0)	50.0 (25.0)	**<0.001**
1st tertile (25–33 years)	41.2%	29.5%	22.8%	**<0.001**
2nd tertile (34–46 years)	37.3%	34.5%	22.8%	
3rd tertile (47–65 years)	21.5%	36.0%	54.4%	
Educational level				
Low	44.6%	37.0%	25.4%	**<0.001**
Medium	40.7%	41.6%	52.9%	
High	14.6%	21.4%	21.8%	
Employment status (% unemployed)	57.9%	54.8%	53.2%	**0.017**
Marital status (% living in couple)	44.5%	56.6%	58.1%	**0.017**
Smoking status				
Never smokers	46.4%	59.4%	61.3%	**<0.001**
Former smokers	7.5%	7.1%	15.8%	
Current smokers	46.1%	33.5%	22.9%	
Use of supplements (% users)	14.7%	14.5%	29.0%	0.097
Having children (% yes)	65.1%	72.8%	74.2%	0.142
Number of children	2.0 (1.0)	2.0 (1.0)	2.0 (1.0)	0.275
Body Mass Index, kg/m^2	26.30 (9.10)	23.34 (4.30)	23.05 (5.10)	**<0.001**
Underweight	2.5%	8.2%	9.7%	**<0.001**
Normal weight	54.3%	66.3%	61.5%	
Overweight	28.1%	18.3%	19.1%	
Obese	15.1%	7.2%	9.7%	
Physical activity				
Poor	13.1%	17.0%	19.2%	0.344
Intermediate	82.1%	71.6%	73.2%	
Ideal	4.8%	11.4%	7.6%	
Menopause (% yes)	11.6%	19.3%	20.8%	0.072

This table is adapted from Maugeri et al., Int. J. Environ. Res. Public Health 2019

3. The effect of lifestyles on LINE-1 methylation levels: a cross-sectional study in healthy women

3.1 Background

Progresses in the field of genetics, especially after the conclusion of the Human Genome Project, raised questions about the effects of gene-environment interaction on human health. For these reasons, several studies have been developed to investigate molecular mechanisms underpinning the effects of lifestyles – and in particular dietary interventions - against aging and age-related diseases [221]. In the last years, it has been shown that environmental factors can potentially modify DNA methylation process, leading to altered gene expression and genome instability both in exposed individuals and in future generations [224]. This process almost exclusively occurs within CpG islands [44], of which nearly 80% happen in repetitive sequences scattered throughout the human genome, such as LINEs and Short Interspersed Nuclear Elements (SINEs) [46]. In general, CpG islands located within LINE-1 sequences and their methylation levels correlate with the global genomic DNA methylation level [316]. For these reasons, LINE-1 methylation has been widely used as a surrogate marker of global DNA methylation [51] in the research on cancer, cardiovascular and neurodegenerative diseases [54-56, 317]. Recent studies have proposed a remarkable link between epigenetic and environmental exposure, suggesting how nutrients, pollutants and other environmental factors can influence the turnover of epigenetic marks [318].

With respect to diet, several nutrients - folate, polyphenols, selenium, retinoids, fatty acids, isothiocyanates and allyl compounds - and bioactive compounds of fruit, vegetables and spices can influence epigenetic mechanisms by inhibiting enzymes and substrates involved [223, 297, 319-321]. To date, there is a growing interest in investigating overall diet or dietary patterns, rather than focusing on single foods or nutrients. To the best of our knowledge, Zhang and colleagues were the first to report a positive association between *a posteriori* prudent dietary pattern – rich of vegetables and fruits - and LINE-1 methylation in a cancer-free population [322]. In line, we demonstrated the positive association of the adherence to the MD with the increased LINE-1 methylation level, counteracting the harmful effect of particulate matter exposure [97].

With respect to other habits, it would be also interesting to uncover epigenetic mechanisms associated with raised BMI and obesity, due to their potential role in development of obesity from the early stages of life [323]. It has been well established the involvement of DNA methylation, aberrant miRNA expression, histone modification and nucleosome release in obesity and associated comorbidities [324-326]. Overweight and obesity are delineated by an excessive accumulation of

body fat, which results in BMI greater than or equal to 25 kg/m^2 and 30 kg/m^2, respectively [327]. According to WHO, nearly 2 billion adults were overweight in 2016, out of which approximately 650 million were obese [328]. In line, more than one adult in ten were obese in 2016, with a prevalence that tripled in the last four decades [328], probably due to the increased intake of energy-dense foods and sedentary nature of human life [328]. Overweight and obesity also account for an important burden for public health [329], because raised BMI is often associated with an increased risk of cardiovascular and musculoskeletal diseases, diabetes, and some cancers [330]. Moreover, raised BMI could have adverse consequences on women of childbearing age, with higher risk of adverse pregnancy outcomes for women with excessive weight gain prior and during pregnancy [176, 331-336]. Additionally, children born from overweight or obese women were not only at higher risk of being born SGA [173, 333, 335, 337] and PTB [338], but also to develop metabolic disorders later in life [339-341].

To meet the need of determining the impact of lifestyles on DNA methylation levels, we performed an observational cross-sectional study in women from Catania, Southern Italy. Firstly, we evaluated correlations between food intakes and LINE-1 methylation. Next, we defined posteriori dietary patterns that characterized dietary habits of women and investigated their association with leukocyte LINE-1 methylation level. Finally, we assessed the association of BMI and obesity with LINE-1 methylation level.

3.2. Methods

3.2.1 Study design

Women with no history of severe diseases (i.e., cancer, diabetes, cardiovascular, neurodegenerative, and autoimmune diseases) were selected from those who referred for routine medical examination to three clinical laboratories in Catania (Italy) from 2010 to 2017. The study protocol was in accordance with the Declaration of Helsinki and approved by the ethics committees "Catania" and "Catania 2" with the following protocol numbers: 52/2010/VE, 16/2015/CECT2, and 227/2011/BE. All women who met inclusion criteria were invited to participate, after being informed of all aspects of the research protocol. Those who agreed to participate in the study had to sign a written informed consent. Sociodemographic and lifestyle information were collected by trained epidemiologists using a structured questionnaire. We included non-pregnant women, aged 12-87 years (median= 36 years), with complete assessment of social and behavioral characteristics and anthropometric measures. Education was categorized as low (i.e., primary school diploma or none), medium (i.e., bachelor's degree or higher). Women were also classified, according to their employment status, as employed (part-time and full-time employment) or unemployed (housewives and retired). Smoking

status was categorized as current, former, or never. BMI was calculated as the ratio between weight (kg) and squared height (m^2), and participants were categorized into underweight, normal weight, overweight and obesity according to the WHO criteria [293].

At the same time, women provided a blood sample for DNA extraction and LINE-1 methylation assessment. Women with incomplete information on anthropometric measures and those who did not provide a blood sample were excluded from the current analysis.

3.2.2 Dietary assessment

Dietary data were collected by a validated 95-item semi-quantitative FFQ, with the previous month as reference period [97, 268, 297, 298]. For each food item, women were asked to report frequency of consumption (twelve categories from "almost never" to "two or more times a day") and portion size (small, medium, or large), using a photograph atlas to minimize inaccuracies. Food intakes were calculated by multiplying frequency of consumption by daily portion size of each food group. Moreover, food intakes were adjusted for total energy intake using the residual method [301] .

Prior to further analyses, food items were classified into 39 predefined food groups based on the similarity of nutrient profiles or culinary usage, while individual food items that constituted a distinct item on their own (e.g., pasta, pizza, or eggs) or that represent a particular dietary pattern (e.g., alcoholic drinks and fries) were preserved [268, 278]. *A posteriori* dietary patterns were derived by Principal Component Analysis (PCA) followed by varimax rotation on energy-adjusted food group intakes. The number of dietary patterns was defined based on eigenvalues >2.0, Scree plot examination, and interpretability of components. To characterize each dietary pattern, we considered factor loadings with absolute value ≥0.3. For each dietary pattern, factor scores were calculated by summing the products between observed energy-adjusted food group intakes and their factor loadings and categorized according to tertile distribution (1st tertile = low adherence, 2nd tertile = medium adherence, or 3rd tertile = high adherence).

3.2.3 LINE-1 methylation analysis

In the last decades, several methods have been proposed to determine levels of global genomic DNA methylation. These methods are based on high performance liquid chromatography-ultraviolet, liquid chromatography coupled with tandem mass spectrometry, enzyme-linked immunosorbent assay, or pyrosequencing of bisulfite converted DNA [342]. Alternatively, determining the methylation level of CpG sites located within LINE-1 sequences can be used as a surrogate marker of global DNA methylation level [316]. To allow comparison with our previous studies in this field of research, we evaluated the methylation level of three CpG sites within the LINE-1 sequence (GenBank Accession No. X58075) [97, 297]. Thus, DNA extraction and the

assessment of LINE-1 methylation were performed using standardized protocols [343]. In brief, DNA was extracted from leukocytes using the QIAamp DNA Mini Kit (Qiagen, Milan, Italy). Next, bisulphite conversion of 40 ng of the extracted DNA was performed using the EpiTect Bisulfite Kit (Qiagen, Milan, Italy). Specifically, the assessment of methylation levels was performed on three CpG sites within the LINE-1 sequence (GenBank Accession No. X58075). To do that, the LINE-1 sequence was amplified by Hot Start Polymerase chain reaction (PCR) on the Eppendorf Mastercycler (Eppendorf, Milan, Italy). The PCR reaction was conducted in a final volume of 25 μl, containing 1.5 μl of bisulfite-converted DNA, 12.5 μl of PyroMark PCR Master Mix 2×, 2.5 μl of Coral Load Concentrate 10×, and 2 μl of primers (0.2 μM for each). The sequences of forward and reverse-biotinylated primer were 5′-TTTTGAGTTAGGTGTGGGATATA-3′) and 5′-biotin AAAATCAAAAAATTCC CTTTC-3′, respectively [97]. The PCR conditions were the following: 1 cycle at 95°C for 15 min, 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. Finally, the PCR products were sequenced by pyrosequencing on the PyroMark Q24 instrument (Qiagen, Milan, Italy), using 0.3 mM of the sequencing primer 5′-AGTTAGGTGTGGGATATAGT-3′. For each CpG site, methylation level was calculated as the percentage of methylated cytosines over all cytosines. All the protocols were performed according to the manufacturers' instructions, and each sample was analysed in triplicate. All the assays included a positive (100% methylated DNA) and a negative (0% methylated DNA) control, while failed assays were repeated. Intra-observer coefficient of variability between replicates was 2.2% (SD=1.0%), as previously reported [103]. For each sample, LINE-1 methylation level was calculated as the mean of methylation level of the three CpG sites.

3.2.4 Statistical analysis

Prior to analysis, quantitative variables were tested for normality using the Kolmogorov-Smirnov test. To study the effect of dietary patterns on LINE-1 methylation levels, descriptive statistics were used to summarize categorical variables (using frequency and percentage) and quantitative variables (using median and IQR). Categorical variables were expressed frequency (percentage) and compared using the Chi-square test. In particular, we first conducted correlation analysis between food intakes and LINE-1 methylation, using Spearman test followed by Bonferroni correction for 39 tests ($p < 0.001$ after Bonferroni correction). Next, we investigated the associations between dietary patterns and LINE-1 methylation levels using linear regression models on log-transformed data. In particular, we applied an unadjusted model (Model 1), followed by an adjusted model that included all the variables collected in our study (Model 2).

To study the effect of obesity on LINE-1 methylation, all variables were compared across BMI categories using the Chi-squared test for categorical variables and the Kruskal–Wallis test for quantitative variables. After that, the association of BMI with LINE-1 methylation was examined by simple linear regression, and further adjusting for age, educational level, employment status, marital status, parity, menopause, and smoking status. Similarly, the association of BMI categories with LINE-1 methylation was examined using normal weight as the reference group in unadjusted and adjusted linear regression models. Results were reported as β coefficients and their standard error (SE). All the above-mentioned analyses were two-sided and p-values < 0.05 were considered statistically significant, except of those reported after Bonferroni correction.

3.3 Results

3.3.1 Study population

The study population consisted of 488 women, aged 15-85 years, with a complete assessment of anthropometric measures and leukocyte LINE-1 methylation. With respect to educational level, 35.2% had a primary school diploma, while 47.1% a secondary school diploma, and 17.6% a degree. Overall, 44.1% of women were part-time or full-time employed, while 50.4% lived in couple. Approximately 70% had at least a child, while less than 10% were menopausal. With respect to behaviours, 57.3% of women did not smoke, while 11.7% of them were former smokers and about one third (31.0%) were current smokers. The median total energy intake was 1935 kcal, and 17.4% used dietary supplements.

3.3.2 Dietary patterns of study population

The analysis of the effect of dietary patterns on LINE-1 methylation levels is based on a subgroup of 349 women from the above-mentioned study population, aged 12-87 years (median = 36 years), in which we derived two major dietary patterns with eigenvalues ≥2.0, explaining 17.2% of total variance among 39 predefined food groups. **Figure 1** shows factor loadings, which represent the correlation between each food group and dietary pattern. The "Prudent" dietary pattern was characterized by high intake of potatoes, cooked and raw vegetables, legumes, soup, and fish. **Table 1** shows that age increased from the bottom to the top tertile of adherence to the "Prudent" dietary pattern. Likewise, women in the top tertile had a higher total energy intake than those in the bottom tertile. By contrast, women in the top tertile were less likely to use folic acid supplements than those in the bottom tertile. We also observed slight but significant difference in distribution of educational level across tertiles.

By contrast, the "Western" dietary pattern was characterized by high intake of canned fish, vegetable oil, processed meat, salty snacks, alcoholic drinks and dipping sauces, and low intake of

fruits. Contrary to the "Prudent" dietary pattern, age decreased from the bottom to the top tertile of adherence to the "Western" dietary. With respect to other socio-demographic variables, no significant differences were evident. Interestingly, total energy intake showed a U-shaped distribution, with lower levels in the second tertile than in the others. Moreover, being in the top tertile of adherence to the "Western" dietary pattern was associated with higher prevalence of never smokers and obesity.

3.3.3 Correlations between food intakes and LINE-1 methylation

Overall, LINE-1 methylation levels displayed a skewed distribution with a median level of 65.0 %5mC (IQR = 7.5 %5mC). We first reported significant correlations between intake of 39 food groups and LINE-1 methylation after Bonferroni correction (**Figure 2**). Interestingly, we observed that whole-meal bread, cereals, fish, fruit, raw and cooked vegetables, legumes, soup, potatoes, fries, rice, and pizza positively correlated with average LINE-1 methylation. By contrast, vegetable oil negatively correlated with average LINE-1 methylation levels. **Figure 2** also displays similar correlations between food intakes and LINE-1 methylation at CpG site 1, 2 and 3.

3.3.4 Association between dietary patterns and LINE-1 methylation

Our analysis pointed out differences in LINE-1 methylation levels according to adherence to dietary patterns (**Figure 3**). LINE-1 methylation levels at the three CpG sites increased from the bottom to the top tertile of adherence to the "Prudent" dietary pattern (**Figure 3a**). Particularly, women in the top tertile exhibited higher LINE-1 methylation levels in CpG site 1 (median = 81.0 %5mC; IQR = 3.0 %5mC), CpG site 2 (median = 54.0 %5mC; IQR = 6.0 %5mC) and CpG site 3 (median = 64.0 %5mC; IQR = 6.0 %5mC) than those in the bottom tertile (median = 80.0 %5mC; IQR = 4.0 %5mC; median = 51.0 %5mC; IQR = 21.0 %5mC; median = 59.5 %5mC; IQR = 16.5 %5mC, respectively). In line, women in the top tertile of adherence to the "Prudent" dietary pattern showed higher average LINE-1 methylation level (median = 66.7 %5mC; IQR = 4.67 %5mC) than those in the bottom tertile (median = 63.1 %5mC; IQR = 12.3 %5mC) ($p < 0.001$). By contrast, no differences in LINE-1 methylation levels across tertiles of adherence to the "Western" dietary pattern were evident (**Figure 3b**).

Finally, the increasing trend of LINE-1 methylation across tertiles of adherence to the "Prudent" dietary pattern was confirmed by a linear regression analysis on log-transformed data, both considering the unadjusted model (Model 1), and further adjusting for age, educational level, employment and smoking status, folic acid supplement, total energy intake and BMI (Model 2) (**Table 2**). Particularly, being in the top tertile of adherence to the "Prudent" dietary pattern was associated with higher LINE-1 methylation levels at CpG site 1 ($\beta = 0.009$; SE = 0.003; $p = 0.001$),

CpG site 2 (β = 0.030; SE = 0.005; p < 0.001) and CpG site 3 (β = 0.034; SE = 0.003; p < 0.001), after adjusting for covariates. In line, we observed that women in the top tertile exhibited higher average LINE-1 methylation levels that those in the bottom tertile (β = 0.022; SE = 0.003; p < 0.001). By contrast, no association between "Western" dietary pattern and LINE-1 methylation was evident, in the unadjusted (Model 1) and adjusted (Model 2) models (**Table 3**). Among the covariates considered in the regression model, only increasing age (p < 0.001) and total energy intake (p < 0.001) were positively associated with LINE-1 methylation level.

3.3.5 Comparisons across BMI categories

According to their BMI (median of 23.3 kg/m2), 488 women were categorized as underweight (6.4%), normal weight (57.6%), overweight (23.6%), or obese (12.5%). We compared the above-mentioned characteristics across these BMI categories (**Table 4**). Notably, the median age and the proportion of menopausal women increased from the underweight (p < 0.001) to the obese (p = 0.023). In line with increasing age, also the proportion of women who lived in couple (p < 0.001) and those who had at least a child (p = 0.004) increased. With respect to social factors, the proportion of women with low educational level increased from the underweight to the obese group, as well as of those who were unemployed (p-values < 0.001). Regarding behaviors, the proportion of current smokers decreased from the underweight to the obese category (p < 0.001), while no differences were evident for total energy intake and use of dietary supplements.

3.3.6 The relationship between BMI and LINE-1 methylation

We first established the relationship between BMI and LINE-1 methylation. As showed in the scatter plot reported in **Figure 4,** the percentage of LINE-1 methylation decreased by 0.125 for each unit increase of BMI (SE = 0.057; p = 0.029). Accordingly, we observed that LINE-1 methylation tended to decrease from the underweight to the obese category (p = 0.048) (**Figure 5**). Thus, median LINE-1 methylation level was 69.7 (IQR = 10.0) in underweight, 68.7 (IQR = 10.0) in normal weight, 67.3 (IQR = 10.7) in overweight, and 65.0 (IQR = 9.5) in obese women.

3.3.7 The association of obesity with LINE-1 methylation

Finally, we established the association of BMI and its categories with LINE-1 methylation level. As shown in **Table 5**, we first adjusted the negative relationship between BMI and LINE-1 methylation for the potential effect of covariates. Notably, LINE-1 methylation significantly decreased by 0.145 for each unit increase of BMI (SE = 0.058; p = 0.013). Moreover, we used normal weight women as the reference group to evaluate the association between BMI categories and LINE-1 methylation. In the unadjusted model, obese women showed lower LINE-1 methylation level than their counterpart (β = -1.971; SE = 0.876; p = 0.025), while no significant differences were evident for underweight

or overweight women. Interestingly, the negative association between obesity and LINE-1 methylation continued to be significant (β = -2.050; SE = 0.868; p = 0.019) after adjusting for age, educational level, employment and marital status, parity, menopause, and smoking habits.

3.4 Discussion

The present study aimed to investigate the relationship of lifestyles – especially diet and obesity – with LINE-1 methylation in healthy women from Southern Italy.

With respect to diet, previous studies reported that higher intake of vegetables and/or fruits decreased the risk of LINE-1 hypomethylation [297, 322]. We first partially confirmed these findings, suggesting how the intake of healthy foods - whole meal bread, cereals, fish, fruit, raw and cooked vegetables, legumes, and soup - positively correlated with LINE-1 methylation. The biological explanation of this relationship could be recognized in the wide variety of nutrients and bioactive compounds provided by fruits and vegetables - including phytochemicals, vitamins, minerals, and fibres - which in turn modulate pathways associated with epigenetic mechanisms [344, 345]. By contrast, we showed that intake of vegetable oil seemed to be negatively correlated with LINE-1 methylation, partially supporting a previous study on the association between whole peripheral blood fatty acids and DNA methylation measured as total level of 5-methyldeoxycytosine [346]. However, further research is needed to better investigate the effect of dietary fat intake on LINE-1 methylation.

Due to the growing interest in understanding how dietary patterns may affect DNA methylation in humans, Zhang and colleagues investigated the association between dietary patterns and leukocyte LINE-1 methylation in 149 individuals with no history of cancer [322]. First, they applied PCA on 13 food groups to derive the "Prudent" and the "Western" dietary patterns. While only the intake of dark green vegetables seemed to be significantly associated with LINE-1 methylation, the analysis of dietary patterns showed a positive association between the prudent dietary pattern and LINE-1 methylation in a dose-response manner [322]. In our subgroup of 349 women with no history of severe diseases, we identified two dietary patterns by applying PCA on 39 food groups. Similar to Zhang and colleagues, we derived the ""Prudent" and the "Western" dietary patterns. Interestingly, LINE-1 methylation levels at the three CpG sites and their average increased from the bottom to the top tertile of adherence to the "prudent" dietary pattern. After adjusting for covariates, we confirmed the association between high adherence to the "Prudent" dietary pattern and higher LINE-1 methylation levels at CpG site 1 and CpG site 3. In line with these findings, women with high adherence to the "prudent" dietary pattern also had higher average LINE-1 methylation levels

that those with low adherence. By contrast, no association between "Western" dietary pattern and LINE-1 methylation was evident, also adjusting for covariates.

On the other hand, our study demonstrated a negative relationship between BMI and LINE-1 methylation. In our cohort of 388 women with no history of severe diseases, we observed lower methylation level among obese women if compared with their normal weight counterpart, as partially reported in the comprehensive review published by Samblas and colleagues in 2019 [323]. Several limitations, however, occurred in previous studies, which were heterogeneous in terms of study design, DNA source, methylation marker under investigation, and outcome of interest [323]. Thus, findings were not easy to interpret, and obesity was associated with DNA methylation both positively and negatively, depending on the genes or DNA sequences under study [323]. To the best of our knowledge, few studies investigated the association between obesity and LINE-1 methylation. Carraro and colleagues reported a positive association of waist circumference and BMI with methylation level in blood samples from 40 health professionals aged 20-59 years [347]. By contrast, a longitudinal analysis of the Bogota School Children Cohort stated a negative association between adiposity measures and LINE-1 methylation in blood samples from children aged 5–12 years [348]. A negative association was also observed in our study, which for the first time investigated the relationship between BMI, obesity, and LINE-1 methylation in a large population of healthy women. These controversies might be partially described by the fact that DNA methylation could be affected by several factors - demographic, behavioral, and physiological [323] - as we have just demonstrated through the association between healthy dietary patterns and higher LINE-1 methylation level. In line with these findings, there was also evidence that weight loss interventions might significantly increase LINE-1 methylation level in blood samples [349, 350]. Moreover, it has been proposed that LINE-1 methylation level prior to the intervention might significantly predict the amount of weight loss [350]. Despite these interesting suggestions, however, there were also studies that produced inconclusive or opposite results [351, 352].

This study had several strengths. Compared to previous studies, we obtained results from a larger cohort of individuals with no history of severe diseases. Moreover, data collection was performed through validated tools, which enabled us to investigate the effect of dietary patterns on LINE-1 methylation applying PCA on 39 food groups. With respect to DNA methylation analysis, we applied the pyrosequencing of bisulfite-treated DNA, which is a replicable methodology and the "gold standard" to evaluate LINE-1 methylation levels [353, 354]

This study had also some limitations. Its observational cross-sectional design did not allow to assess causality. Moreover, dietary data collection was performed using FFQs, which did not exclude

measurement errors. Nowadays, FFQs still remain widely used as the primary dietary assessment tool in epidemiological studies [315]. Despite its limitations, the FFQ has been previously developed and validated among a similar cohort of women from Southern Italy [296]. In addition, our PCA-derived dietary patterns were consistent with previous studies [268, 355-358]. Additionally, we used information on BMI and its classification, even if other anthropometric measures and adiposity indexes should have been also considered.

Moreover, we performed LINE-1 methylation analysis on leukocyte DNA, which included several cell type subsets. Finally, we cannot completely ignore the effect of unmeasured residual factors, such as ethnicity, drinking, physical activity, and environmental exposures. Moreover, the association between lifestyles and LINE-1 methylation may be also affected by genetic factors (e.g., polymorphisms in the *MTHFR* gene), which in turn may interact with methylation process

Table 1. Characteristics of study population by adherence to dietary patterns

Characteristics	Prudent				Western			
	1st tertile	2nd tertile	3rd tertile	p-value	1st tertile	2nd tertile	3rd tertile	p-value
Age, years	32.0 (19.0)	35.0 (19.0)	46.0 (34.0)	<0.001	41.0 (34.0)	40.0 (24.0)	30.0 (17.0)	<0.001
Educational level								
Low	34.5%	18.8%	23.3%		24.1%	23.1%	29.3%	
Medium	37.9%	53.8%	50.9%	0.049	47.4%	49.6%	45.7%	0.832
High	27.6%	27.4%	25.9%		28.4%	27.4%	25.0%	
Employment status (% unemployed)	62.1%	47.0%	53.4%	0.069	53.4%	55.6%	53.4%	0.933
Smoking status								
Never smokers	67.8%	69.2%	62.6%		63.5%	65.5%	70.7%	
Former smokers	11.3%	13.7%	14.8%	0.757	14.8%	19.8%	5.2%	0.011
Current smokers	20.9%	17.1%	22.6%		21.7%	14.7%	24.1%	
Use of folic acid supplement (% users)	19.0%	18.8%	7.8%	0.024	19.8%	16.2%	9.5%	0.083
Total energy intake, kcal	1693.0 (3581.0)	1940.0 (614.0)	2142.0 (563.0)	<0.001	2028.0 (682.0)	1781.0 (2610.0)	2065.0 (744.0)	0.001
Body Mass Index, kg/m²	23.3 (6.8)	23.9 (5.8)	24.1 (23.0)	0.971	23.0 (4.8)	25.0 (5.7)	23.0 (8.1)	0.067
Body Mass Index categories								
Underweight	3.5%	6.9%	5.2%		3.5%	2.6%	9.6%	
Normal weight	60.9%	50.9%	53.0%	0.403	65.2%	47.9%	51.8%	0.002
Overweight	19.1%	28.4%	29.6%		21.7%	35.9%	19.3%	
Obese	16.5%	13.8%	12.2%		9.6%	13.7%	19.3%	

This table is adapted from Barchitta et al., Nutrients 2019

Table 2. Linear regression analysis of the association between adherence to the prudent dietary pattern and LINE-1 methylation level

Regression model	LINE-1 methylation	1st tertile	2nd tertile		3rd tertile		p-trend
			β (SE)	p-value	β (SE)	p-value	
Model 1	CpG site 1	*Ref.*	0.001 (0.002)	0.599	0.008 (0.002)	<0.001	<0.001
	CpG site 2	*Ref.*	0.009 (0.010)	0.348	0.011 (0.009)	0.234	0.233
	CpG site 3	*Ref.*	0.011 (0.007)	0.120	0.019 (0.006)	0.003	0.003
	Average	*Ref.*	0.006 (0.005)	0.263	0.012 (0.005)	0.017	0.017
Model 2	CpG site 1	*Ref.*	0.001 (0.002)	0.990	0.009 (0.003)	0.001	<0.001
	CpG site 2	*Ref.*	0.015 (0.004)	0.001	0.030 (0.005)	<0.001	<0.001
	CpG site 3	*Ref.*	0.016 (0.003)	<0.001	0.034 (0.003)	<0.001	<0.001
	Average	*Ref.*	0.009 (0.002)	<0.001	0.022 (0.003)	<0.001	<0.001

This table is adapted from Barchitta et al., Nutrients 2019

Table 3. Linear regression analysis of the association between adherence to the western dietary pattern and LINE-1 methylation level

Regression model	LINE-1 methylation	1st tertile	2nd tertile		3rd tertile		p-trend
			β (SE)	p-value	β (SE)	p-value	
Model 1	CpG site 1	Ref.	0.001 (0.002)	0.828	-0.003 (0.002)	0.276	0.262
	CpG site 2	Ref.	-0.009 (0.008)	0.310	-0.002 (0.009)	0.838	0.835
	CpG site 3	Ref.	-0.008 (0.006)	0.202	-0.002 (0.007)	0.753	0.743
	Average	Ref.	-0.005 (0.005)	0.316	-0.002 (0.005)	0.702	0.690
Model 2	CpG site 1	Ref.	0.002 (0.002)	0.523	-0.001 (0.003)	0.676	0.760
	CpG site 2	Ref.	0.005 (0.005)	0.282	-0.003 (0.005)	0.549	0.837
	CpG site 3	Ref.	0.007 (0.004)	0.067	-0.001 (0.004)	0.834	0.705
	Average	Ref.	0.004 (0.003)	0.101	-0.001 (0.003)	0.647	0.986

This table is adapted from Barchitta et al., Nutrients 2019

Table 4. Characteristics of the study population across categories of the body mass index

Characteristics	Underweight (n=31)	Normal weight (n=281)	Overweight (n=115)	Obese (n=61)	P-value
Age, years	30 (11)	39 (18)	46 (22)	44 (21)	<0.001
Educational level					
Low	19.3%	30.4%	46.2%	53.9%	
Medium	49.1%	46.9%	44.0%	34.8%	<0.001
High	31.6%	22.7%	9.9%	11.2%	
Unemployed	45.6%	51.3%	59.9%	74.2%	<0.001
Living in couple	18.6%	46.1%	68.3%	75.6%	<0.001
Having children	36.8%	70.5%	80.9%	78.9%	0.004
Menopause	0.0%	15.2%	21.8%	15.8%	0.023
Smoking status					
Never smokers	47.7%	54.0%	60.4%	61.8%	
Former smokers	7.0%	7.9%	15.9%	13.5%	<0.001
Current smokers	45.6%	38.1%	23.6%	24.7%	
Total energy intake, kcal	2014 (705)	1923 (650)	1935 (708)	1950 (778)	0.335
Users of supplements	11.6%	15.7%	16.3%	15.6%	0.905

This table is adapted from Maugeri et al., Disease Markers 2021

Table 5. Linear regression analyses between BMI, its categories, and LINE-1 methylation level

Model[a]	BMI	B coefficient	Standard error	P-value
Unadjusted	**Continuous**	-0.125	0.057	0.029
	Categories			
	Underweight	0.194	1.173	0.868
	Normal weight		*Ref.*	
	Overweight	0.170	0.687	0.803
	Obese	-1.971	0.876	0.025
Adjusted	**Continuous**	-0.145	0.058	0.013
	Categories			
	Underweight	-0.015	1.161	0.990
	Normal weight		*Ref.*	
	Overweight	-0.108	0.687	0.875
	Obese	-2.050	0.868	0.019

This table is adapted from Maugeri et al., Disease Markers 2021

Figure 1. Bar graph of factor loadings characterizing dietary patterns

This figure is adapted from Barchitta et al., Nutrients 2019

Figure 2. Correlation matrix between food intakes and LINE-1 methylation level

	CpG 1	CpG 2	CpG 3	Average
White bread	-0,02	0,03	0,06	0,04
Wholemeal bread	0,08	**0,24**	**0,20**	**0,21**
Cereals	0,09	**0,17**	**0,17**	0,16
Butter and Margarine	-0,10	-0,10	-0,10	-0,11
Milk	-0,03	-0,05	-0,03	-0,04
Yogurt	-0,03	0,05	0,06	0,05
Olive oil	0,15	0,17	0,16	0,17
Vegetable oil	-0,13	**-0,20**	-0,16	**-0,18**
Soft cheese	-0,06	0,03	0,06	0,03
Hard cheese	0,00	0,11	0,11	0,10
Eggs	0,07	0,16	0,17	0,16
Processed meat	-0,08	-0,04	0,00	-0,02
Red meat	-0,08	-0,06	-0,05	-0,06
White meat	0,08	0,08	0,10	0,10
Offal	-0,04	0,04	0,04	0,03
Fish	0,13	0,17	**0,22**	**0,20**
Shellfish	0,12	0,04	0,11	0,08
Canned fish	-0,03	-0,02	-0,03	-0,02
Fruit	0,16	**0,24**	**0,29**	**0,26**
Fruit salad	0,02	0,12	0,10	0,11
Raw vegetable	0,13	**0,18**	**0,23**	**0,21**
Cooked vegetable	**0,26**	**0,29**	**0,39**	**0,35**
Legumes	**0,27**	**0,23**	**0,32**	**0,30**
Soup	**0,22**	**0,24**	**0,29**	**0,28**
Potatoes	0,10	**0,20**	**0,18**	**0,20**
Fries	0,13	0,17	**0,22**	**0,19**
Rice	0,09	0,15	**0,19**	**0,17**
Pasta	0,06	0,12	0,13	0,12
Pizza	0,14	**0,26**	**0,27**	**0,26**
Nuts	-0,10	0,10	0,14	0,09
Sweets	-0,10	0,10	0,12	0,08
Salty snacks	-0,08	0,02	-0,01	0,00
Dipping sauces	-0,13	-0,13	-0,15	-0,15
Wine	0,12	0,07	0,08	0,09
Beer	0,09	0,01	0,01	0,03
Alcoholic drinks	-0,04	-0,08	-0,10	-0,09
Coffee	0,01	-0,14	-0,14	-0,13
Tea	0,05	0,05	0,05	0,06
Fruit juice	0,02	0,06	0,04	0,04

Results are reported as Spearman's correlation coefficient and those with Bonferroni-corrected p-value<0.001 are indicated in bold font.

This figure is adapted from Barchitta et al., Nutrients 2019

Figure 3. Comparison of LINE-1 methylation level across tertiles of adherence to (a) the prudent and (b) western dietary patterns
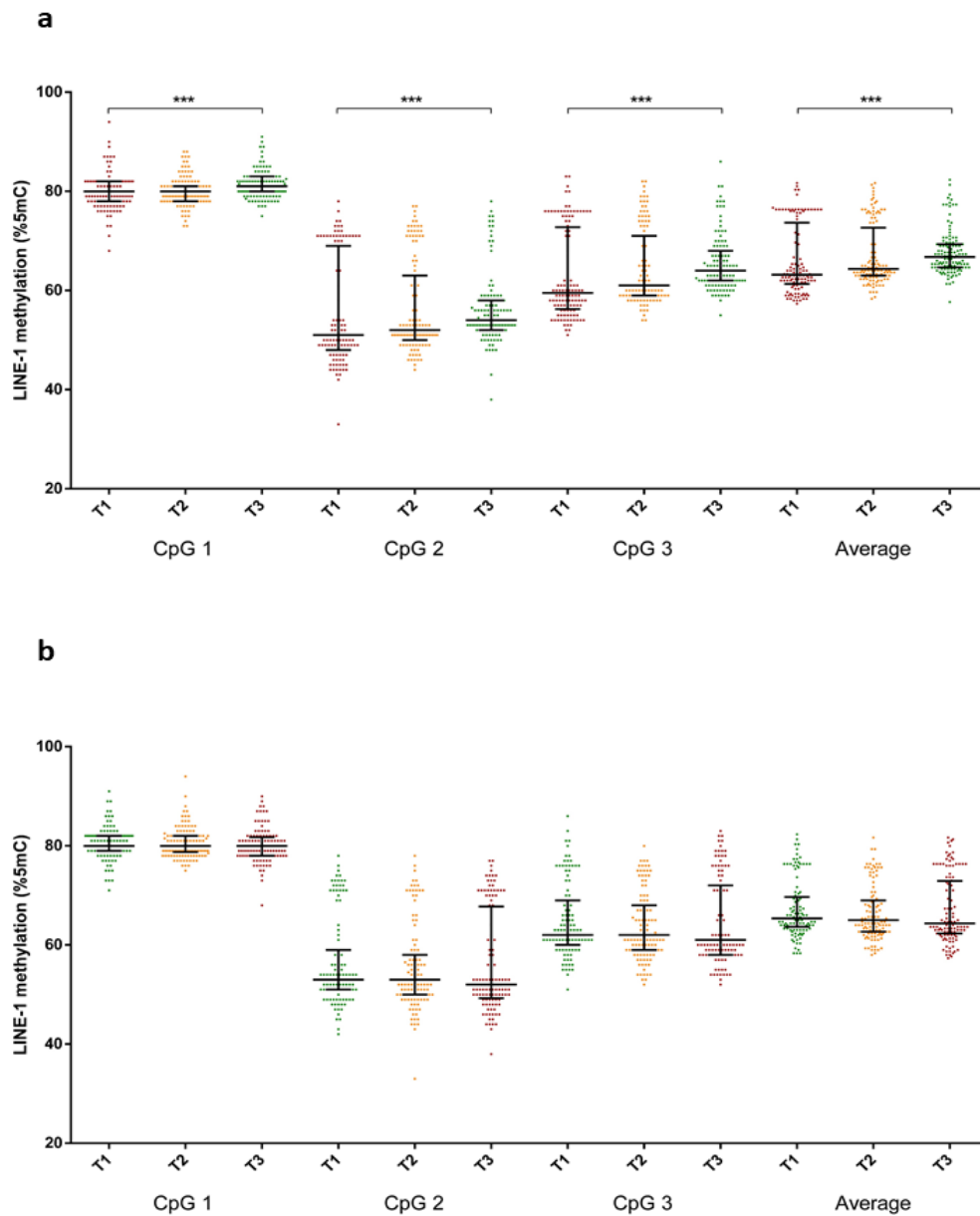
***p<0.001 based on the Kruskal–Wallis test.

This figure is adapted from Barchitta et al., Nutrients 2019

Figure 4. Scatter plot of the relationship between body mass index and LINE-1 methylation
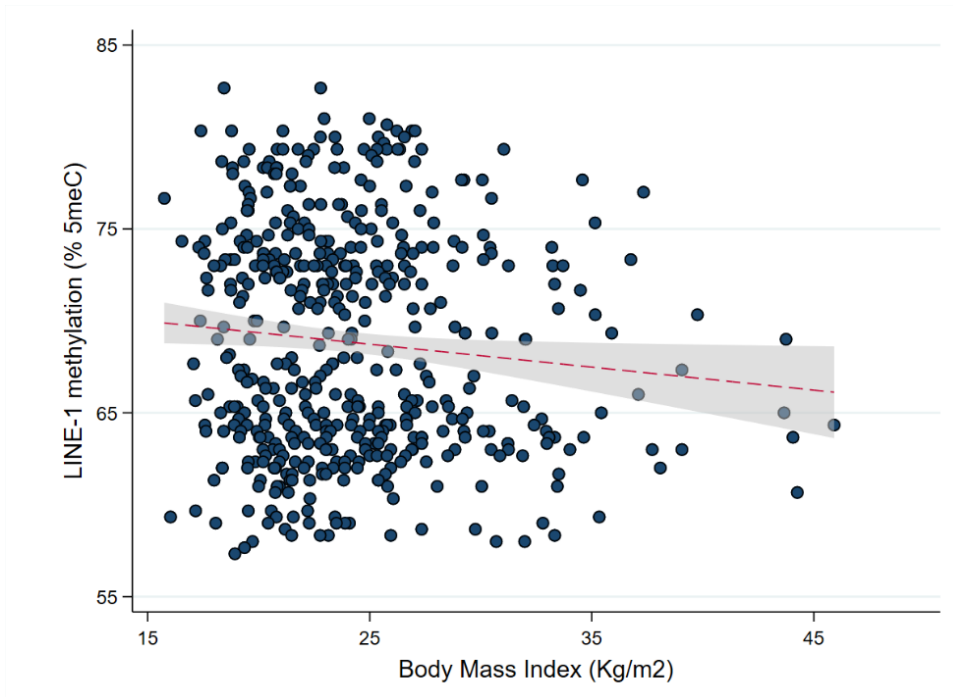
The red line represents the linear regression line with its 95% confidence interval.

This table is adapted from Maugeri et al., Disease Markers 2021

Figure 5. Violin plot showing the distribution of LINE-1 methylation level across categories of body mass index

This table is adapted from Maugeri et al., Disease Markers 2021

4. The role of behaviors as mediators of the relationship between socioeconomic status and LINE-1 methylation level

4.1 Background

Social determinants represent a crucial target for Public Health interventions due to their involvement in health inequalities between and within countries [359]. Earlier onset of age-related chronic diseases and higher risk of death are generally associated with low socioeconomic status (SES) – usually referred as educational level, employment, and income [360, 361]. People with socio-economic disadvantaged tend to be prone worse with regards to non-communicable diseases risk factors, including unhealthy behaviors [360-364]. The promotion of healthy lifestyles, therefore, plays a crucial role in the prevention of several diseases [365], as well as in reducing health disparities.

Social disadvantages and unhealthy behaviors can occur either *in utero* or during lifetime, inducing sustainable biological changes involved in individual non-communicable diseases risk profile [366, 367]. However, the involved molecular mechanisms are still not fully clarified. For this reason, uncover the epigenetic mechanisms underpinning this relationship might help to explain the effect of SES on human health. However, even if previous studies have reported that socioeconomic disadvantage significantly affected DNA methylation process, social inequalities alone do not completely explain socioeconomic difference in DNA methylation level. With this in mind, recent studies have been conducted to investigate the effects of behaviors on global DNA methylation - using LINE-1 sequences as a surrogate marker - in exposed individuals and in future generations [224]. As previously discussed, several lines of evidence demonstrated how dietary factors [297, 320, 321, 343, 368], smoking habits [369, 370], physical activity [371] and weight status [372] might affect LINE-1 methylation levels.

In this scenario, our hypothesis was that the behaviors – including diet, physical activity, smoking habits and weight status - might act as mediator of the association between SES and LINE-1 methylation. For this reason, we conducted a cross-sectional study on women from Catania (Italy) to assess socioeconomic inequalities in LINE-1 methylation levels, and to examine whether lifestyles were potential mediators of this difference.

4.2 Methods

4.2.1 Study design

The current cross-sectional study recruited women from those who referred for routine medical examination to three clinical laboratories in Catania (Italy) from 2010 to 2017. Healthy non-pregnant women, with no history of severe diseases - including cancer, diabetes, cardiovascular, neurodegenerative, and autoimmune diseases - were included. Participants were fully informed of all aspects of the research protocol, which was conducted in accordance with the Declaration of Helsinki and invited to sign a written informed consent to participate in the study. The protocol was approved by the ethics committees (Ethics Committees *"Catania"* and *"Catania 2"*) of the involved institutions (*Azienda Ospedaliero - Universitaria "Policlinico - Vittorio Emanuele"* and *Azienda Sanitaria Provinciale* of Catania, Italy) with the following protocol numbers: 52/2010/VE, 16/2015/CECT2, and 227/2011/BE. At recruitment, information on socioeconomic characteristics and lifestyles were collected by trained interviewers using structured questionnaires. Moreover, a blood sample was requested from all participating women. Next, each whole blood sample was centrifuged at 2500 rpm for 15 min, and the buffy coat was immediately frozen at -20 °C until further analyses.

4.2.2 Socioeconomic status assessment

Educational level was categorized as: low (i.e., primary school or none), medium (i.e. vocational or another secondary school) or high (i.e. university or vocational postsecondary school) level. Employment status was categorized as full-time employment, part-time employment, and unemployment (including housewives and retired). For statistical analysis, educational level and employment status were used as proxies for SES [373, 374], with low educational level and unemployment as reference groups.

4.2.3 Behavioral data collection

Height and weight were measured at recruitment using standardized procedures, and BMI was obtained as the ratio between weight (kg) and squared height (m^2) [293]. Information on smoking habits - collected using a questionnaire – allowed us to categorize women as never, former, and current smokers. With respect to physical activity, it was assessed using the long form of the IPAQ-L [294]. Women were classified as inactive (i.e. no moderate or vigorous activity), moderately inactive and moderately active, and active (≥150 min/week moderate or ≥75 min/ week vigorous or ≥150 min/ week moderate + vigorous), according to the American Heart Association criteria [295]. Dietary information was collected using a semi-quantitative FFQ, previously described [97, 297, 343, 363]. Adherence to Mediterranean diet was assessed using the 9-point index of MDS and

categorized as low (MDS range: 0–3), medium (MDS range: 4–6), or high (MDS range: 7–9) [302, 303].

4.2.4 LINE-1 methylation analysis

DNA samples were extracted from buffy coats using the QIAamp DNA Mini Kit (Qiagen, Italy) according to the manufacturer's protocol. Methylation analysis was performed on three CpG sites within the LINE-1 sequence (GenBank Accession No. X58075) [97, 297] to allow comparison with our previous studies in this field of research. Bisulphite conversion of 40 ng of each DNA sample was performed using the EpiTect Bisulfite Kit (Qiagen, Italy). PCR was conducted in a reaction volume of 25 μl, which contained 1.5 μl of bisulfite-converted DNA, 12.5 μl of PyroMark PCR Master Mix 2×, 2.5 μl of Coral Load Concentrate 10×, and 2 μl of the forward primer (5′-TTTTGAGTTAGGTGTGGGATATA-3′) and the reverse-biotinylated primer (5′-biotin-AAAATCAAAAAATTCCCTTTC-3′) (0.2 μM for each) [97, 102, 297, 343]. Hot start PCR conditions were as follows: 1 cycle at 95°C for 15 min, 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. As described elsewhere [102, 103, 297], pyrosequencing of PCR product was performed with 0.3 mM of the sequencing primer (5′-AGTTAGGTGTGGGATATAGT-3′) using the PyroMark Q24 instrument (Qiagen, Italy). All assays were conducted in triplicate including positive (100% methylated DNA) and negative (0% methylated DNA) controls, while failed assays were repeated. Intra-observer coefficient of variability between replicates was 2.2% (SD=1.0%), as previously reported [103]. For each CpG site, methylation level was calculated as the ratio between methylated cytosines and the sum of methylated and unmethylated cytosines. LINE-1 methylation level was computed as the average methylation level of the three CpG sites.

4.2.5 Statistical analysis

We first compared LINE-1 methylation across categories of SES proxies (i.e., educational level and employment status). Due to its skewness (p<0.001 based on the Kolmogorov-Smirnov test), LINE-1 methylation level was expressed as median and IQR and compared using the Kruskal–Wallis test. Age-adjusted linear regression models were applied to test the association of SES proxies with LINE-1 methylation, using log-transformed LINE-1 methylation level as the outcome. SES proxies were first added to the model one-by-one, and then included simultaneously. In all the analyses, the lower SES categories were used as the reference to assess associations of high SES with LINE-1 methylation.

Next, we compared behavioral characteristics across different educational levels. Chi-square test was used to compare categorical variables, while continuous variables were compared using the

Kruskal–Wallis test. The association of behaviors with LINE-1 methylation was examined by age-adjusted linear regression models using log-transformed LINE-1 methylation level as the outcome. The predictors that were initially considered separately were: BMI (as continuous variable), smoking status (as ordinal categorical variable: current, former, never), physical activity (as ordinal categorical variable: active, moderately active, moderately inactive, inactive), and MDS (as ordinal categorical score from 0 to 9). We also tested for interaction between predictors that were significantly associated with log-transformed LINE-1 methylation level in the abovementioned age-adjusted linear regression models.

To evaluate the mediation effect, we performed a mediation analysis as described by Preacher and Hayes [375]. The first equation (path a) regressed the mediator (MDS) on the independent variable (educational level). The second equation (path b) regressed the dependent variable (log-transformed LINE-1 methylation) on the mediator. The third equation (path c') regressed the dependent variable on the independent variable, adjusting for the effect of the mediator. Low educational level was used as reference group and age as covariate in all the mediation models. Bias-corrected and accelerated bootstrap confidence intervals (CI) were calculated for indirect effects (a*b). Bootstrapping (5000 samples) was conducted. The percentage mediated was expressed as the percentage of the total effect (path c) accounted for by the indirect effect (a*b).

4.3 Results

4.3.1 Study population

The present cross-sectional study included 349 women aged 25-64 years and with a complete assessment of SES, lifestyles, and LINE-1 methylation. Overall, 25.5% of women had a low educational level, 47.6% were in the middle educational level and 26.9% in the higher one. Moreover, 54.2% of women were unemployed while 21.2% and 24.6% were part-time or full-time employed, respectively.

4.3.2 Socioeconomic differences in LINE-1 methylation

We first compared LINE-1 methylation levels across categories of SES, considering SES in terms of educational level (**Figure 1a**) and employment status (**Figure 1b**). We noted that LINE-1 methylation level increased with increasing educational level ($p < 0.001$). We confirmed our results after applying a linear regression analysis on log-transformed data, which showed the increasing trend of LINE-1 methylation across educational levels in the age-adjusted model ($\beta = 0.016$; SE = 0.003; $p < 0.001$). Similarly, employed women exhibited higher LINE-1 methylation level than their counterpart ($p = 0.002$). This trend was confirmed by age-adjusted linear regression analysis on log-transformed LINE-1 methylation level ($\beta = 0.007$; SE = 0.002; $p = 0.003$). However, when

SES indicators were evaluated simultaneously, only educational level exhibited a statistically significant association with LINE-1 methylation ($\beta = 0.016$; SE = 0.003; $p < 0.001$).

4.3.3 Association between educational level and behaviors

We next evaluated whether behaviors mediate the effect of SES on LINE-1 methylation, using educational level as a proxy indicator. To do that, as shown in **Table 1**, we first compared BMI, MDS, smoking status and physical activity according to educational level. Notably, BMI decreased with increasing educational level ($p < 0.001$). Moreover, highly educated women were more likely to be normal weight ($p < 0.001$) and to adhere to MD ($p = 0.018$). Furthermore, women with high educational level were less likely to perform physical activity ($p = 0.012$) than their less educated counterpart. Instead, no social differences in smoking status were evident ($p = 0.508$).

4.3.4 The effects of behaviors on LINE-1 methylation

We next applied age-adjusted linear regression analyses to assess the effects of behaviors on LINE-1 methylation. Notably, we observed a positive association between adherence to MD – evaluated by MDS score – and LINE-1 methylation ($\beta = 0.006$; SE = 0.001; $p < 0.001$). Moreover, former ($\beta = 0.014$; SE = 0.007; $p = 0.037$) and non-smokers ($\beta = 0.012$; SE = 0.005; $p = 0.020$) showed higher LINE-1 methylation levels than current smokers. By contrast, no association with BMI and physical activity was evident (p-values > 0.05). We also did not demonstrate an interaction between adherence to MD and smoking status on LINE-1 methylation level (p-value for interaction = 0.498).

4.3.5 Mediation analysis

Finally, we tested the mediating effect of women habits on the relationship between educational level and LINE-1 methylation. As shown in **Figure 2**, we observed a positive association of high educational level with MDS ($\beta = 0.669$; 95%CI = 0.173-1.165; $p < 0.01$) and LINE-1 methylation level ($\beta = 0.033$; 95%CI = 0.022-0.043; $p < 0.001$). Notably, there was a significant indirect effect of high educational level on LINE-1 methylation through the adherence to MD ($\beta = 0.003$; 95%CI = 0.001-0.006). The mediator could account for 9.5% of the total effect. We obtained similar results also using employment status as indicator of SES.

By contrast, we did not observe any mediation when evaluating the indirect effect of medium educational level on LINE-1 methylation ($\beta = 0.001$; 95%CI = -0.001 - 0.004). A plausible explanation is that medium educational level was not significantly associated with adherence to MD ($\beta = 0.312$; 95%CI = -0.127 – 0.752). Similarly, none of the other habits were associated with a statistically significant mediation in the relationship between educational level and LINE-1 methylation.

4.4 Discussion

The present cross-sectional study aims to investigate potential behaviors as mediators of socioeconomic differences in leukocyte LINE-1 methylation. In our analysis, we first evaluated the association of SES and LINE-1 methylation, using educational level and employment status as proxy indicators. Women in the highest socioeconomic classes showed higher LINE-1 methylation level than their counterpart. Yet, educational level seemed the strongest predictor of LINE-1 methylation when both SES indicators were evaluated simultaneously. This was in line with the current belief that life experiences affect epigenetic marks in specific loci of the genome [376]. In particular, several studies conducted on animals showed the relationship of social stress and isolation with changes in CpG methylation of promoter regions [376, 377]. In addition, epidemiological studies stated that adverse exposures in early life was related to aberrant methylation profiles in adolescents and adults [378, 379]. More recently, Fiorito and colleagues observed a graded relationship between SES and accelerated biological aging [373, 374], using two alternative predictors of accelerated biological aging based on DNA methylation at 353 CpG sites and 71 CpG sites, respectively [373, 374]. These predictors, named 'epigenetic clocks', allow to estimate biological aging as the difference between DNA methylation age and chronological age.

In our study, women with high educational level were also more likely to be normal weight, to perform more physical activity and to adhere more to MD than those with low educational level. For this reason, we also evaluated whether lifestyle-related behaviors might affect LINE-1 methylation levels. In line with previous studies [97, 297, 343], we observed that women who highly adhered to MD exhibited higher LINE-1 methylation levels that their unhealthy counterpart. These findings supported the hypothesis that adherence to MD might partially explain and mediate socioeconomic differences in LINE-1 methylation level. To test it, we assessed changes in the effect of educational level on LINE-1 methylation, adjusting for confounder (i.e., age) and mediator (i.e., adherence to MD). In particular, we considered adherence to MD as a mediator due to its relationship with educational level and, simultaneously, with LINE-1 methylation. However, only ~10% of socioeconomic difference in LINE-1 methylation could be explained by adherence to MD, while remaining difference was likely due to unmeasured factors. In fact, our analyses demonstrated that none of the other behaviors constituted a potential mediator of the relationship between educational level and LINE-1 methylation. Similarly, the above-mentioned study aimed to investigate the role of behaviors in mediating the association between SES and biological aging [373, 374]. However, their mediation analysis failed in demonstrating a significant reduction of the association magnitude due to the inclusion of behaviors in the model [373, 374]. They stated that

the association was robust to adjustment for mediators, with a partial effect attenuation when including smoking status in the model [373, 374]. In our study, we observed that former and non-smokers showed higher LINE-1 methylation level than current smokers. Although molecular mechanisms underpinning this relationship are not clearly understood yet, our findings were consistent with the notion that cigarette smoke affects DNA methylation [380]. However, cigarette smoking did non interact with adherence to MD, and therefore the latter cannot counteract the detrimental effect on LINE-1 methylation observed among current smokers. Moreover, the lack of relationship between educational level and smoking status excluded one of the requirements for a significant mediating effect.

Our study had some limitations. Firstly, the cross-sectional design did not allow us to understand the causality between diet and LINE-1 methylation. With respect to the use of LINE-1 methylation as a surrogate marker of DNA methylation, the heterogeneity in LINE-1 methylation levels across CpG sites and tissues [381-385] hindered the comparisons between different studies. Despite these issues, aberrant LINE-1 methylation still remains an important molecular mechanism in the research on non-communicable diseases [54-56]. Finally, data collection was performed by subjective methods that did not preclude inaccuracy. However, even if the FFQ used for dietary assessment was prone to a degree of misreporting [315], this tool has been developed and validated among women from Southern Italy [296], and current findings were consistent with previous studies conducted on similar cohorts [268, 355-358]. Moreover, we cannot evaluate the potential effect of additional social factors (e.g., income, household size and composition), confounders (e.g., genetic variants and environmental exposure), and mediators (e.g., drinking habits).

To our knowledge, this is the first study evaluating the role of behaviors as mediators in the relationship between SES and LINE-1 methylation. Our findings confirm previous studies that SES is a determinant of health, also through biological changes such as epigenetic mechanisms. Notably, we suggest that behaviors – and especially the adherence to MD – might mediate this association, encouraging public health interventions to promote healthy dietary habits in social disadvantaged people. However, further prospective studies should be recommended to confirm this evidence taking into account additional social factors and behaviors.

Table 1. Population characteristics by educational level

Characteristics	Educational level			p-value
	Low (n=89)	Medium (n=166)	High (n=94)	
Age, years	40 (27)	36 (25)	34 (17)	0.739
BMI	26.0 (7.5)	23.5 (4.9)	22.3 (5.0)	**<0.001**
BMI categories				
Underweight	1.1%	7.9%	4.3%	**<0.001**
Normal weight	42.0%	53.3%	69.9%	
Overweight	31.8%	28.5%	15.1%	
Obese	25.0%	10.3%	10.8%	
Mediterranean Diet Score	4 (3)	4 (3)	4 (3)	0.291
MDS categories				
Low	39.8%	42.7%	35.1%	**0.018**
Medium	52.4%	43.8%	50.0%	
High	7.8%	13.5%	14.9%	
Smoking status				
Current	20.5%	22.9%	15.1%	0.508
Former	15.9%	12.7%	11.8%	
Never	63.6%	64.5%	73.1%	
Physical activity				
Inactive	8.1%	19.4%	28.6%	**0.012**
Moderate active	91.9%	73.1%	68.6%	
Active	0%	7.5%	2.9%	

This table is adapted from Maugeri et al., Scientific Reports 2020

Figure 1. Comparison of LINE-1 methylation level by educational level (A) and employment status (B)
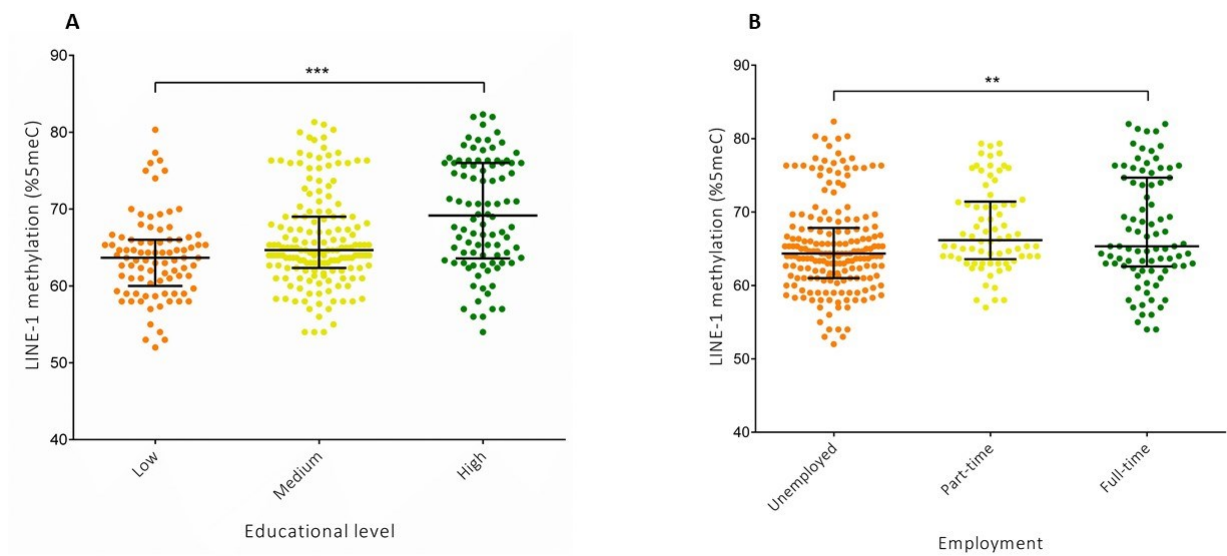
** p < 0.01 and *** p < 0.001, based on the Kruskal–Wallis test.

This figure is adapted from Maugeri et al., Scientific Reports 2020

Figure 2. Analysis of the mediating effect of Mediterranean Diet Score in the association between high educational level and LINE-1 methylation

β= 0.669 (95%CI= 0.173 – 1.165) **

β= 0.005 (95%CI= 0.002 – 0.007) ***

Adherence to MD

Educational Level (high vs. low)

LINE-1 Methylation

Total effect
β= 0.033 (95%CI= 0.022– 0.043) ***

Indirect effect
β= 0.003 (95%CI= 0.001-0.006); Pm=9.5%
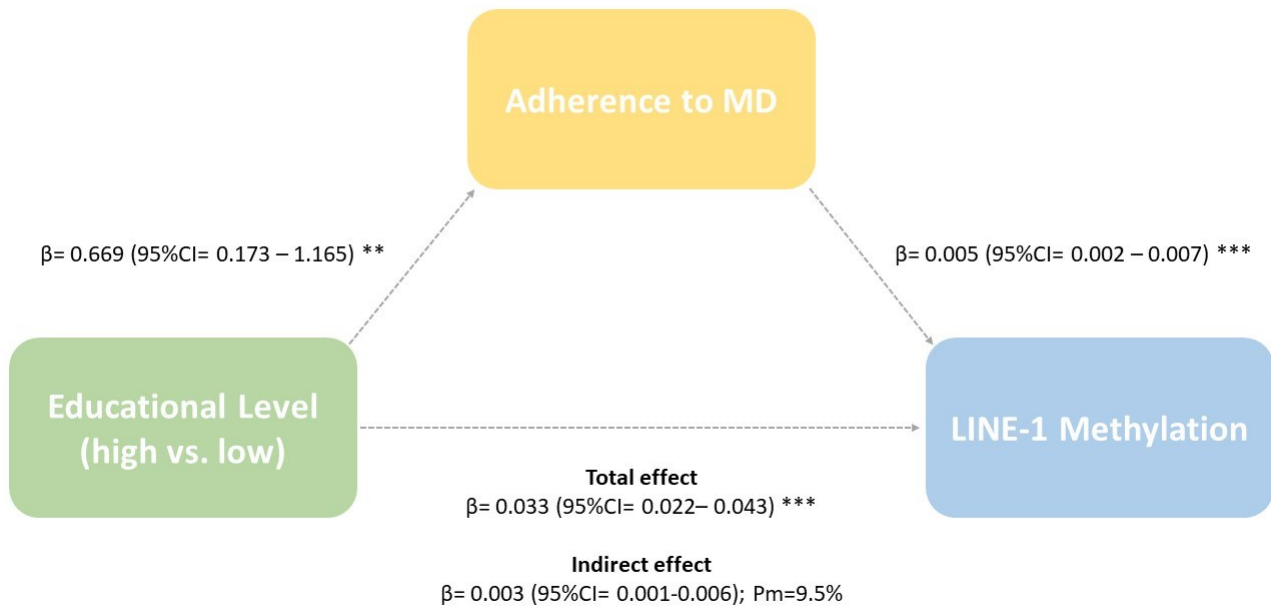
Mediation analysis was conducted using the procedure described by Preacher and Hayes [375]. Low educational level was used as reference group and age as covariate in all the regressions. Bias-corrected and accelerated bootstrap confidence intervals (CI) were calculated for indirect effects (a*b). Bootstrapping (5000 samples) was conducted. The percentage mediated was expressed as the percentage of the total effect (path c) accounted for by the indirect effect (a*b). ** $p < 0.01$ and *** $p < 0.001$.

This figure is adapted from Maugeri et al., Scientific Reports 2020

5. The main determinants of women choices before and during pregnancy

5.1 Background

The determinants of health often influence our choices during lifetime. Among lifestyles, nutrition plays a critical role in determining maternal and infant health. During the preconception, perinatal and breastfeeding periods, a well-balanced diet is essential for fetal development and neonatal growth [228, 296]. By contrast, unhealthy food choices – such as inadequate intake of micronutrients – lead to maternal nutrient deficiencies and might affect the risk of adverse outcomes (i.e., PTB and SGA) [184]. In line, mounting evidence suggests a firmly interplay between newborn and mother metabolisms, which in turn involve nutrient stores and intakes [180-182]. For this reason, the WHO and the Food and Agricultural Organization (FAO) suggested several dietary recommendations and strategies for the prevention of adverse pregnancy outcomes [156] [164-166]. Similarly, the guidelines of the Italian Ministry of Health suggest that maternal diet should be as varied as possible [155]. In this context, folate – a water-soluble vitamin B present in fruits, legumes, cereals, and green leafy vegetables – is required for placental growth [386] and neural tube formation [387]. Folate acts as a methyl donor for important molecular pathways, such as methylation, synthesis and DNA repair, replication and cell maintenance [388]. For these reasons, inadequate folate concentrations are associated with a number of birth defects - including anencephaly, spina bifida and encephalocoele [185] - that can cause lifelong problems affecting health, growth and learning [185]. Folate requirement is usually of 400 μg/day among women of childbearing age and of 600 μg/day during the periconceptional period [389]. To meet this need, in 1998, the United States began the fortification of cereal flour enriched with folic acid [390], next followed by several countries policies for folate fortification [391]. Nowadays, developed countries considered the supplementation of folic acid – the synthetic form of this vitamin B - as a strategy to ensure the correct fetal development [392]. However, folic acid supplementation was often insufficient in the preconception period, with several negative effects on maternal-child health [393]. Thus, further studies are needed to investigate the relationship of social and behavioral determinants with the adherence to recommendations, also increasing the awareness about benefits of folic acid supplementation [394-397]. Given the importance of this issue, previous studies investigated the relationships of socio-demographic factors and other behaviors with dietary intakes of nutrients in women of childbearing age [398-404]. However, preventive strategies should be based on the promotion of healthy dietary patterns, rather than on single nutrients or food groups.

Despite of recommendations, maternal dietary habits seem to be influenced by socio-demographic factors (i.e., socio-economic status, educational level, employment status) and behavioral factors (i.e., smoking habits) similar to general populations [400, 403, 405, 406]. In line, several studies demonstrated that maternal food choices are affected by age, educational level, and smoking habits in several populations [398-404]. The latter, along with parity and BMI, have been also associated with the use of supplements in European populations [407].

Additionally, to increase the awareness about benefits of healthy behaviors differing from nutrition, public health strategies are also moving towards a life-course immunization approach, focusing on childhood and women of childbearing age. Since several diseases can adversely affect pregnancy, Measles – Mumps – Rubella (MMR) and HPV vaccines are recommended for women of childbearing age [408, 409]. Furthermore, active immunization during pregnancy represents a remarkably promising strategy to protect mothers and their offspring against vaccine-preventable diseases (VPDs). Several lines of evidence pointed up vaccines' safety and effectiveness during pregnancy, not only for women but also for developing fetus [410-414]. Through maternal immunization, acquired antibodies can be transferred to the fetus, conferring passive immunity until the first infant immunization [415]. With this in mind, the Italian National Immunization Plan (Piano Nazionale Prevenzione Vaccinale 2017-19; PNPV) strongly advised to administer booster doses of seasonal Influenza vaccine at any time-point during pregnancy, and vaccine against Diphtheria – Tetanus - and acellular Pertussis (DTaP) between the 27th and the 36th week of every pregnancy [416]. However, the rates of coverage for the above-mentioned vaccines among pregnant women remain very low worldwide [414, 417, 418]. Similarly, infant and childhood vaccine uptake rates are not enough to control VPDs. To prevent measles outbreaks occurred in the past decade, several countries have enacted mandatory childhood immunization legislation [419-421]. In July 2017, Italian immunization strategies on infants extended the number of mandatory vaccines from four - Diphtheria, Tetanus, Poliomyelitis, Hepatitis B vaccines - to ten, also including Pertussis, *Haemophilus influenzae* type b, MMR and Varicella vaccines [422, 423]. In this scenario, vaccine hesitancy – defined as "a delay in acceptance or refusal of vaccines despite availability of vaccinations services" – is believed to be responsible for decreasing vaccine coverage [424, 425]. Although socio-demographic and socio-economic factors can influence vaccination choice, disinformation, and lack of confidence in vaccination play a key role in determining low vaccine uptake among women [414, 425-427]. Thus, including transparency in policy-making decisions and providing vaccines information to the public and health providers could be counter vaccine hesitancy at the population level [428].

To meet the need to understand how determinants of health often influence women choices about healthy behaviors, we used data from the ongoing prospective "Mamma & Bambino" study, which enrols mother–child pairs from Catania, Italy. Firstly, we described the prevalence of dietary folate intake and its determinants among pregnant women, also evaluating folic acid supplement use. Moreover, we investigated the effect of folate intake and folic acid supplement use on neonatal outcomes in a subgroup of women who completed pregnancy. Next, we evaluated the association of social determinants and lifestyles with maternal dietary patterns. Finally, we evaluated vaccination adherence among mothers and their children, in order to uncover the main determinants involved in the vaccination choice and to underline the need for improving maternal knowledge about immunization programs.

5.2 Methods

5.2.1 Study design

In the current analyses, we used data from the Mamma & Bambino project. The ongoing mother-child cohort, settled in Catania (Italy), aims to understand the effects of social, environmental, behavioral, and molecular factors on mother-child health. Study design and protocols have been fully described elsewhere [298, 300] (and also at the website http://www.birthcohorts.net). From 2015, this cohort prospectively recruits pregnant women during their prenatal genetic counselling (median gestational age = 16 weeks) at the Azienda Ospedaliera Universitaria "Policlinico-Vittorio Emanuele" (Catania, Italy). We excluded women with plurality, pre-existing medical conditions, or pregnancy complications (i.e., autoimmune and/or chronic diseases, preeclampsia, gestational hypertension, and diabetes), as well as with intrauterine fetal death and congenital malformations. The study protocol was approved by the ethics committees of the involved institutions (Ethics Committees Catania 1 and 2; Protocol Numbers 227/BE and 275/BE, 186/EMPO and 83/EMPO) and performed according to the Declaration of Helsinki. All women were fully informed of the purpose and procedures and gave written informed consent.

5.2.2 Data collection

Information on mothers and their children are collected through telephone interviews at the delivery and with planned follow-ups at 1 and 2 years. At the recruitment, a structured questionnaire is administered by trained epidemiologists to collect socio-demographic and behavioral information. Educational level is categorized as low-medium (primary school, i.e., ≤8 years of school) and high education level (high school education or greater, i.e., >8 years of school). Employment status was classified as unemployment - including students and housewives - and employment - including part-

time and full-time. Women were also classified in those who lived alone or in couple, and in those who had children or not. With respect to smoking status, women were categorized as non-smokers, former smokers, or current smokers. Women reported their weight and height before pregnancy and pre-gestational BMI was calculated as weight in Kg divided by height in m^2 and categorized according to the WHO criteria [429]. Women were also asked about dietary restrictions or food intolerances.

Moreover, to evaluate vaccination adherence among mothers and their children, self-reported maternal vaccination status was collected during the follow-up interview at delivery, while children vaccination status was collected from their mothers during the follow-up interviews at 1 and 2 years. The structured questionnaire aimed to collect information referring maternal vaccination status, time of vaccination and grounds for refusal for the following vaccines: i) MMR; ii) HPV; iii) DTaP; iv) Varicella and v) Influenza. Moreover, we collected information referring children vaccination status, time of vaccination and any booster shots for the following vaccines: i) Hexavalent; ii) MMRV and iii) Pneumococcal.

5.2.3 Assessment of dietary data and dietary patterns

Dietary data - referred to the month preceding the recruitment - were obtained by a 95-item semi-quantitative Food Frequency Questionnaire (FFQ) [268, 296]. For each food item, women reported the frequency of consumption (i.e., twelve categories from "almost never" to "two or more times a day") and portion size (i.e., small, medium, and large using an indicative photograph atlas). Dietary data were further converted into monthly and daily food intakes, multiplying the frequency of consumption for the portion size (g). Dietary folate intake was calculated using the table of food composition of the US Department of Agriculture (http://ndb.nal.usda.gov/), adapted to typical Italian consumption. Inadequate folate intake was defined as an intake < 600 μg/day of dietary folate equivalents (DFEs) [430].

To identify dietary patterns, it has been suggested to use *a priori* and/or *a posteriori* method. The first one explores the data using predefined combinations of foods in a dietary index, such as MDS [431]. Thus, adherence to the MD was evaluated using the 9-item MDS, as previously described [302, 303]. For this reason, ranged from 0 (non-adherence) to 9 (perfect adherence), women were classified as low-adhered (MDS ≤ 3), medium-adhered (MDS = 4–6), or high-adhered (MDS > 6) to MD [304]. On the other hand, a *posteriori* approaches explore the available data post hoc by either cluster or factor analysis. In this way, the nutritional variables are reduced to a smaller number of variables [431]. We performed the PCA analysis to derive dietary patterns, followed by Varimax rotation on energy-adjusted food intakes [268, 278]. In particular, we determined the

number of dietary patterns according to scree plot examination, eigenvalues >2.0 and interpretability. Factor loadings with an absolute value ≥0.25 were used to define food groups that characterized each dietary pattern. Women received a factor score for each of the derived factor, that indicated their adherence to each dietary pattern. Factor scores were calculated as the sum of products between the energy-adjusted food group intakes and their factor loadings. Adherence to each dietary pattern was classified as low (1st tertile), medium (2nd tertile) and high (3rd tertile) adherence, based on the distribution of factor scores.

5.2.4 Use of folic acid supplements

Women were also asked to report the use of folic acid supplements, alone or with other multivitamin supplements, before and during the first trimester of pregnancy. Italian recommendation suggests that women who plan to become pregnant should use folic acid supplements for 4 weeks before and until 12 weeks after conception [432]. Accordingly, women were classified as i) non-users, if they did not use folic acid supplements; ii) insufficient users, if they did not take folic acid supplements as recommended, and iii) recommended users.

5.2.5 Neonatal outcomes

At birth, gestational age and neonatal anthropometric measures were assessed among women who completed singleton pregnancy. At recruitment, gestational age was assessed by ultrasound evaluation, in order to define preterm birth as spontaneous delivery before 37 weeks. According to sex-specific national reference charts, birth weight and length were used to assess birth weight for gestational as follows: small for gestational age (SGA, birth weight < 10th percentile for gestational age), adequate for gestational age (AGA), or large for gestational age (LGA, birth weight > 90th percentile for gestational age) [433].

5.2.6 Statistical analysis

Statistical analyses were performed using SPSS software version 26.0 (SPSS, Chicago, IL, USA). Descriptive statistics - using frequency or median and IQR - were used to characterize mothers. Frequency or median and range were used to describe the main characteristics of children. Prior to analysis, the normal distribution of continuous variables was checked using the Kolmogorov–Smirnov test. Next, continuous variables underlying skewed distribution were compared using the Mann-Whitney U test for comparisons between two groups, and the Kruskal-Wallis test for comparisons between three groups. Categorical variables were compared using Chi-squared test. Next, logistic regression models were used: i) to identify main determinants of inadequate folate intake and folic acid supplement use. The models included variables that were significantly

associated with inadequate folate intake or folic acid supplement use in the univariate analysis; ii) to investigate the association of population characteristics (i.e., age, education level, employment status, smoking, pre-gestational BMI, use of folic acid supplements and use of multi-vitamin and/or multi-mineral supplements) with high adherence to each dietary pattern (3rd tertile vs. 1st and 2nd tertiles); iii) to identify main determinants of vaccination choice.

Results were reported as OR and 95% CI. All statistical tests were two-sided, and p-values < 0.05 were considered statistically significant.

5.3 Results

5.3.1 Dietary folate intake among pregnant women

In the current analysis, we included pregnant women – recruited from 2015 to 2019 – with complete assessment of dietary folate intakes and information about folic acid supplementation. Particularly, the subgroup included 397 pregnant women, aged 15–50 years old (median = 37 years), 282 out of which completed pregnancy at the time of this study.

In general, the average dietary folate intake was 533.4 μg/day (median = 516.3 μg/day; range = 68.9–2633.5 μg/day). Moreover, near 65.0% of women (n = 257) did not meet the current recommendation of 600 μg/day during pregnancy. **Figure 1** shows women distribution according to dietary folate intake and the use of supplements. **Table 1** shows women characteristics according to their dietary folate intake. Women who did not follow dietary recommendation were more likely to be smokers (p = 0.028) and with higher pre-gestational BMI (p = 0.029) than their counterpart. With respect to dietary practises, we observed that women who did not meet dietary recommendation were more likely to follow dietary restrictions (p = 0.003) and less likely to adhere to MD (p < 0.001). Notably, after applying logistic regression analysis, we demonstrated that following dietary restrictions (OR = 2.180; 95%CI = 1.085–4.378; p = 0.029), being a smoker (OR = 1.457; 95%CI = 1.046–2.030; *p* = 0.026), and low adherence to MD (OR = 3.194; 95%CI = 1.958–5.210; p < 0.001) were the main determinants of inadequate folate intake. In the subsample of 282 women who completed pregnancy, we also observed a higher percentage of SGA and LGA among those with inadequate folate intake (p < 0.001).

5.3.2 Use of folic acid supplements

Next, we evaluated the use of folic acid supplements among women of the Mamma & Bambino cohort. As shown in **Figure 2A**, only 2.8% of women did not take supplements. In this scenario, 74.8% of women were classified as insufficient users before pregnancy, while 22.4% met the recommendations both before and during pregnancy. **Figure 2B** shows the comparison about supplement use between women with inadequate folate intake and those with adequate folate intake.

We noted higher proportions of non-users and recommended users among women with inadequate folate intake than their counterpart. By contrast, a higher proportion of insufficient users has been reported among women with adequate dietary folate intake. However, these differences were not statistically significant. With respect to neonatal outcomes, we did not observe differences in the proportion of preterm birth (p = 0.430) and inadequate birthweight for gestational age (p = 0.770).

5.3.3 Determinants of folic acid supplement use among women with inadequate folate intake

Among 257 women who did not meet the recommendation of dietary folate intake, we next identified the main determinants of folic acid supplement use. Univariate analysis demonstrated that women who did not take supplements were less educated (p < 0001) and with lower MDS (p = 0.047) than supplement users **(Table 2).** Notably, logistic regression analysis confirmed that women with low educational level were more likely to not us folic acid supplements than their more educated counterpart (OR = 5.574; 95%CI = 1.487–21.435; p = 0.012).

5.3.4 Use of supplements and neonatal outcomes

Among 184 women with inadequate folate intake, we investigated the relationship between folic acid supplement use and neonatal outcomes. In this subsample, median gestational duration was 39 weeks, with 9.8% of preterm deliveries. With respect to neonatal anthropometric measures, median values of birth weight and length were 3.25 Kg (range = 1.0–4.75 Kg) and 50.0 cm (range = 41–56 cm), respectively. Moreover, approximately 84.1% of newborns were AGA, 5.5% were SGA and 10.4% were LGA. Compared with women who met supplement recommendation, **Figure 3A** showed a higher proportion of SGA newborns among those who did not take supplements before pregnancy and those who did not take supplements before and during pregnancy (p = 0.009). By contrast, the proportion of AGA newborns was the highest among women who took supplements both before and during pregnancy. Moreover, as shown in **Figure 3B,** the proportion of preterm births was higher among non-users and insufficient users of folic acid supplements. However, these differences were not statistically significant.

5.3.5 Dietary patterns among pregnant women

In the current analysis, we included 332 pregnant women (median age = 37 years) with complete assessment of dietary data. In general, 19.3% of women were less-educated, while 41.9% of them were unemployed. Moreover, 20.3% of pregnant women were current smokers. According to pre-gestational BMI (median = 22.7 Kg/m^2), 6.9% of women were underweight, 66.5% were normal weight, 17.2% were overweight and 9.4% were obese. While nearly 95% of women used folic acid supplements, 39.1% of them used multi-vitamin or multi-mineral supplements.

With respect to dietary patterns, the two-derived ones – named "western" and "prudent" - can explain 15.6% of total variance among the 39 food groups. **Figure 4** shows factor loadings retained to characterize each dietary pattern: the "western" dietary pattern was characterized by high intake of red meat, fries, dipping sauces, salty snacks, and alcoholic drinks, while the "prudent" dietary pattern was characterized by high intake of potatoes, raw and cooked vegetables, legumes, rice, and soup.

5.3.6 The association of socio-demographic characteristics and lifestyle with dietary patterns

As shown in **Table 3**, women with high adherence to the western dietary pattern were younger (p < 0.001) and less-educated (p = 0.022), compared with women who reported low or medium adherence. By contrast, women with high adherence to the prudent dietary pattern were more likely to use folic acid supplementation (p = 0.033) and exhibited lower pre-gestational BMI (p = 0.019), compared with women who reported low or medium adherence. Logistic regression analysis confirmed that high adherence to the western dietary pattern was negatively associated with age (OR = 0.885; 95%CI = 0.829-0.945; p = 0.001) and positively with low educational level and smoking (OR = 1.617; 95%CI = 1.006-3.374; p = 0.047 and OR = 1.812; 95%CI= 1.004-3.269; p = 0.048, respectively) (**Table 4**). By contrast, we confirmed a negative association between pre-gestational BMI and the adherence to the prudent dietary pattern (OR = 0.920; 95%CI = 0.865-0.978; p = 0.007). However, no associations of socio-demographic characteristics and lifestyles with the prudent dietary pattern were evident.

5.3.7 Vaccination choice among women

From the "Mamma & Bambino" cohort, we included 220 mother-child pairs with complete assessment of sociodemographic characteristics and vaccination status. With respect to social characteristics, 86.4% of women reported a high education level, while 56.8% were employed.

Firstly, we investigated the vaccination choice of recruited women at delivery. **Figure 5A** shows that the percentage of vaccinated women was heterogeneous in relation to the type of vaccine, from 8.3% (i.e., vaccine against HPV) to 65.6% (i.e., vaccine against DTaP). We next assessed whether women were vaccinated in their childhood, prior or during pregnancy. As shown in **Figure 5B**, the two different times of vaccination indicated by women were the childhood and the period immediately prior to pregnancy, while none of women has planned vaccinations during pregnancy. For instance, among vaccinated women the majority were vaccinated for MMR (84.1%), DTaP (96.4%) and Varicella (90.5%) during childhood, while a higher proportion of women were vaccinated for HPV (72.2%) before pregnancy. Specifically, we noted that 62.6% women were vaccinated for Influenza in the period immediately prior to pregnancy (**Figure 5B**). However, as

shown in **Figure 5C**, not all women were vaccinated during their lifetime due to different grounds for refusal. Among women who have not undergone the MMR vaccine, the majority declared that they have already contracted the diseases, while only a small proportion was not fully informed about vaccination programs or afraid to get vaccinated (3.8% and 0.8%, respectively). Similarly, 95.4% of unvaccinated women for Varicella have already contracted it, while only a small proportion was not fully informed about vaccination programs or afraid to get vaccinated (3.5% and 1.2%, respectively). By contrast, the proportions of unvaccinated women for HPV (97.0%), DTaP (97.2%) and Influenza (97.0%) were higher among pregnant women who were not fully informed about vaccination programs.

5.3.8 Vaccination choice among children

We next explored the vaccination status among children, using data collected during the follow-up interviews. Median age of children at the last available follow-up interview ranged from 11 to 24 months. The percentages of vaccinated children were high for Hexavalent (99.5%), Pneumococcal (98.6%) and MMRV (84.1%) vaccines (**Figure 6A)**. Specifically, most children received the first dose of vaccine at a median of 3.0 months (2.0 – 12.0) for the Hexavalent and the Pneumococcal, and at a median of 13.0 month (12.0 – 36.0) for the MMRV, according to the recommended period (**Figure 6B**). We also noted high proportion of children who received the booster shots for Hexavalent (98.1%) and Pneumococcal (96.7%) vaccines (**Figure 6C**).

5.3.9 Association between age and vaccination choice

Afterwards, we aimed to investigate the relationships of age, educational level, and employment status with vaccination choice among mothers. As shown in **Figure 7**, unvaccinated women against MMR, HPV and DTaP were older if compared with their vaccinated counterparts. Notably, results from logistic regression analysis further confirmed that increasing age was associated with higher odds of not being vaccinated for MMR (OR = 1.12; 95%CI = 1.04-1.21; p = 0.003), HPV (OR = 1.18; 95%CI = 1.07-1.30; p = 0.001) and DTaP (OR = 1.09; 95%CI = 1.01-1.81; p = 0.044). Moreover, the positive association between age and no-vaccination for MMR (OR = 1.12; 95%CI = 1.04-1.21; p = 0.004), HPV (OR = 1.20; 95%CI = 1.08-1.33; p = 0.001) and DTaP (OR = 1.09; 95%CI = 1.01-1.18; p = 0.040) remained significant after adjusting for educational level and employment status. However, no association between social factors and vaccination choice was evident.

5.4 Discussion

As stated above, the determinants of health often influence our choices about healthy behaviors. Among lifestyles, nutrition in women of childbearing age plays a critical role in determining maternal and infant health. Degree of folate deficiency differs between and within countries, with higher prevalence in those without folic acid fortification of cereal-grain products [434, 435]. In line with previous studies [102, 268, 296, 297, 436], two out of three pregnant women from our cohort did not meet current recommendation.

Firstly, we aimed to uncover the main determinants of dietary folate intake in order to encourage public health strategies against folate deficiency. In particular, women with dietary restrictions and low adherence to MD were more likely to report inadequate folate intake, which in turn was higher among current smokers if compared with former or non-smoking women. During the periconceptional period, also the supplementation of folic acid represents an important strategy against adverse pregnancy outcomes [392]. However, the prevalence of folic acid supplementation remains often inadequate in several countries [395, 438], as demonstrated by previous study in which only 3% of Italian pregnant women used folic acid supplements as recommended [439]. Accordingly, we observed that ~75% of pregnant women did not take supplements as recommended (i.e., 4 weeks before conception until 8 weeks after). By contrast, in other European countries, prevalence of recommended users reached 50% [440].

Our analysis demonstrated that, among women with inadequate folate intake, those who reported low educational level were more likely to not use folic acid supplements than their counterpart. Among social factors, younger age [395], low income [441], educational level [438], and employment status [396] might affect the use of folic acid supplements, probably due to the reduced level of knowledge and awareness among the more disadvantaged groups [397]. For these reasons our findings, together with previous results, underline the need for identifying people at the highest risk for folate deficiency and, in turn, for increasing the prevalence of folic acid supplementation.

Additionally, we also evaluated the effects of folic acid supplements among women with inadequate folate intake. Our results showed a higher proportion of SGA births among women who did not take supplement before pregnancy and those who did not take supplement before and during pregnancy. Although the majority of studies demonstrated that the use of folic acid supplements before and during pregnancy reduced the risk of SGA [442-448], others reported an opposite [449, 450] or null effect [451, 452]. Thus, further research is needed to better understand the relationship between folic acid supplement use and the risk of adverse pregnancy outcomes.

In this scenario, we also described the effects of dietary patterns on maternal and neonatal health, and the main determinants involved in this relationship. To our knowledge, our study is the first evaluating the relationships between *a posteriori* dietary patterns (i.e., "prudent" and "western" dietary patterns), social determinants, and lifestyles in pregnant women from the Mediterranean area. Previous studies reported the association between high adherence to "western" dietary patterns and adverse maternal-neonatal outcomes, including GDM [453], PTB [195] and reduced birth length [195]. However, no associations with hypertension [454] and early foetal growth [455] have been established. In our cohort, young pregnant women with low educational level adhered more to the "western" dietary pattern, as previously demonstrated [398, 399]. Results from other cohorts showed that healthy dietary patterns were positively associated with education level and age [401, 402], which in turn were negatively associated with unhealthy dietary patterns [403].

In line with previous studies [404, 456], we also observed that current smokers tend to have unhealthier dietary habits than former- or non-smokers, probably due to a reduction of the senses of smell and an unhealthier lifestyle [457, 458]. Overall, our analysis confirmed the importance of exploring the relationships of dietary patterns, socio-demographic factors, and lifestyles during the peri-conceptional period.

With respect to the determinants of health, they are involved in several women choices – apart from food choices - during their reproductive age. For instance, public health increasingly seeks to focus on childhood and women of childbearing age [408, 409]. Focusing on the Italian scenario, we aimed to assess the vaccination choice among mother-child pairs enrolled in "Mamma & Bambino" cohort. Firstly, we evaluated the vaccination status of recruited women at delivery, reporting higher proportion of vaccinated women for DTaP (65.6%), than those vaccinated for MMR (38.1%), Influenza (20.1%), and Varicella (19.4%). Vaccinations against the above-mentioned diseases can be considered useful for promoting health among women of childbearing age [416, 459].

Moreover, in line with PNPV content, HPV vaccination should be recommended for adolescents aged 12 to 13 years old. However, we reported that only 8.3% of women were vaccinated for HPV, and nearly 73% of them before pregnancy. By contrast, among vaccinated women, 84.1%, 96.4% and 90.5% were vaccinated for MMR, DTaP and Varicella during childhood. With respect to Influenza, we reported that 62.6% women were vaccinated in the period immediately prior to pregnancy. In spite of these findings, we observed that no women have planned vaccinations during pregnancy. However, it is well known that infections during pregnancy have related to increased risk of adverse pregnancy outcomes, longer hospitalization periods, and higher mortality rate [414]. In Italy, women should be vaccinated for DTaP between the 27th and the 36th week of every

pregnancy, regardless of prior DTaP history. Additionally, influenza vaccination is recommended for all pregnant women in any trimester [459]. In line with our findings, however, vaccination coverage among pregnant women remains steadily very low worldwide [417, 418]. In this context, the so-called "vaccine hesitancy" is a widespread behavior influenced by several factors, including: i) confidence, as low level of trust in vaccine; ii) complacency, as low awareness of vaccine usefulness and safety; iii) convenience, as lack of accessible healthcare system [426, 460]. For instance, in our study none of women reported to be informed about the recommended vaccines during the pregnancy, suggesting the need to improve women's level of knowledge during the maternal counselling. Rates of low coverage are more determined by the complacency rather than socio-demographic and/or socio-economic factors [461]. However, social determinants can vary among countries of different income levels, supporting the idea that their effects on vaccination choice should be considered for designing appropriate interventions [427]. We noted that unvaccinated women against MMR, HPV and DTaP were older if compared with their vaccinated counterparts. Notably, also after adjusting for educational level and employment status, logistic regression model showed that increasing age was associated with higher odds of not being vaccinated for MMR, HPV and DTaP. By contrast, no association between these social determinants and vaccination choice was evident. Our findings could be explained by the fact that women were more likely to get vaccinated during childhood, and younger women have benefited from better vaccination programs compared to the older ones. Thus, we supposed that age is not to be considered as direct determinant of vaccination choice, but it should suggest the need to improve the quality of vaccination offering.

Our findings should be interpreted with cautions due to some limitations, including the cross-sectional design, which does not allow us to demonstrate the causality of the relationships. With respect to dietary folate intake, low sample size in the subgroups did not allow us to adjust for potential confounders. Moreover, we cannot exclude bias from residual unknown or unmeasured factors that might affect social determinants, lifestyles, and maternal diet. Moreover, future studies should evaluate folate status by measuring folate blood concentration and reviewing the size and morphology of blood cells. Secondly, data relied on self-reported interviews, which cannot completely exclude reporting errors. Furthermore, dietary assessment was referred to the first month of gestation and we were not able to account for changes in maternal dietary habits during pregnancy.

In conclusion, further research will be also directed to uncover the complex relationship between healthy/unhealthy lifestyles and adverse outcomes in both mother and child, with a focus on the

previously proposed role of gene-diet interaction [296, 298, 462] and epigenetic mechanisms [55, 102, 297, 463-465]. Moreover, our results raise the need of strategies for the promotion of healthy dietary habits among women of reproductive age, as well as for improving mothers' knowledge about vaccines and their related preventable diseases.

Table 1. Characteristics of pregnant women according to folate intake

Characteristics	Inadequate Folate Intake (n = 257)	Adequate Folate Intake (n = 140)	*p*-Value
Age, years	37.0 (5.0)	37.5 (4.0)	0.375
Educational level			
Low	19.3%	18.7%	
Medium	51.4%	43.6%	0.214
High	29.3%	37.7%	
Employed	56.4%	58.6%	0.679
Living in couple	92.3%	93.8%	0.973
Having children	68.5%	68.6%	0.985
Food intolerance (% yes)	16.7%	10.0%	0.150
Dietary restriction (% yes)	19.8%	8.6%	**0.003**
Smoking status			
Non-smoker	55.0%	57.6%	
Former smoker	15.7%	23.5%	**0.028**
Smoker	29.3%	18.8%	
Pregestational BMI, Kg/m^2	23.2 (4.9)	22.1 (5.4)	**0.029**
Pregestational BMI categories			
Underweight	5.5%	8.6%	
Normal weight	63.7%	66.4%	0.353
Overweight	20.3%	13.6%	
Obese	10.2%	11.4%	
MDS	4 (2)	5 (2)	**<0.001**
Adherence to MD			
Low	45.1%	20.0%	
Medium	52.9%	63.6%	**<0.001**
High	1.9%	16.4%	
Preterm birth[c]	8.4%	7.2%	0.722
Birthweight for gestational age			
SGA	13.7%	4.8%	
AGA	67.4%%	88.4%	**<0.001**
LGA	18.9%	6.9%	

This table is adapted from Barchitta et al., Int. J. Environ. Res. Public Health 2020

Table 2. Characteristics of folate deficient women according to supplement use

Characteristics	Non-Users (n = 9)	Insufficient Users (n = 188)	Recommended Users (n = 60)	*p*-Value
Age, years	38.0 (8.0)	37.0 (4.0)	37.0 (4.0))	0.884
Educational level				
Low	44.4%	22.9%	1.7%	
Medium	55.6%	44.1%	40.0%	**<0.001**
High	0%	33.0%	58.3%	
Employed	55.6%	52.7%	68.3%	0.103
Living in couple	90.1%	91.2%	94.2	0.878
Having children	66.7%	70.2%	63.3%	0.603
Food intolerance (% yes)	0%	17.0%	18.3%	0.264
Dietary restriction (% yes)	33.3%	18.6%	21.7%	0.514
Smoking status				
Non-smoker	55.6%	55.1%	66.1%	
Former smoker	33.3%	21.9%	27.1%	0.078
Smoker	11.1%	23.0%	6.8%	
Pregestational BMI, Kg/m²	22.7 (3.2)	23.6 (5.3)	22.2 (3.9)	0.256
Pregestational BMI categories				
Underweight	0%	5.9%	5.0%	
Normal weight	66.7%	60.4%	73.3%	0.575
Overweight	33.3%	20.9%	16.7%	
Obese	0%	12.3%	5.0%	
MDS	3 (3)	4 (2)	4 (2)	**0.047**
Adherence to MD				
Low	66.7%	46.8%	36.7%	
Medium	33.3%	51.1%	61.7%	0.424
High	0%	2.1%	1.7%	

This table is adapted from Barchitta et al., Int. J. Environ. Res. Public Health 2020

Table 3. Characteristics of study population by adherence to dietary patterns

Population characteristics	Western dietary pattern				Prudent dietary pattern			
	1°tertile	2°tertile	3°tertile	p-value	1°tertile	2°tertile	3°tertile	p-value
Age, years	38.0 (4.0)	38.0 (4.0)	36.0 4.0)	**<0.001**	38.0 (4.0)	37.0 (4.0)	37.0 (4.0)	0.675
Gestational age, weeks	14.7 (4.0)	16.0 (5.0)	16.0 (5.0)	0.777	16.0 (2)	16.0 (4.0)	15.0 (5.0)	**0.001**
Educational level, % of low-medium	20.0%	11.7%	26.4%	**0.022**	22.7%	19.8%	15.5%	0.389
Employment status, % of unemployed	40.9%	36.9%	47.3%	0.291	42.7%	39.6%	42.7%	0.865
Smoking status, % of current smokers	20.9%	18.0%	22.2%	0.731	20.9%	18.0%	22.2%	0.731
Use of folic acid supplements, % of users	94.5%	95.5%	94.5%	0.972	91.8%	93.6%	99.1%	**0.033**
Use of multi-vitamins or multi-minerals, % of users	40.7%	35.1%	40.9%	0.606	38.5%	32.4%	45.9%	0.123
Pre-gestational BMI, kg/m^{2*}	22.5 (4.9)	22.7 (5.1)	22.9 (5.2)	0.704	23.0 (5.1)	23.2 (4.7)	21.9 (4.8)	**0.019**
Pre-gestational BMI								
Underweight	7.3%	7.2%	6.4%		5.5%	8.1%	7.3%	
Normal weight	63.6%	69.4%	66.1%	0.687	64.5%	65.8%	68.6%	0.575
Overweight	17.3%	13.5%	21.1%		20.9%	13.5%	17.4%	
Obese	11.8%	9.9%	6.4%		9.1%	12.6%	6.4%	

This table is adapted from Maugeri et al., Ann Ig 2019

Table 4. Logistic regression analysis of the association between population characteristics and high adherence to dietary patterns

Population characteristics	Western Dietary Pattern			Prudent Dietary Pattern		
	OR	95%CI	p-value	OR	95%CI	p-value
Age (continuous)	0.885	0.829-0.945	**0.001**	1.011	0.951-1.076	0.718
Pre-gestational BMI (continuous)	1.002	0.950-1.056	0.947	0.920	0.865-0.978	**0.007**
Educational level (low-medium)	1.617	1.006-3.374	**0.047**	0.700	0.346-1.417	0.322
Employment status (unemployed)	1.066	0.635-1.792	0.808	1.358	0.812-2.271	0.243
Smoking status (current smokers)	1.812	1.004-3.269	**0.048**	1.224	0.662-2.263	0.519
Use of folic acid supplements (users)	0.917	0.422-1.996	0.828	0.351	0.095-1.302	0.118
Use of multivitamin supplements (users)	0.782	0.472-1.296	0.340	0.750	0.460-1.222	0.248

This table is adapted from Maugeri et al., Ann Ig 2019

Figure 1. The distribution of women according to dietary folate intake and use of supplements
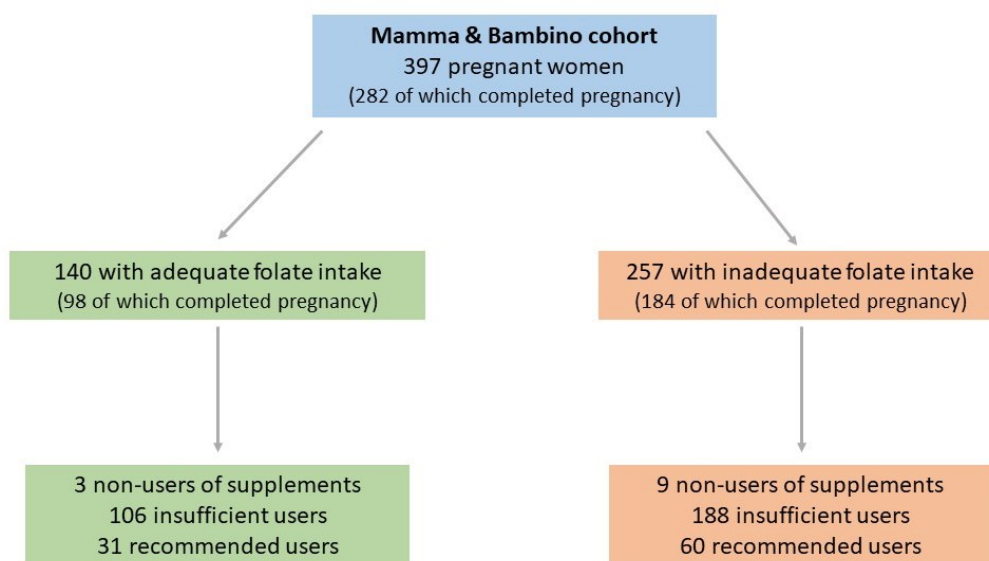
This figure is adapted from Barchitta et al., Int. J. Environ. Res. Public Health 2020

Figure 2. Use of folic acid supplements among pregnant women
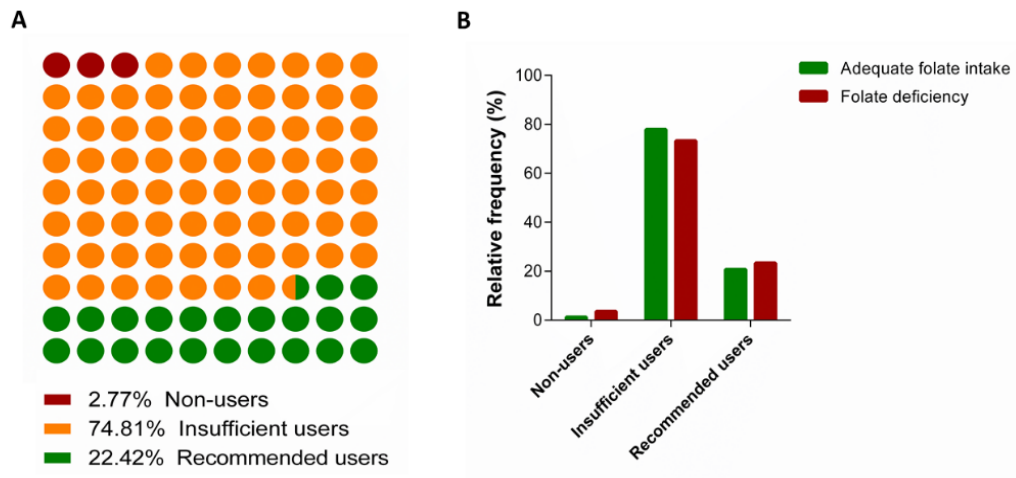
(A) Panel A shows proportions of non-users, insufficient users, and recommended users among the overall cohort. **(B)** Panel B shows the categories of folic acid supplement according to folate intake.

This figure is adapted from Barchitta et al., Int. J. Environ. Res. Public Health 2020

Figure 3. Neonatal adverse outcomes and folic acid supplement use among women with folate deficiency
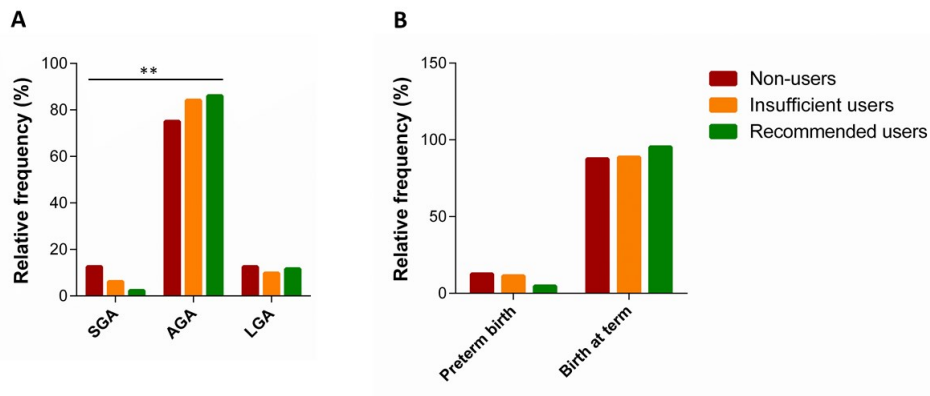
(A) Panel A shows the distribution of small for gestational age (SGA), adequate for gestational age (AGA) and large for gestational age (LGA) infants. **(B)** Panel B shows the distribution of preterm and at term birth. ** p-value <0.01.

This figure is adapted from Barchitta et al., Int. J. Environ. Res. Public Health 2020

Figure 4. Radar graph of factor loadings characterizing each dietary pattern
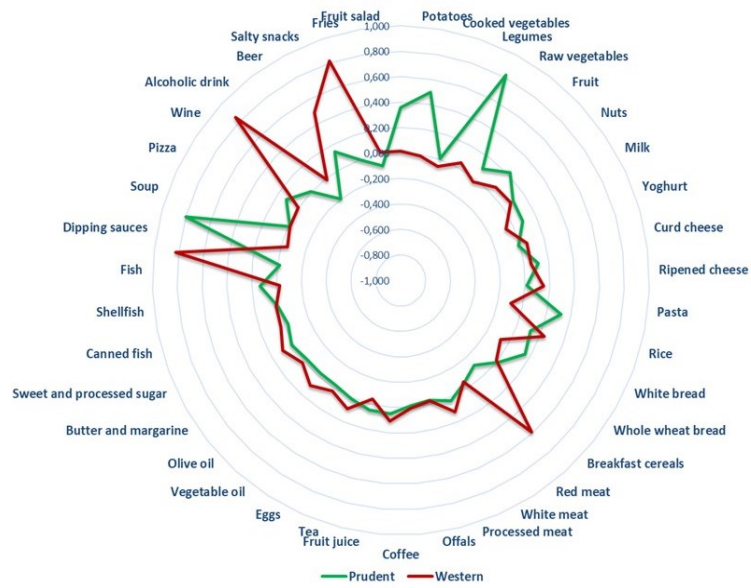
Red line represents the distribution of factor loadings related to the western dietary pattern. Green line represents the distribution of factor loadings related to the prudent dietary pattern.

This figure is adapted from Maugeri et al., Ann Ig 2019

Figure 5. Vaccination choice among pregnant women
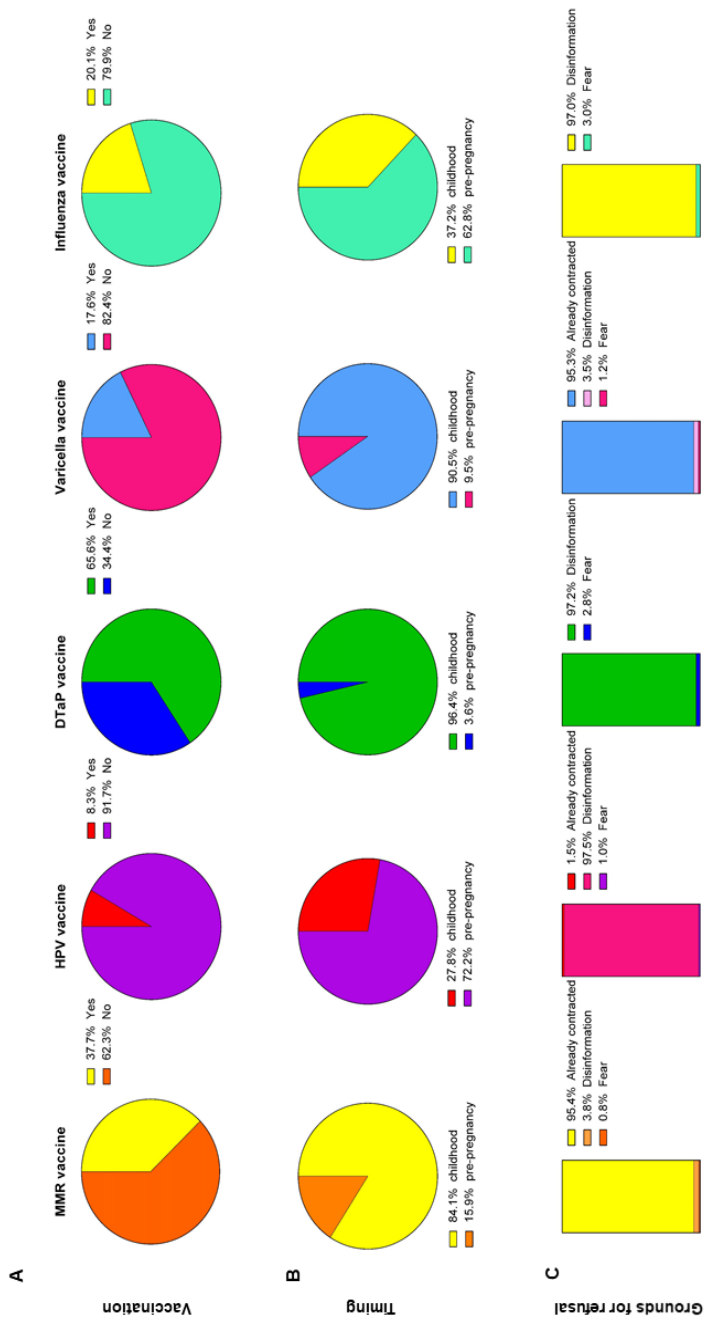
This panel shows (A) the proportion of vaccinated women for MMR, HPV, DTaP, Varicella, and Influenza; (B) the proportion of women vaccinated in the childhood or during the pre-pregnancy period; (C) and grounds of refusal among non-vaccinated women.

This figure is adapted from Barchitta et al., Vaccines 2021

Figure 6. Vaccination choice among children
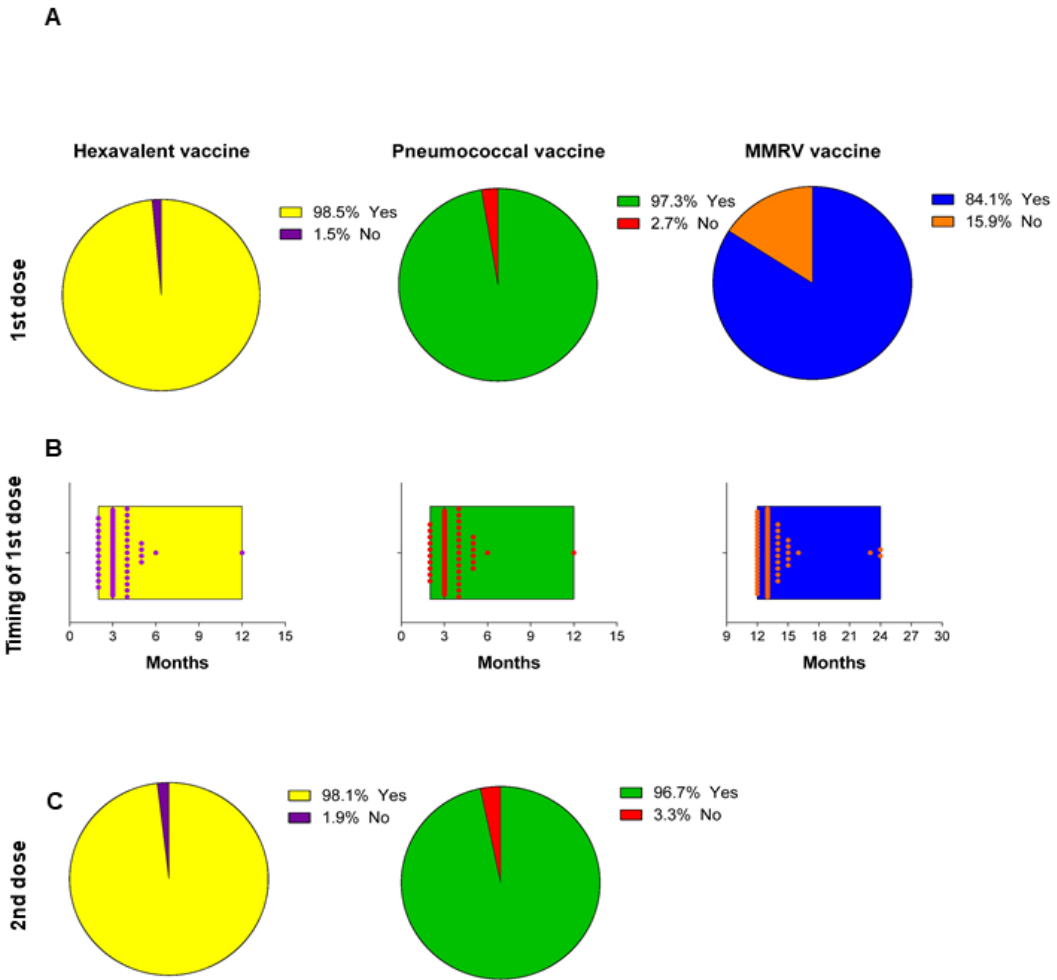
This panel shows (A) the proportion of children who received the first dose of Hexavalent, Pneumococcal, and MMRV vaccines; (B) the timing of receiving the first dose; (C) the proportion of children who received the second dose.

This figure is adapted from Barchitta et al., Vaccines 2021

Figure 7. Comparison of age between vaccinated and non-vaccinated women
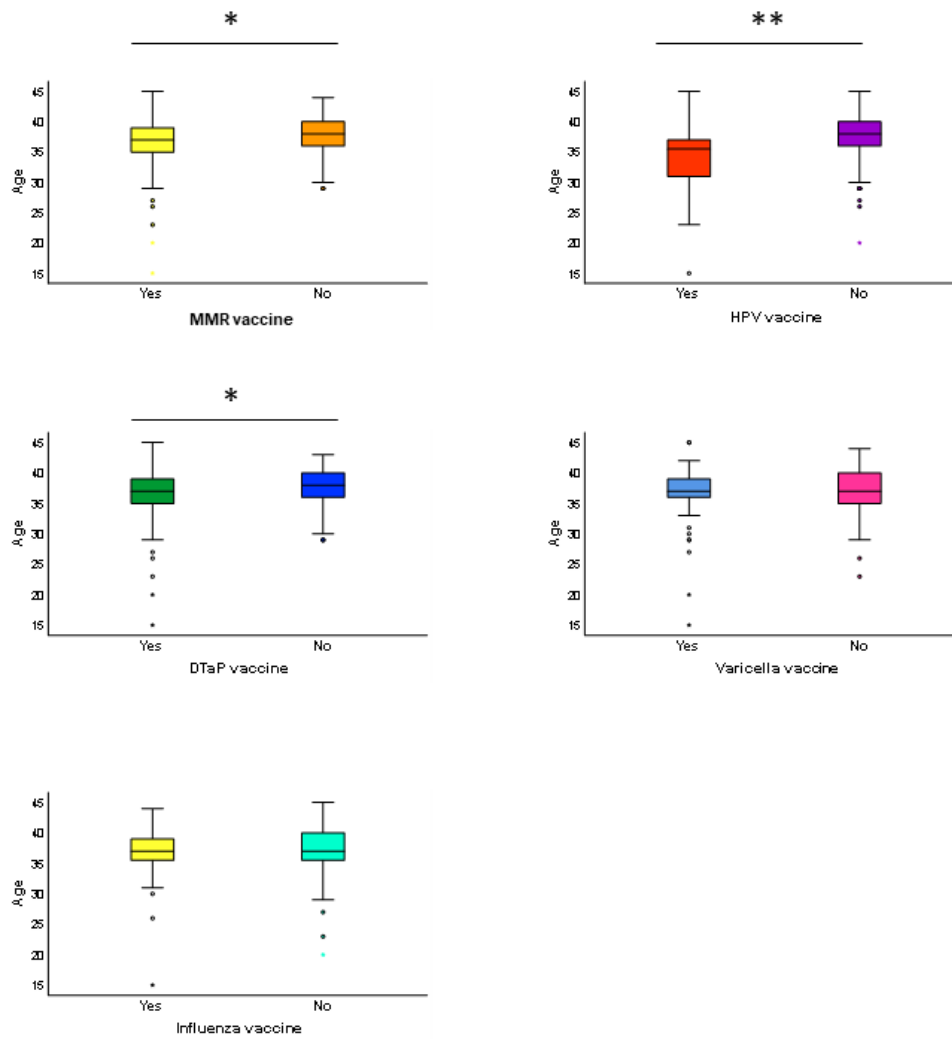
These box plots show the distribution of age between vaccinated and non-vaccinated women. Age was compared using the Mann-Whitney U test. * p-value <0.05; ** p-value<0.01.

This figure is adapted from Barchitta et al., Vaccines 2021

6. The effect of dietary habits on pregnancy outcomes

6.1 Background

Despite great efforts against the obesity epidemic worldwide, the WHO reported that more than two billion adults were overweight or obese in 2014 [466]. The first thousand days of life - from conception to the end of the second year - are a critical period for the obesity development. In early pregnancy, in fact, excessive GWG is a risk factor for increased birth weight, which is associated with higher risk of obesity both in childhood and adulthood [467, 468]. Interestingly, there has been found a link between low birth weight and higher body fat percentage/abdominal obesity in adolescents [469]. In general, excessive or reduced GWG may affect both maternal and new-born health. Indeed, excessive GWG has been associated with an increased risk of hypertension [172], diabetes [173], caesarean section [174], postpartum weight retention [175] and obesity [176]. In newborns, inadequate GWG has been associated with neonatal and infant mortality, preterm birth, and fetal growth retardation [470]. Promotion programs, addressing lifestyle behaviors and dietary habits, should be encouraged to prevent weight loss and to control excessive weight gain [471]. Although evidence is currently limited, these interventions should be promoted during the periconceptional period. The study of dietary patterns during pregnancy represents one of the suitable approaches to assess the effect of diet on maternal and neonatal health. Previous studies evaluated the association of dietary patterns with pre-pregnancy BMI and/or GWG [472-475], but none of them has focused on Southern Europe populations. Since dietary patterns differ across countries, it is crucial to identify country-specific dietary patterns that may be associated with maternal and neonatal outcomes. With this in mind, the ongoing prospective "Mamma & Bambino" study aims to explore the effect of preconception, perinatal and early life exposure on maternal and neonatal health. We supposed that the adherence to healthy dietary habits during the early phase of pregnancy might improve adequate GWG. Thus, we used data from the Mamma & Bambino cohort to identify major maternal dietary patterns and to investigate their association with pre-pregnancy BMI and GWG.

6.2 Methods

6.2.1 Study design

Study design and protocols of the "Mamma & Bambino" study are described in the paragraph 5.2.1. For this analysis, we included pregnant women who completed their pregnancy, while all the mothers with plurality, pre-existing diseases, pregnancy complications, pre-term induced delivery or caesarean section, intrauterine foetal death and congenital malformations were excluded.

6.2.2 Definitions of pre-pregnancy body mass index and gestational weight gain

At recruitment, we collected information on maternal pre-pregnancy weight and height, in order to calculate pre-pregnancy BMI as weight in kilograms divided by height in meters squared and to classify it according to WHO criteria [429]. At delivery, maternal weight, length of gestation and anthropometric measures (i.e., birth weight and birth length of newborns) were collected from clinical records. Total GWG was calculated by subtracting the self-reported pre-pregnancy weight from the weight at delivery. According to the IOM guidelines, we defined adequate GWG as 12.5-18 kg for underweight, 11.5-16 kg for normal weight, 7-11.5 kg for overweight, and 5-9 kg for obese women [476].

6.2.3. Assessment of Dietary patterns

Dietary assessment was performed as described in the paragraph 5.2.3. In particular dietary assessment was referred to the early phase of pregnancy (i.e., from the beginning to the 16 week of gestation). Diet information - obtained from the FFQ - were converted into monthly and daily food intakes, multiplying the frequency of consumption for the portion size (g). Total energy intake was calculated using the table of food composition of the US Department of Agriculture (http://ndb.nal.usda.gov/), adapted to typical Italian food consumption. Food intakes were adjusted for total energy intake using the residual method [301]. Principal component analysis (PCA) was *a posteriori* method used to extract dietary patterns, as described elsewhere [268, 278]. We determined the number of retained dietary patterns as described in the paragraph 5.2.3, according to scree plot examination, eigenvalues >2.0, and interpretability. Factor loadings with an absolute value ≥0.2 were retained to define food groups characterizing each dietary pattern. For each dietary pattern, factor scores were calculated as the sum of products between observed energy-adjusted food group intakes and their factor loadings. According to factor loading distribution, adherence to each dietary pattern was categorized as i) low (1st tertile of factor loading), ii) medium (2nd tertile), or iii) high (3rd tertile). To corroborate internal reproducibility, factor analysis was separately replicated in two randomly selected subgroups (n = 100), using the same approach as for the main analysis. Next, we tested the correlation of factors scores between the overall sample and two randomly selected subgroups. Cohen's weighted Kappa were applied to assess the correct ranking ability by comparing tertile distribution in the overall cohort and in two randomly selected subgroups.

6.2.5. Statistical analysis

Statistical analyses were performed using SPSS software (version 22.0, SPSS, Chicago, IL, USA). Descriptive statistics were applied to characterize the study population using frequency or median

and IQR. Prior to analysis, the normal distribution of all variables was checked using the Kolmogorov-Smirnov test. Continuous variables underlying skewed distribution were compared using the Kruskal-Wallis test for comparisons between three or more groups. Categorical variables were compared using Chi-squared test. Linear regression models were performed to explore the association of dietary patterns with pre-pregnancy BMI and GWG, using the first tertile as reference. For each dietary pattern, we also investigated the association of one-standard deviation increase in factor score with BMI and GWG. For pre-pregnancy BMI, the model was adjusted for potential confounders (i.e., age, educational level, employment status, smoking, total energy intake, and gestational age at recruitment). We also tested for interaction between gestational age at recruitment and adherence to dietary pattern on pregestational BMI. For GWG, linear regression models were applied on the overall population and stratified by pre-pregnancy BMI categories. The model was adjusted for potential variables associated with GWG, as age, length of gestation, birth weight, educational level, working status, smoking, parity, newborn sex, and total energy intake. All statistical tests were two-sided, and p values < 0.05 were considered statistically significant.

6.3 Results

6.3.1 Characteristics of the study population

Overall, we included 232 women who completed singleton pregnancy, with a median age of 37 years and enrolled at a median gestational age of 16 weeks. Based on pre-gestational BMI, we classified women as: underweight (8.1%), normal weight (66.2%), overweight (16.7%) and obese (9%). According to pre-gestational BMI and GWG, we noted that 31.2% of women exhibited a reduced GWG, while 27.4% of them an excessive GWG. **Table 1** shows maternal characteristics according to GWG categories. In general, we observed U-shaped distributions of pre-pregnancy weight (p < 0.001) and (BMI p = 0.002) across GWG categories. Thus, women with adequate GWG exhibited lower pre-pregnancy weight and BMI than those with reduced or excessive GWG. Moreover, maternal weight at delivery was higher in women with excessive GWG (p < 0.001), as well as newborn birth weight (p = 0.039).

6.3.2. Dietary patterns and pre-pregnancy body mass index

We first derived two dietary patterns which explain 15.55% of total variance among 39 food groups. The analysis of dietary patterns in two randomly selected subgroups yielded similar results. Indeed, factor scores obtained in the two subgroups well correlated with those obtained in the entire cohort, with a Spearman's correlation coefficient from 0.8 to 0.9. Moreover, we observed almost perfect agreement in the ranking ability between the whole cohort and the selected subgroups, with a weighted kappa from 0.81 to 1.0.

In the whole cohort, we derived two dietary patterns: the "western" dietary pattern - characterized by high intake of red meat, fries, dipping sauces, salty snacks, and alcoholic drinks- and the "prudent" dietary patterns – characterized by high intake of boiled potatoes, cooked vegetables, legumes, pizza and soup. As shown in **Table 2,** higher adherence to the western dietary pattern was associated with decreasing age ($p < 0.001$) and percentage of high-educated women ($p = 0.022$). With respect to the prudent dietary pattern, pre-pregnancy weight and BMI decreased across tertiles ($p = 0.043$ and $p = 0.019$, respectively). As shown in **Table 3,** women who highly adhered to the prudent dietary pattern were less likely to be overweight or obese ($p = 0.007$). Linear regression analysis further confirmed the negative association between pre-pregnancy BMI and the adherence to the prudent dietary pattern ($\beta = -0.631$; SE $= 0.318$; p-trend $= 0.038$), after adjusting for potential confounders (i.e., age, educational level, employment status, smoking, total energy intake and gestational age at recruitment). Particularly, women who highly adhered to the prudent dietary pattern exhibited a ≈1.4 point reduced pre-pregnancy BMI, compared to those with low adherence ($\beta = -1.347$; SE $= 0.598$; $p = 0.024$). No association between pre-pregnancy BMI and the adherence to the western dietary pattern, nor interaction with gestational age at recruitment were evident.

6.3.3. Dietary patterns and gestational weight gain

Although no associations between dietary patterns and GWG were evident the univariate analysis, we applied a linear regression model adjusting for age, weight at delivery, gestational duration, educational level, working, smoking, parity, new-born sex and total energy intake (**Table 4**). In general, we observed a positive trend of GWG across tertiles of the western dietary pattern ($\beta = -1.217$; SE $= 0.487$; $p = 0.013$). A similar trend was observed in obese ($\beta = 7.363$; SE $= 1.808$; $p = 0.005$), with women in the 3rd tertile exhibiting a ≈13.7 Kg increased GWG compared to those in the 1st tertile ($\beta = 13.701$; SE $= 0.887$; $p = 0.041$). By contrast, no association between GWG and adherence to the prudent dietary pattern was found in the overall population. However, we demonstrated an opposite effect across BMI categories. Specifically, we found a positive trend of GWG across tertiles of the prudent dietary pattern among underweight ($\beta = 4.127$; SE $= 1.722$; $p = 0.048$), while a negative trend was evident in overweight ($\beta = -4.209$; SE $= 1.635$; $p = 0.016$) and obese ($\beta = -7.356$; SE $= 2.304$; $p = 0.031$). Particularly, overweight women in the 2nd and 3rd tertiles exhibited a ≈8.0 and ≈9.8 Kg reduced GWG, respectively, compared to those in the 1st tertile ($\beta = -7.975$; SE $= 2.672$; $p = 0.010$; and $\beta = -9.736$; SE $= 4.302$; $p = 0.037$).

6.4 Discussions

To our knowledge, the present study is the first to explore the association of maternal dietary patterns with pre-pregnancy BMI and GWG in Southern Europe. We demonstrated that, in the early

phase of pregnancy, "healthy" dietary habits might promote adequate GWG according to pre-gestational BMI. Interestingly, we first demonstrated that women with high adherence to the prudent dietary pattern reported ≈1.3 point reduced pre-pregnancy BMI than those with low adherence. Moreover, we observed a dual opposite effect of the prudent dietary pattern on GWG across BMI categories. In fact, the adherence to this pattern was positively associated with GWG among underweight, and negatively among overweight and obese. Although some studies did not demonstrate the association between healthy diet and GWG [472, 473], results from the prospective Norwegian Mother and Child Cohort Study showed the association between high adherence to the New Nordic Diet - rich in fruits and vegetables - and lower risk of excessive GWG among underweight [475]. These findings and ours might be attributable to the healthy effect of fruits and vegetables on maternal health and fetal growth.

For instance, US pregnant women who consumed more than 3 servings/day of fruits and vegetables reported significantly lower GWG than those who consumed <3servings/day [477]. Similarly, a cross-sectional analysis within the NHANES project showed greater odds of exceeding GWG recommendations in pregnant women who consumed low amounts of total vegetables [478]. The adherence to a prudent dietary pattern might provide a balanced intake of energy, macro- and micronutrients [477], also promoting an adequate GWG independent of pre-pregnancy BMI. Moreover, women who adhered to a prudent diet were more likely to have healthy habits, which in turn contributed to adequate GWG independent of pre-gestational BMI [480]. In this scenario, our study demonstrated that the adherence to a prudent dietary pattern improved pre-pregnancy BMI and GWG across BMI categories, which in turn have been previously associated with increased birth weight [483]. Of note, our analysis also confirmed that birth weight was higher in infants born from mothers with excessive GWG, compared to those with adequate or reduced GWG.

With respect to the western dietary pattern, pregnant women with high adherence were more likely to be younger and less educated. In line, previous studies demonstrated that older women are more likely to have a healthy lifestyle in general [475] and that healthy food choices are a reflection of an overall healthy behavior [484, 485]. Contrary to the prudent dietary pattern, associations between the adherence to the western dietary pattern and pre-gestational BMI were not found. For this reason, it would be important to develop recommendations on healthy foods to be consumed (e.g., fruit and vegetables), instead of on foods to be avoided during pregnancy. However, among obese women, the high adherence to the western dietary pattern led to a ≈13.7 Kg increased GWG compared to the low adherence. Although few studies investigated the association between maternal unhealthy diet and GWG [472-475, 486, 487], our findings are consistent with previous evidence

demonstrating that unhealthy dietary patterns were associated with higher GWG [472, 473]. A plausible explanation lies in the fact that unhealthy and energy-dense foods (e.g. fries, dipping sauces, salty snacks and alcoholic drinks) could increase total energy intake, confirming the positive association between energy intake and GWG [488].

Our study had some limitations. We cannot assess the causality of the association between dietary patterns and pre-pregnancy BMI in our cross-sectional study. Since we calculated GWG at delivery, instead of at each trimester, we did not investigate the effect of dietary patterns on GWG trajectories. Self-reported weight assessment restricted the power of our work, and reporting errors of weight cannot be completely excluded. Moreover, further research should evaluate components of GWG, including total body water, fat-free mass, and fat mass. Furthermore, due to the limited sample size in underweight and obese groups, we were not able to investigate the effect of adherence to dietary patterns on adequate GWG, assessed as dichotomous outcomes.

With respect to dietary assessment, the PCA-derived dietary patterns only explained 15.55% of total variance among food groups. However, we used well-established criteria to derive dietary patterns, consistent with those reported by previous studies [268, 278]. Furthermore, since dietary assessment was referred to the early phase of pregnancy, we were not able to account for changes in maternal dietary pattern, especially those related to alcoholic drinks [494]. Accordingly, previous studies did not take into account the alcohol component in their dietary assessment [474]. However, in our cohort, the western dietary pattern was characterized also by the intake of alcoholic drinks, underlining that some pregnant women did not follow the recommendations on avoiding alcohol intake during pregnancy. Despite an increased energy intake, however, dietary patterns may not change largely during pregnancy [492, 493]. Finally, we cannot exclude the possibility of bias from residual confounders that might affect both maternal dietary patterns and GWG (e.g., physical activity, sedentary lifestyle, unmeasured socio-demographic factors, psychosocial conditions, illness, and vomiting).

Thus, we demonstrated that a western dietary pattern did not affect pre-gestational BMI but increased GWG, especially in obese women. In contrast, the prudent dietary pattern ameliorated pre-gestational BMI and GWG, with different effect across BMI categories. Indeed, the adherence to this pattern was positively associated with GWG among underweight, and negatively among overweight and obese. Thus, the promotion of healthy dietary habits, even during the periconceptional period, represents a potential strategy to maintain an adequate weight independent of pre-gestational BMI.

Table 1. Characteristics of women from the Mamma & Bambino cohort (n=232) according to gestational weight gain categories

Characteristics	Reduced GWG (n=73)	Adequate GWG (n=95)	Excessive GWG (n=64)	p-value
Age	37.0 (4.0)	38.0 (5.0)	37.0 (4.0)	0.546
Educational level (low-medium %)	15.1%	13.4%	17.2%	0.804
Working (%)	58.9%	63.9%	54.7%	0.495
Smoking (%)	15.1%	17.7%	22.2%	0.553
Pre-pregnancy weight	61.0 (13.3)	59.0 (13.0)	64.0 (17.8)	<0.001
Pre-pregnancy BMI	23.1 (4.4)	21.6 (3.8)	24.2 (6.5)	0.002
Pre-pregnancy BMI categories				
Underweight	6.8%	8.2%	9.4%	0.001
Normal weight	68.5%	77.3%	46.9%	
Overweight	15.1%	7.2%	32.8%	
Obese	9.6%	7.2%	10.9%	
Weight at delivery	68.0 (10.0)	72.0 (14.0)	82.0 (16.7)	<0.001
Length of gestation	39.0 (2.0)	39.0 (2.0)	39.2 (2.0)	0.701
Birth weight	3.2 (0.6)	3.2 (0.6)	3.3 (0.6)	0.039
Birth length	50.0 (2.0)	50.0 (2.0)	50.0 (2.0)	0.286

This table is adapted from Maugeri et al., Nutrients 2019

Table 2. Characteristics of women from the Mamma & Bambino cohort (n=232) according to adherence to western dietary pattern

Characteristics	1st tertile	2nd tertile	3rd tertile	p-value
Age	38.0 (5.0)	38.0 (4.0)	36.0 (3.0)	<0.001
Gestational age	16.0 (3.0)	16.0 (4.0)	16.0 (2.0)	0.777
Educational level (low-medium %)	20.0%	11.7%	26.4%	0.022
Working (%)	59.1%	63.1%	52.7%	0.291
Smoking (%)	17.4%	16.2%	27.5%	0.074
Use of folic acid supplements (%)	95.1%	94.7%	94.7%	0.949
Use of other multivitamin supplements (%)	44.4%	33.3%	42.1%	0.334
Pre-pregnancy weight	60.0 (14.2)	62.5 (15.0)	60.0 (15.0)	0.923
Pre-pregnancy BMI	22.3 (4.4)	22.7 (5.0)	22.8 (5.5)	0.704
Pre-pregnancy BMI categories				
Underweight	7.3%	7.2%	6.4%	0.687
Normal weight	63.6%	69.4%	66.1%	
Overweight	17.3%	13.5%	21.1%	
Obese	11.8%	9.9%	6.4%	
Weight at delivery	71.5 (16.5)	74.0 (16.0)	74.0 (14.0)	0.636
Length of gestation	39.0 (2.0)	39.0 (2.0)	39.0 (2.0)	0.976
Birth weight	3.2 (0.6)	3.2 (0.7)	3.3 (0.5)	0.800
Length	50.0 (2.0)	50.0 (1.0)	50.0 (2.0)	0.391
GWG	11.5 (7.2)	13.0 (7.0)	13.0 (9.0)	0.056
GWG classification				
Reduced	36.6%	28.0%	28.9%	0.162
Adequate	41.5%	48.0%	34.2%	
Excessive	22%	24%	36.8%	

This table is adapted from Maugeri et al., Nutrients 2019

Table 3. Characteristics of women from the Mamma & Bambino cohort (n=232) according to adherence to prudent dietary pattern

Characteristics	1st tertile	2nd tertile	3rd tertile	p-value [a]
Age [b]	38.0 (5.0)	37.0 (4.0)	37.0 (4.0)	0.675
Gestational age [b]	16.0 (1.0)	16.0 (3.0)	15.0 (5.0)	0.001
Educational level (low-medium %) [c]	22.7%	19.8%	15.5%	0.389
Working (%)	57.3%	60.4%	57.3%	0.865
Smoking (%)	20.9%	18.0%	22.2%	0.731
Use of folic acid supplements (%)	91.7%	93.8%	98.7%	0.210
Use of other multivitamin supplements (%)	59.7%	61.0%	59.0%	0.966
Pre-pregnancy weight [b]	63.0 (12.0)	60.5 (14.2)	58.5 (14.0)	0.043
Pre-pregnancy BMI [b]	23.2 (4.7)	22.7 (4.7)	21.8 (5.1)	0.019
Pre-pregnancy BMI categories				
Underweight	5.5%	8.1%	7.3%	0.007
Normal weight	64.5%	65.8%	70.8%	
Overweight	20.9%	17.4%	14.5%	
Obese	9.1%	8.7%	7.4%	
Weight at delivery [b]	74.0 (17.0)	73.5 (14.2)	72.0 (15.0)	0.551
Length of gestation [b]	39.0 (2.0)	39.0 (2.0)	39.0 (2.0)	0.562
Birth weight [b]	3.2 (0.6)	3.2 (0.6)	3.3 (0.7)	0.522
Length [b]	50.0 (2.0)	50.0 (2.0)	50.0 (2.0)	0.935
GWG [b]	12.0 (8.0)	12.0 (6.2)	13.0 (7.5)	0.830
GWG classification				
Reduced	31.9%	34.1%	27.8%	0.823
Adequate	37.5%	40.2%	45.6%	
Excessive	30.6%	25.6%	26.6%	

This table is adapted from Maugeri et al., Nutrients 2019

Table 4. Linear regression of the association between dietary patterns and gestational weight gain, stratified by body mass index categories

Dietary patterns	Total			Underweight			Normal weight			Overweight			Obese		
	β	SE	p-value	B	SE	p-value	B	SE	p-value	β	SE	p-value	β	SE	p-value
Western															
1st tertile	*Ref*			*Ref*			*Ref*			*Ref*			*Ref*		
2nd tertile	1.369	0.971	0.161	1.198	5.516	0.848	1.218	0.992	0.223	5.003	4.152	0.250	2.549	3.967	0.636
3rd tertile	1.542	1.072	0.152	2.308	10.321	0.860	0.961	1.116	0.392	1.917	3.637	0.605	13.701	0.887	**0.041**
Trend	1.217	0.487	**0.013**	-0.425	1.651	0.804	0.372	0.542	0.493	2.695	1.828	0.152	7.363	1.808	**0.005**
Prudent															
1st tertile	*Ref*			*Ref*			*Ref*			*Ref*			*Ref*		
2nd tertile	-0.353	1.019	0.730	-5.149	1.351	0.163	0.895	1.098	0.417	-7.975	2.672	**0.010**	-5.730	2.156	0.131
3rd tertile	0.184	1.067	0.863	5.382	1.678	0.274	-0.003	1.142	0.998	-9.736	4.302	**0.037**	-10.730	4.156	0.061
Trend	0.118	0.513	0.818	4.127	1.722	**0.048**	0.046	0.538	0.932	-4.209	1.635	**0.016**	-7.356	2.304	**0.031**

This table is adapted from Maugeri et al., Nutrients 2019

7. The effect of genetic variants on pregnancy outcomes

7.1 Background

Worldwide, two-thirds of the risk of adverse pregnancy outcomes depend on maternal behaviors, with a key role of maternal nutrition during the pre-conceptional and gestational periods [495]. However, uncovering the main genetic risk factors of adverse pregnancy outcomes represents one of the main challenges for public health, since they conferred about a third of this risk [168-170]. In spite of improvements in health care, adverse maternal and neonatal outcomes are a major public health problem [160, 496, 497]. For instance, PTB represents the first cause of death among newborns and the second among children under five years [158]. The major risk factors associated with PTB are maternal age, short inter-pregnancy interval, multiple gestation, drug abuse, smoking, infections, low maternal pre-pregnancy weight or inadequate GWG [498-501]. However, also genetic susceptibility has been taken into account [161], with novel genomic variants that affect maternal and neonatal health [162].

Overall, vitamin D is crucial for adult health [502] and its deficiency during pregnancy is associated with potential maternal and neonatal adverse outcomes [503]. In humans, Vitamin D is mostly provided by the endogenous cutaneous synthesis of pre-vitamin D3, obtained from 7-dehydrocholesterol through the exposure to ultraviolet radiation [504]. However, other sources of Vitamin D include dietary intakes of oily fish, fortified margarines and some breakfast cereals, while smaller amounts are also present in red meat and egg yolk [505]. The primary form of Vitamin D in these foods is vitamin D3 [506], which is used in fortified foods in U.S.A. and Canada [507, 508]. While Vitamin D3 represents almost 95% vitamin D serum levels [509], Vitamin D2 is also provided by foods and supplements [510].Therefore, serum levels of 25-hydroxylated vitamin D2+D3 (25OHD) characterize the vitamin D pool of the body. With respect to Vitamin D absorption, it follows the same process of other fat-soluble vitamins. After being incorporated into micelles, vitamin D reaches intestinal epithelium where it is encapsulated into chylomicrons which enter the lymphatic circulation. In the liver, Vitamin D3 is metabolized by a number of mitochondrial and microsomal P450 enzymes [511], acting as 25 hydroxylases, into hydroxycholecalciferol [25(OH)D], which in turn passes into general circulation and binds to a specific carrier protein, named as vitamin D binding protein (DBP). Particularly, 25(OH)D serum levels vary across populations, depending on latitude, pollution, concealing clothing, sun exposure, gender, dietary habits [512]. Once in kidney, 25(OH)D can undergo several hydroxylation reactions, catalyzed by 1α-hydroxylase and 24-hydroxylase, which give rise respectively to the

active component, named 1,25-dihydroxycholecalciferol [1,25(OH)D] or calcitriol, and the inactive form 24,25-dihydroxycholecalciferol [24,25(OH)D] [513]. Many other tissues also retain the 1 α-hydroxylase enzyme, allowing the local conversion of 25(OH)D to the active 1,25(OH)2D [514]. The endocrine effects of 1,25(OH)D concern mineral metabolism, prevention and treatment of rickets and regulation of bone health [515]. Moreover, Vitamin D is also considered an important regulatory factor of the immune system, mainly for macrophages, B lymphocytes, T lymphocytes, dendritic cells (DCs) and thymocytes [516].

During pregnancy, maternal vitamin D is provided to the fetus, also regulating placental function [504]. Maternal vitamin D deficiency may influence maternal and neonatal outcomes, such as recurrent pregnancy losses, preeclampsia, gestational diabetes, PTB, LBW and SGA [517]. However, it is not fully understood if adequate vitamin D levels may reduce the risk of adverse outcomes [517-520].

In this scenario, growing interest concerns the effect of genetic variants affecting vitamin D metabolism and functions. Vitamin D activity is mediated by the nuclear vitamin D receptor (VDR), which acts as a high-affinity ligand-activated transcription factor [521]. *VDR* gene is located on the chromosome 12q12-14, with high expression in several human tissues (i.e. skin epithelium, osteoblasts and chondrocytes, muscles, cells from the immune system and placenta) [522]. Since VDR is a ligand-activated transcription factor which binds to genes with promoters characterized by a vitamin D response element (VDRE), it is involved in the transcription of more than 900 genes. Moreover, VDR interacts with other transcriptional factors, such as the retinoid X receptor (RXR), to form a heterodimer [523]. *VDR* gene, which includes two promoter regions, eight coding exons and six untranslated exons, might be commonly affected by SNPs, which change the activity of the vitamin D-VDR complex [524, 525]. Recently, some studies investigated the association between VDR polymorphisms and the risk of adverse outcomes, such as PTB, LBW and SGA births [157, 161, 526-534]. The most common described di-allelic *VDR* gene polymorphisms are: *BsmI* (rs1544410) and *ApaI* (rs7975232) on the last intron, *FokI* (rs2228570) and *TaqI* (rs731236) on the coding exons [524, 528]: while *TaqI* and *FokI* consist of a single base change (A to G and G to A in exons 9 and 2, respectively), *BsmI* and *ApaI* are located in the last intron of the sequence and result from a single base change (G to A and A to C, respectively). However, evidence is still inconclusive, with high heterogeneity across studies [535]. With this in mind, our study aimed to investigate the association of maternal VDR polymorphisms with neonatal anthropometric measures and the risk of PTB, even considering dietary intake of vitamin D. Next, we performed a systematic review to evaluate the effect of VDR polymorphisms on PTB risk and on neonatal anthropometric

measures. Finally, we validated our results by pooling them with those described in previous studies through a meta-analysis.

7.2 Methods

7.2.1 Study design

The "Mamma & Bambino" cohort is designed to explore the effect of preconception, perinatal and early life exposure on maternal and infant health. Full details on study design and protocol are reported in the paragraph 5.2.1.

In the current analysis, we included mother-child pairs with complete information on sociodemographic characteristics, lifestyle and vitamin D intake, pregnancy outcomes and maternal VDR genotype distributions. Primary outcomes were gestational duration and PTB, as spontaneous delivery before 37 weeks. Secondary outcomes were birth weight and length. Specifically, birth weight was classified as LBW (< 2.5 Kg) and macrosomia (≥ 4.0Kg). Birth weight for gestational age was defined as SGA, AGA or LGA according to sex-specific national reference charts [433]. We included women who completed pregnancy, excluding those with pre-existing diseases, pregnancy complications, pre-term induced delivery, intrauterine fetal death, plurality, and congenital malformations.

7.2.2 VDR genotyping

Maternal DNA was extracted from peripheral blood samples using QIAamp DNA Mini Kit according to the manufacturer protocol (Qiagen, Milano). VDR polymorphisms were genotyped using the following commercially available TaqMan SNP Genotyping Assays (Applied Biosystem): *ApaI* rs7975232 (C_28977635_10), *TaqI* rs731236 (C_2404008_10), *BsmI* rs1544410 (C_8716062_10), *FokI* rs2228570 (C_12060045_20). All reactions were performed in triplicate on the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystem) deploying conditions set by the manufacturer. Allele determination was carried out using QuantStudio™ 7 Flex System Software.

7.2.3 Assessment of vitamin D intake

Vitamin D intake was assessed by a validated 95-item semi-quantitative Food Frequency Questionnaire (FFQ), as described in the paragraph 5.2.3.

Vitamin D intake was calculated using the USDA Nutrient Database (http://ndb.nal.usda.gov/) adapted to the Italian food consumption. The use of multimineral or multivitamin supplements containing vitamin D was verified, but the vitamin D intake was based only on food sources, as the FFQ was not designed to establish the vitamin D intake by supplementation.

7.2.4 Statistical analyses

Statistical analyses were conducted using SPSS software (version 22.0, SPSS, Chicago, IL). Descriptive statistics were used to characterize the study population, using frequency, mean and SD, or median and IQR. The Chi-square test was performed to determine if genotype distributions in mothers with full-term delivery were deviated from the Hardy-Weinberg Equilibrium (HWE). Prior to analysis, the normal distribution of all variables was checked using the Kolmogorov-Smirnov test. Based on skewed distribution, the Mann–Whitney U test or the Kruskal–Wallis test were used to compare maternal and neonatal quantitative variables across VDR genotypes. Chi-squared test was used to compare categorical. Linear and binary regression models were performed to evaluate the associations of VDR polymorphisms with pregnancy outcomes, using the non-mutated genotypes as reference. Regression models were adjusted for potential confounders (i.e., age, smoking, educational level, employment status, pre-gestational BMI, GWG, vitamin D intake, use of vitamin D supplements, type of delivery and parity). Post-hoc statistical power analysis was performed using Epi Info™ software (version 7). All statistical tests were two-sided, and p values < 0.05 were considered statistically significant.

7.2.5 Systematic review and Meta-analysis: Search strategy

We carried out a systematic literature search in the PubMed-Medline and Web of Science databases to identify epidemiological studies evaluating the association of *BsmI, ApaI, FokI* and *TaqI* polymorphism with anthropometric measures and incidence of PTB, LBW and SGA births. The search strategy comprised the terms (Vitamin D receptor OR VDR) AND (variation OR polymorphism OR mutations OR SNP) AND (Preterm Birth OR birthweight OR birth weight OR birth length OR Low Birth Weight OR Intrauterine Growth Retardation OR Fetal Growth Retardation OR Small for Gestational Age). The databases were searched from inception to February 2018 without language restriction, excluding abstracts and unpublished studies. The preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines were followed.

7.2.6 Systematic review and Meta-analysis: Selection criteria

Two researchers independently assessed the articles, and any inconsistencies were resolved through discussion. Studies included were consistent with the following criteria: i) observational studies or randomized control trials (RCTs) ii) on pregnant women of any gestational age iii) without pregnancy complications, iv) focusing on the association of *FokI, ApaI, TaqI* and *BsmI* VDR polymorphisms with PTB, LBW, and SGA. Moreover, studies were selected if they provide sufficient information on the numbers or genotype frequencies in cases and controls in order to

estimate ORs and 95% CIs. By contrast, the exclusion criteria were as follow: i) systematic reviews or meta-analyses; ii) abstracts and unpublished studies; iii) studies with insufficient or lack of data to estimate ORs and 95% CIs, after attempting to contact the corresponding authors via e-mail; iv) investigating the association with other VDR polymorphisms v) or with other adverse pregnancy outcomes; vi) studies with no control group.

7.2.7 Systematic review and Meta-analysis: Study selection and data extraction

Two researchers independently extracted the main information of the selected articles: first Author's last name, year of publication, country, ethnicity and number of participants, sample type, phenotype of the cases evaluated, genotyping method and genotype distributions in cases and controls, p-values for HWE in controls. Primary outcome was PTB since lack of data avoided the quantitative analysis for birth weight, birth length, LBW and SGA.

7.2.8 Procedures of meta-analysis

All statistical analyses were performed using the Review Manager software (Version 6.1).

Strength of association between VDR polymorphisms and PTB was estimated as ORs (95% CIs) under three models: the allelic model (2 vs. 1), the dominant model (22 and 12 vs. 11) and the recessive model (22 vs. 11 and 12). The significance of pooled OR was determined by the Z test. The Q test was used to measure heterogeneity across studies, with significant statistical heterogeneity as p <0.1. Since the Q test only shows the presence of heterogeneity and not its magnitude, we also applied the I^2 statistic to estimate the percentage of outcome variability that can be attributed to heterogeneity across studies. An I^2 value of 0% denotes no observed heterogeneity, whereas, 25%, 50% and 75% are considered as "low", "moderate" and "high" heterogeneity, respectively [536]. We also estimated the between-study variance using tau-squared (t) statistics [537]. We applied the fixed-effects model - using the Mantel–Haenszel method - when heterogeneity was negligible, while we performed the random-effects models – using DerSimonian-Laird method – if heterogeneity was significant. The presence of publication bias was evaluated by Begg's test and Egger's regression asymmetry test [538, 539]. Except for the Q test, p< 0.05 was considered statistically significant, and all tests were 2-sided.

7.3 Results

7.3.1 "Mamma & Bambino" cohort

Our analysis included 187 women from the "Mamma & Bambino" cohort (median age = 37 years), enrolled at a median gestational age of 16 weeks. According to pre-gestational BMI, we observed

that 30.9% of women exhibited reduced GWG, while 27.1% of them excessive GWG. In general, gestational duration was 39 weeks (IQR = 2), while median birth length and weight were 50.0 cm (IQR = 2) and 3.2 Kg (IQR = 0.6), respectively. With respect to anthropometric measures and neonatal outcomes, nearly 8% of newborns were underweight and 7.5% was diagnosed with macrosomia. Moreover, 80.2% of newborns were AGA, while the proportion of SGA and LGA were 8% and 11.8%, respectively. Regarding maternal habits, only 10.7% of pregnant women reported the use of multimineral/multivitamin supplements containing vitamin D. Notably, vitamin D intake was not associated with neonatal anthropometric measures nor with PTB risk (**Table 1**).

As shown in **Table 1,** we also compared women with PTB (n = 17; 9%) with those with full-term delivery (n = 170; 91%). We did not find difference in terms of maternal socio-demographic characteristics, pre-gestational BMI, GWG, use of vitamin D supplements and behaviours. As expected, PTB newborns reported lower birth length (47.5 cm vs. 50.0 cm; $p < 0.001$) and weight (2.44 Kg vs. 3.2 Kg; $p < 0.001$), with a higher proportion of underweight compared to full-term new-borns (58.8% vs. 2.9%; $p < 0.001$).

As shown in **Figure 1A**, only *FokI* and *TaqI* VDR polymorphisms were in HWE. The comparison of neonatal anthropometric measures across *TaqI* genotypes pointed out that birth weight increased with increasing number of G allele (AA = 3.2 Kg vs. AG = 3.2 Kg vs. GG = 3.4; p=0.020). However, we did not confirm this difference after adjusting for covariates. The comparison of maternal and neonatal characteristics across *FokI* genotypes demonstrated that both gestational duration (p = 0.040) and birth weight (p = 0.010) decreased with increasing number of A allele. Accordingly, as shown in **Figure 1B**, the proportion of PTB increased with increasing number of A allele (GG = 5.2% vs. AG = 8.3% vs. AA = 33.3%; p = 0.001), whereas no statistically significant differences were reported for *BsmI, ApaI* and *TaqI*. In line, we established that the risk of PTB increased with the number of A allele both in the age-adjusted (OR = 3.010; 95% CI = 1.457-6.215; p = 003) and in the multivariable-adjusted models (OR = 4.015; 95% CI = 1.649-9.771; p = 0.002). Specifically, mothers who carried the AA genotype exhibited a higher PTB risk both in the age-adjusted (OR = 7.389; 95% CI = 2.308-23.660; p = 0.001) and in the multivariable-adjusted models (OR = 12.049; 95% CI = 2.606-55.709; p = 0.001), if compared to the GG and GA genotypes.

7.3.2 Systematic review: study characteristics

PRISMA flow diagram shows the detailed steps of study selection (**Figure 2**). A total of 67 articles were selected from the databases, while 18 duplicates were excluded. After reading titles and/or abstracts, 25 articles were excluded and 24 underwent full-text screening. Based on selection criteria, we excluded 13 studies, whereas 11 studies were included in the systematic review. We did

not find RCTs focusing on the association of VDR polymorphisms with PTB, LBW, and SGA. For this reason, we included only observational studies in the systematic review.

A total of 5 studies were from European countries [161, 528, 530, 532, 534], 4 from America [526, 527, 529, 533], and 1 from Asia [157] and Australia [531], respectively. Overall, sample sizes of mothers ranged from 189 to 615, while sample size of infants varied from 90 to 506. PTB was the most reported outcome (n = 4) [157, 161, 526, 528], while 3 studies used birth weight [529], LBW [527] or SGA [533] as primary outcome. Two studies collected maternal and cord blood samples [157, 528], while 5 of them genotyped VDR polymorphisms in cord blood [530-534]. Moreover, 3 studies genotyped VDR polymorphisms in maternal blood samples [161, 526, 529] and 1 of them in placental samples [527]. Given that the majority studies analysed more than one polymorphism, *FokI* was analysed by 7 studies [157, 526-529, 531, 533], BsmI by 8 studies [157, 161, 528, 530-534], *ApaI* by 5 studies [157, 161, 528, 529, 531, 532] and TaqI by 7 studies [157, 161, 528-532]. The most common genotyping method was restriction fragment length polymorphism analysis (RFLP) (n = 6) [157, 528-532, 534], followed by TaqMan SNP Genotyping Assays (n = 4) [161, 526, 529, 533] and Sequenom MassARRAY (n = 1) [527].

7.3.3 Systematic review: VDR polymorphisms and neonatal anthropometric measures

In 2011, Swamy and colleagues conducted a prospective study on 615 pregnant women, evaluating the association between 38 VDR polymorphisms and several neonatal outcomes. In brief, they showed that 8 of 38 SNPs studied - including *ApaI* - were significantly associated with birth weight in black women [529]. In the same year, Silvano and colleagues studied 97 pre-pubertal children from 0 to 12 years to assess phenotypes that better characterize SGA children who failed to achieve postnatal catch-up growth. At the baseline, they did not report difference in *BsmI* and *FokI* genotype distributions across categories of birth weight for gestational age [533]. Consistently with our results, Workalemahu and colleagues demonstrated that birth weight decreased with increasing number of A allele of the *FokI* polymorphism detected in placenta samples [527].

7.3.4 Systematic review: VDR polymorphisms and PTB risk

Manzon and colleagues genotyped VDR polymorphisms in both maternal and cord blood samples, compared 33 PTB and 98 full-term deliveries [528]. In line with our findings, they stated that women with the A allele of *FokI* were at higher risk of PTB than those with the G allele. By contrast, women with the T allele of TaqI polymorphism showed a lower risk of PTB [528] compared to those with non-mutated allele [528]. However, logistic regression model revealed that only maternal *FokI* variant was associated with the risk of PTB. More recently, the study conducted by Javorsky and colleagues, which compared 104 women with PTB to 85 with full-term delivery,

confirmed that *FokI* polymorphism in women was associated with a higher risk of PTB [526]. Next, Rosenfeld and colleagues added to this knowledge, showing that the proportion of mothers with PTB decreased with increasing number of A allele of *BsmI* polymorphism, after adjusting for some confounders [157]. Interestingly, among women with previous history of spontaneous miscarriage, the risk of PTB was higher if their newborns carried the non-mutated allele of *BsmI* or the mutated allele of *ApaI* [157].

7.3.5 Meta-analyses of the association between VDR polymorphisms and PTB risk

According to post-hoc statistical power analysis, only the study investigating the association between *FokI* polymorphism and PTB reached a statistical power of at least 80%, with a significance level of 0.05. Thus, we aimed to summarize evidence about the association between maternal VDR polymorphisms and PTB. For this reason, as shown in **Table 2**, we pooled our results with those reported by three previously published studies [157, 161, 526]. For the *ApaI* polymorphism, we pooled our results with findings reported by Baczyńska-Strzecha et al. and Rosenfeld et al. [157, 161]. The Q-test and I2 statistics showed no significant heterogeneity across studies under the allelic (p = 0.41; I^2 = 0%), dominant (p = 0.19; I2 = 40%) and recessive (p = 0.61; I^2 = 0%) models. Thus, we used the fixed effect model for the meta-analysis, which did not show association of *ApaI* with PTB under the allelic model (C vs. A: OR = 0.89, 95%CI 0.71-1.12), dominant (CC + AC vs. AA: OR = 0.73, 95%CI 0.53-1.2) and recessive model (CC vs. AA+ AC: OR = 1.20, 95%CI 0.81-1.77) (**Figure 3**).

For the *BsmI* polymorphism, we pooled our results with findings reported by Baczyńska-Strzecha et al. and Rosenfeld et al. [157, 161]. The Q-test and I^2 statistics showed no significant heterogeneity across studies under the allelic (p = 0.31; I2 = 15%), dominant (p = 0.18; I^2 = 42%) and recessive (p = 0.97; I2 = 0%) models. Thus, we used the fixed effect model for the meta-analysis, which showed a significant negative association with PTB under the allelic (A vs. G: OR = 0.74, 95%CI 0.59-0.93) and recessive (AA vs. GG + AG: OR = 0.62, 95%CI 0.43-0.89) models. By contrast, no statistically significant association was evident under the dominant model (AA + AG vs. GG: OR = 0.78, 95%CI 0.54-1.12) (**Figure 4**).

For the *FokI* polymorphism we pooled our results with findings reported by Javorski et al. and Rosenfeld et al. [157, 526]. The Q-test and I^2 statistics showed significant heterogeneity across studies under the allelic (p = 0.002; I^2 = 85%), dominant (p = 0.02; I^2 = 74%) and recessive models (p = 0.001; I^2 = 77%). Thus, we used the random effect model for the meta-analysis, which showed a significant association with PTB under the recessive model (AA vs. GG + AG: OR = 3.67, 95%CI 1.18-11.43). By contrast, pooled ORs under the allelic (A vs. G: OR = 1.90, 95%CI 0.96-3.75) and

dominant (AA + AG vs. GG: OR = 1.65, 95%CI 0.80-3.42) models were not statistically significant (**Figure 5**).

For the *TaqI* polymorphism we pooled our results with findings reported by Baczyńska-Strzecha et al. and Rosenfeld et al. [157, 161]. The Q-test and I^2 statistics showed no significant heterogeneity across studies under the allelic (p = 0.29; I^2 = 19%), dominant (p = 0.51; I^2 = 0%) and recessive (p = 0.13; I^2 = 55%) models. Thus, we used the fixed effect model for the meta-analysis, which did not show significant association with PTB under the allelic (G vs. A: OR = 0.94 ,95%CI 0.75-1.18), dominant (GG + AG vs. AA: OR = 0.88, 95%CI 0.63-1.21), and recessive (GG vs. AA+ AG: OR = 1.00, 95%CI 0.69-1.46) models (**Figure 6**). Overall, we found no evidence of publication bias for meta-analyses of *ApaI* (Begg's p = 0.74; Egger's p = 0.89), *BsmI* (Begg's p = 0.70; Egger's p = 0.19), *FokI* (Begg's p = 0.87; Egger's p = 0.81), and *TaqI* (Begg's p = 0.42; Egger's p = 0.24).

7.4 Discussion

In the "Mamma & Bambino" cohort, maternal *FokI* polymorphism affected both gestational duration and birth weight, which decreased with increased number of the A mutated allele. This is in line with Workalemahu and colleagues that demonstrated how birth weight decreased with increasing number of A allele [527]. Our analyses – considering the age-adjusted and in the multivariable-adjusted models – showed that the risk of PTB increased with increasing number of A allele. Notably, women who carried the AA genotype exhibited a 12-fold increased risk of PTB, independent of potential confounders. A similar risk was also observed by Manzon et al. [528] and Javorsky et al. [526], while Rosenfeld and colleagues did not demonstrate this association. When we pooled our results with findings reported by Javorsky et al. and Rosenfeld et al. [157, 526], we observed a significant positive association between FokI polymorphism and PTB under the recessive model. By contrast, Rosenfeld and colleagues [157] also reported that the proportion of women with PTB decreased with increasing number of mutated allele of *BsmI* polymorphism. Although we did not demonstrate this association, the meta-analysis suggested the protective effect of *BsmI* against PTB under the allelic and recessive models. By contrast, results about the effect of *TaqI* polymorphism on PTB risk are currently inconclusive. Manzon and colleagues demonstrated that the mutated allele of *TaqI* polymorphism conferred a lower PTB risk [528], while we did not confirm this association by pooling our results with those reported by Baczyńska-Strzecha et al. and Rosenfeld et al. [157, 161]. However, since we showed that birth weight increased with increasing number of mutated alleles, we cannot completely exclude the protective effect of *TaqI* polymorphism on foetal growth. With respect to ApaI, a prospective study conducted by Swamy and colleagues demonstrated that *ApaI* polymorphism significantly affected birth weight in black

women, proposing a partial explanation for the observed racial disparity in several pregnancy outcomes [529]. Nevertheless, comparisons should be interpreted with caution due to the heterogeneity across studies in terms of design, sample size and type, ethnicity, geographical diversity, sun exposure, maternal habits and outcomes of interest [157, 161, 526].

The link between VDR expression and neonatal outcome is still unclear. Although several lines of evidence suggest that vitamin D system - including VDR, its ligands and the metabolizing enzymes - plays a key role in innate immunity and implantation [540-544], the effects of VDR and its allelic variants in pregnancy are not yet clarified.

Our study had some limitations. The main weakness of our analysis was the relatively small sample size and the statistical power, with a low number of PTB. Moreover, due to inconsistency of reported outcomes, we cannot perform a meta-analysis of the association between VDR polymorphisms and neonatal anthropometric measures. Indeed, only three studies investigated the effect on neonatal anthropometric measures using birth weight [529], LBW [527] or SGA [533] as primary outcome. Furthermore, additional data regarding other anthropometric measures should be added in future studies. Another weakness was that we did not analyse VDR polymorphisms in neonatal samples, not being able to assess the contribution of foetal *VDR* gene on adverse outcomes. Moreover, the influence of unmeasured variables (e.g., sun exposure, maternal habits, and serum vitamin D levels) cannot be completely excluded. Since the FFQ did not quantify vitamin D from nutritional supplements, we did not have a precise measure of vitamin D intake status. However, the use of vitamin D supplements is not common among women from the "Mamma & Bambino" cohort and no association with PTB risk was evident. Furthermore, the measurement of 25OHD would be useful to evaluate whether there was a relationship between serum levels and dietary intake, including vitamin D supplements.

Despite such limitations, the strictly selection criteria ruled out potential confounders. Moreover, extensive data collection enabled to adjust our results for socio-demographic factors, lifestyle, pre-gestational BMI and GWG, and vitamin D dietary intake. Additional research is recommended to establish the role of vitamin D and related factors in pregnancy, and to develop and validate effective preventive strategies against adverse outcomes.

Table 1. Population characteristics and comparison between preterm and full-term births

Characteristics	Study population (n=187)	PTB (n=17)	Full-term (n=170)	p-value
Age, years	37.0 (4)	37.0 (5)	38.0 (4)	0.648
Gestational age at enrolment, weeks	16.0 (4)	16.0 (4)	16.0 (4)	0.691
Educational level (% low-medium)	13.9%	5.9%	14.7%	0.316
Employment status (% employed)	55.6%	47.1%	56.5%	0.456
Smoking (% current smokers)	18.3%	5.8%	19.5%	0.165
Pre-gestational nutritional status				
Underweight	8.1%	11.8%	7.7%	
Normal weight	65.6%	58.8%	66.3%	0.522
Overweight	17.2%	11.8%	17.8%	
Obese	9.1%	17.6%	8.3%	
GWG, Kg	12.0 (7)	11.0 (10)	12.0 (6.9)	0.630
GWG classification				
Reduced	30.9%	35.3%	30.5%	
Adequate	42%	41.2%	42.1%	0.903
Excessive	27.1%	23.5%	27.4%	
Vitamin D intake, µg/day	3.7 (3.5)	3.7 (4.3)	3.1 (3.6)	0.808
Vitamin D supplements (% users)	10.7%	5.9%	11.2%	0.501
Gestational duration, weeks	39.0 (2)	35.5 (2)	39.0 (2)	<0.001
Sex (% male)	50.3%	47.1%	50.6%	0.781
Birth weight, Kg	3.2 (0.6)	2.44 0.5)	3.2 (0.6)	<0.001
Birth length, cm	50.0 (2)	47.5 (4)	50.0 (2)	<0.001
Type of delivery				
Natural	55.1%	47.1%	55.9%	0.486
Caesarean section	44.9%	52.9%	44.1%	
Underweight (%)	8%	58.8%	2.9%	<0.001
Macrosomia (%)	7.5%	0%	8.2%	0.219
Weight for gestational age				
SGA	8%	5.9%	8.2%	
AGA	80.2%	70.6%	81.2%	0.283
LGA	11.8%	23.5%	10.6%	

This table is adapted from Barchitta et al., Nutrients 2018

Table 2. Characteristics of studies included in the meta-analysis

Authors	Country	Study design	Ethnicity	Sample size	Sample	SNPs	Genotyping method
Baczyńska-Strzecha et al.,2016 [161]	Poland	Case-control	Caucasian	199	Maternal blood	ApaI BsmI TaqI	TaqMan Assay
Javorski et al.,2018 [526]	Brazil	Case-control	Mixed	189	Maternal blood	FokI	TaqMan Assay
Rosenfeld et al.,2017 [157]	Israel	Case-control	Mixed	375	Maternal and fetal blood	ApaI BsmI FokI TaqI	RFLP
Mamma & Bambino Cohort, 2018	Italy	Prospective cohort	Caucasian	187	Maternal blood	ApaI BsmI FokI TaqI	TaqMan Assays

This table is adapted from Barchitta et al., Nutrients 2018

Figure 1. (A) Genotype distribution of VDR polymorphisms and (B) comparison between preterm births (PTB, inner ring) and full-term births (outer ring).

This figure is adapted from Barchitta et al., Nutrients 2018

Figure 2. Flow diagram of study selection
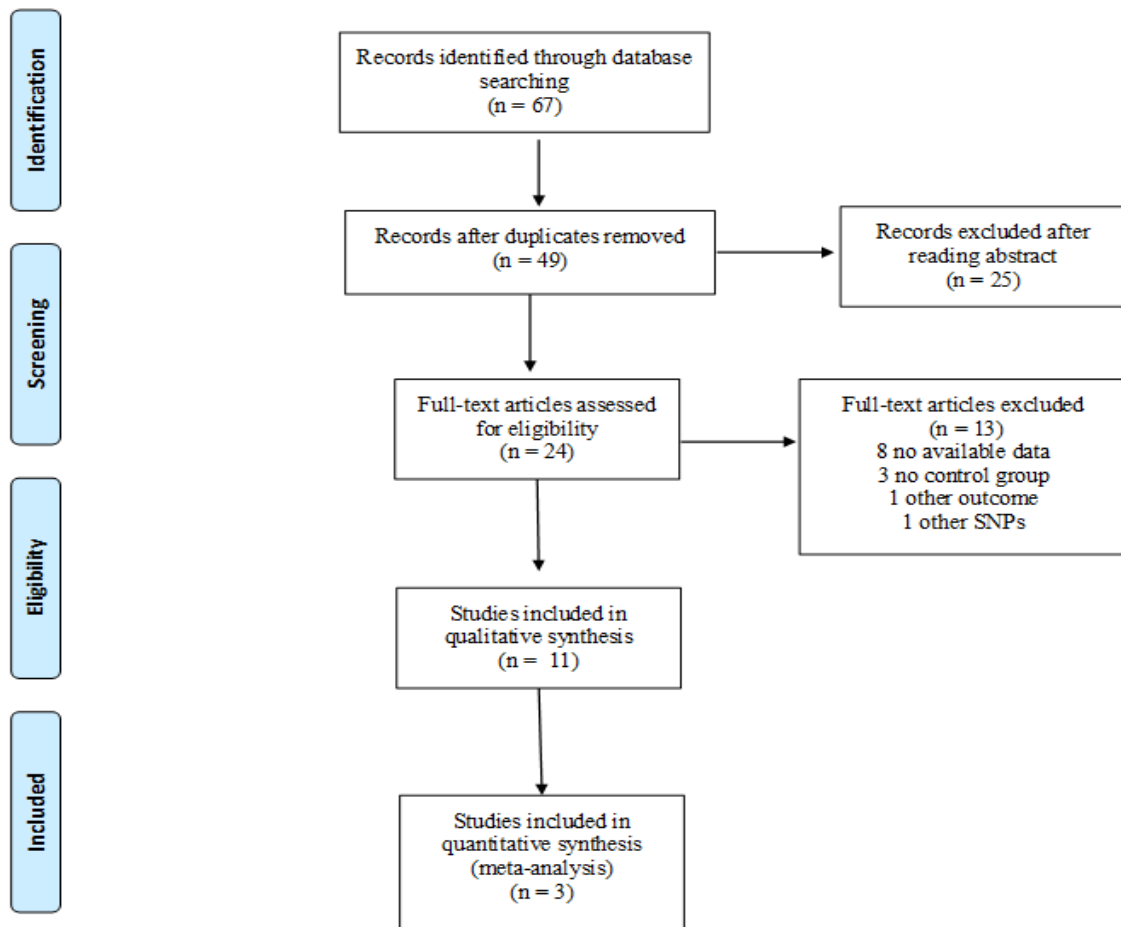
This figure is adapted from Barchitta et al., Nutrients 2018

Figure 3. Forest plots of the association between ApaI polymorphism and preterm birth under the (A) allelic, (B) dominant and (C) recessive models
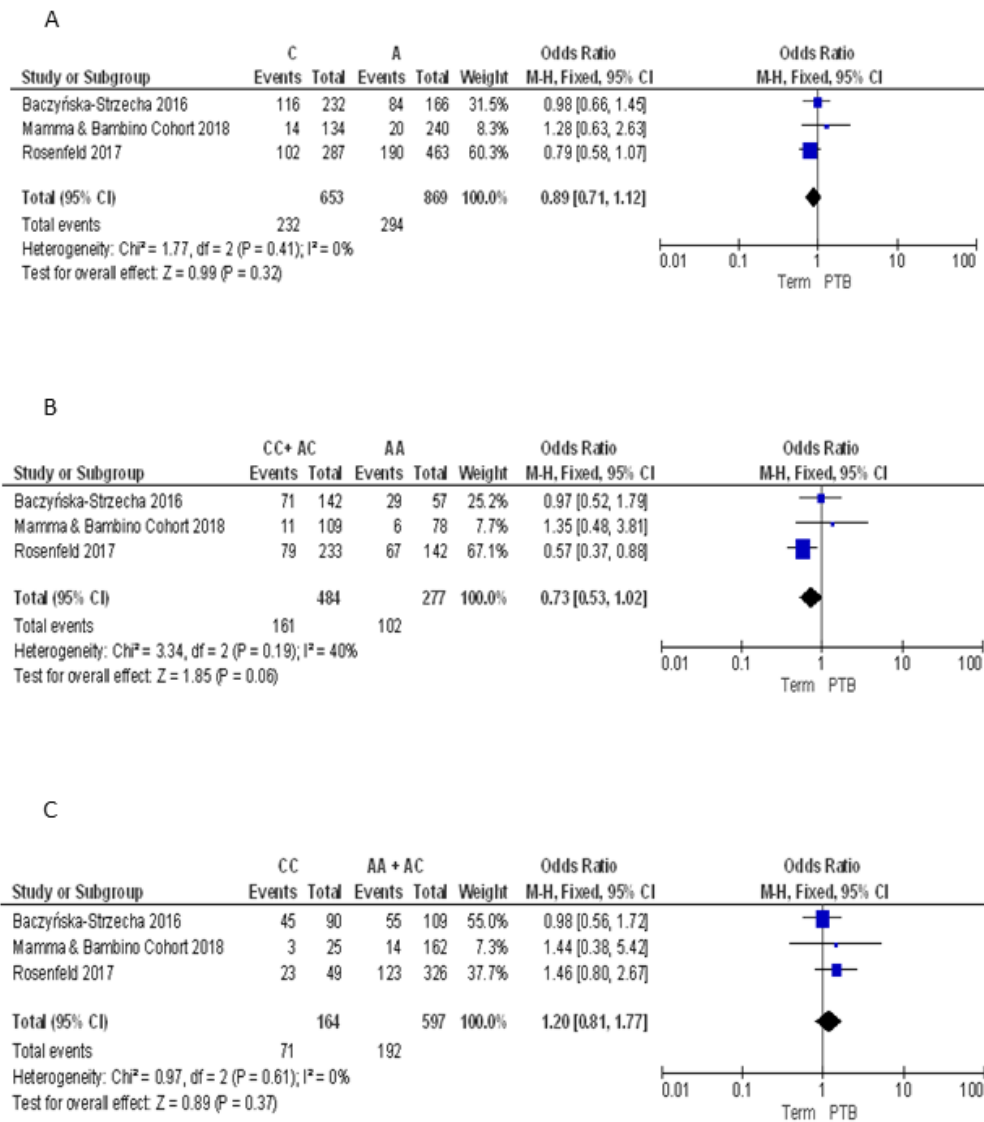
This figure is adapted from Barchitta et al., Nutrients 2018

Figure 4. Forest plots of the association between BsmI polymorphism and preterm birth under the (A) allelic, (B) dominant and (C) recessive models
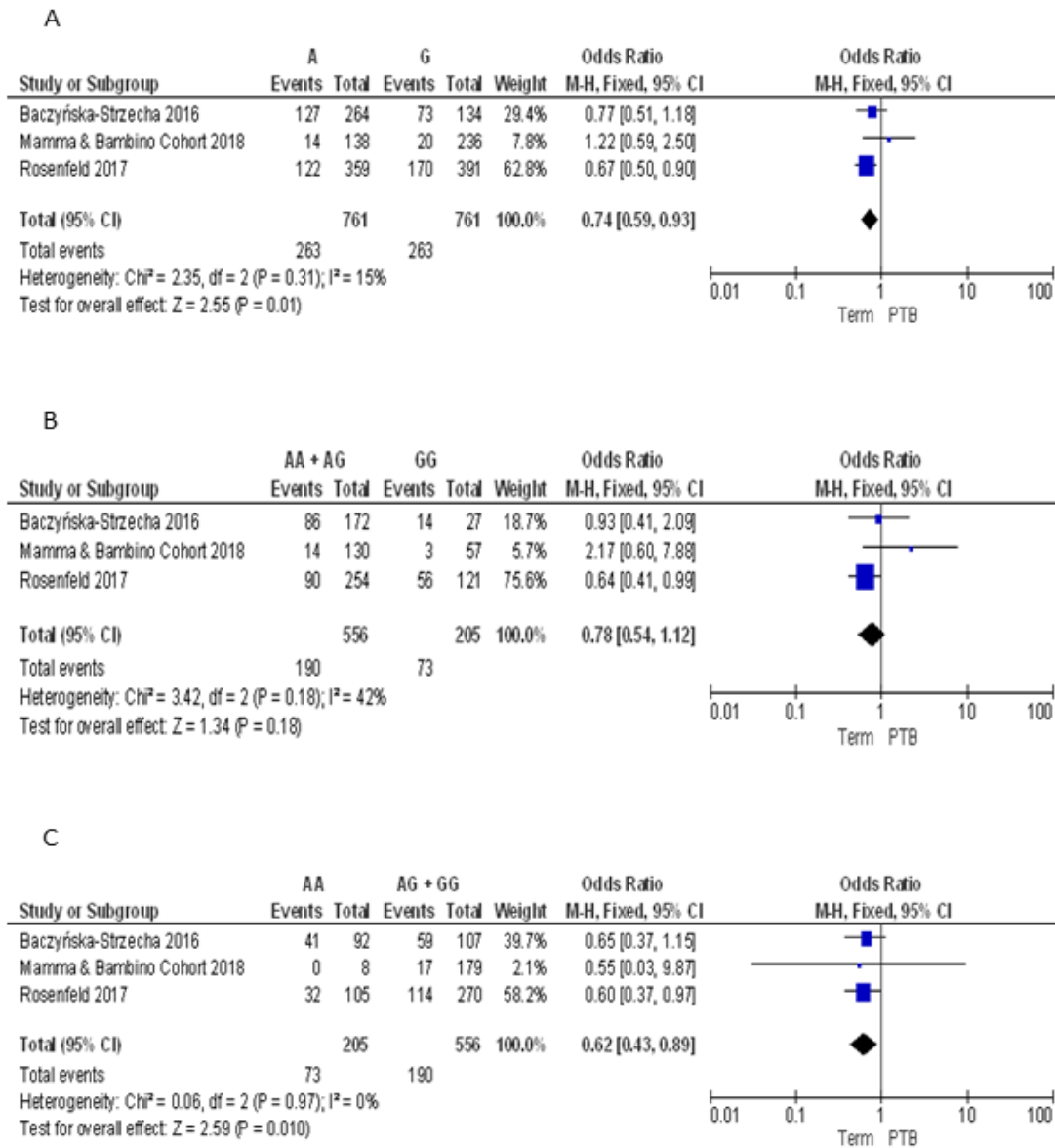
This figure is adapted from Barchitta et al., Nutrients 2018

Figure 5. Forest plots of the association between FokI polymorphism and preterm birth under the (A) allelic, (B) dominant and (C) recessive models
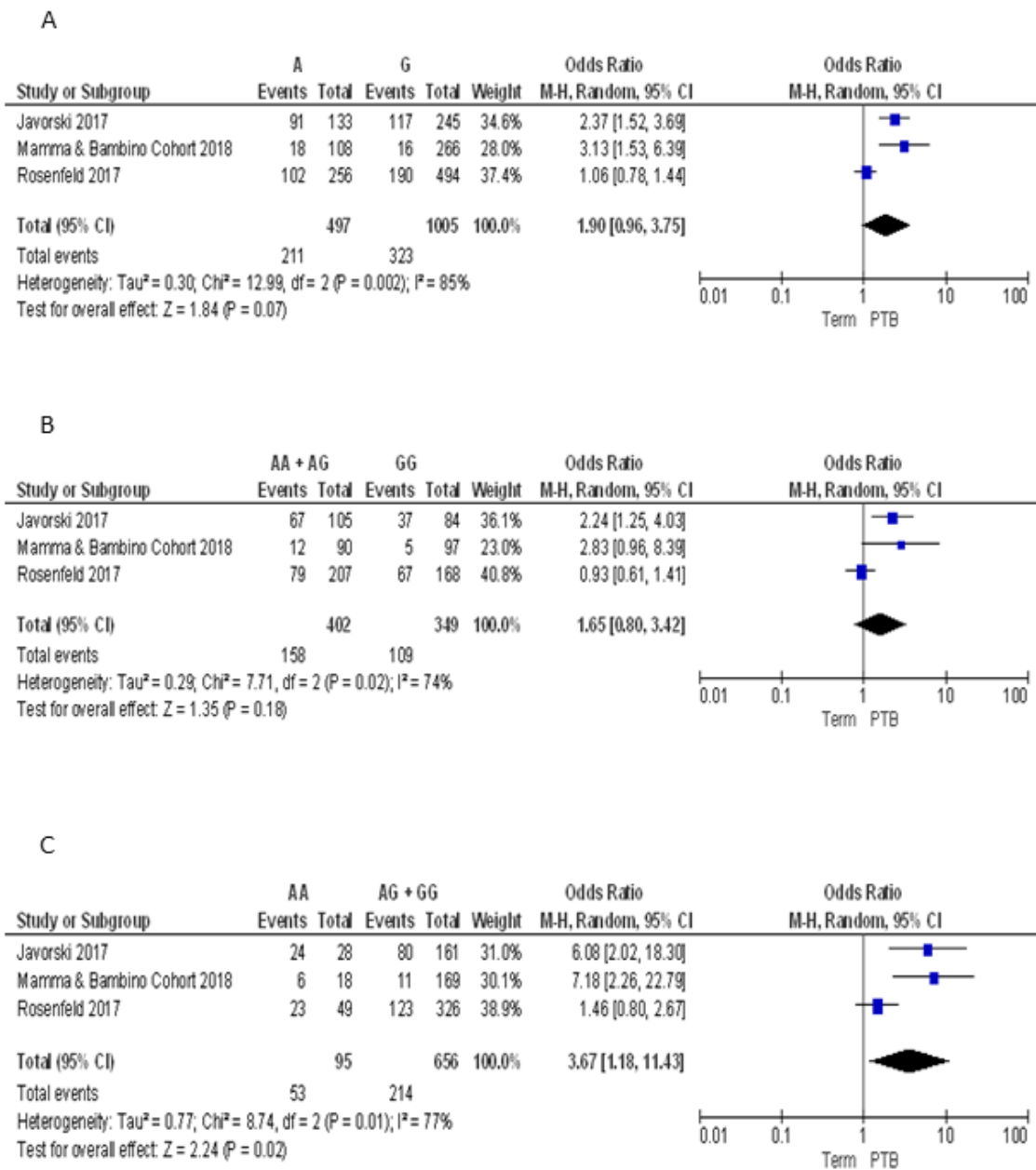
This figure is adapted from Barchitta et al., Nutrients 2018

Figure 6. Forest plots of the association between TaqI polymorphism and preterm birth under the (A) allelic, (B) dominant and (C) recessive models
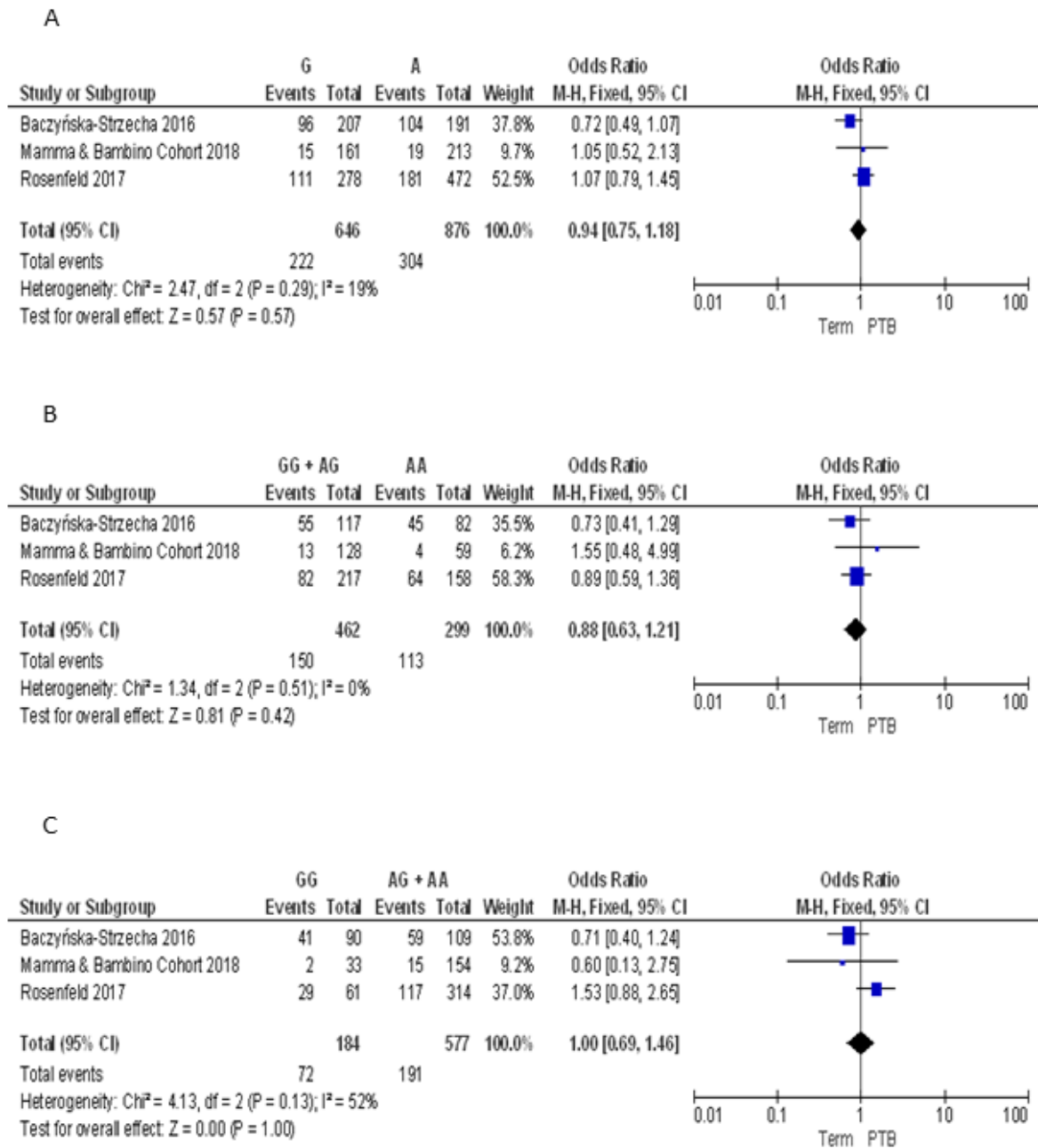
This figure is adapted from Barchitta et al., Nutrients 2018

8. The effects of dietary factors on telomere length

8.1 Background

The human aging is characterized by the repeated adaptation to internal and external stressors during lifetime [59], which results in the interaction between environmental, genetic and epigenetic events [57, 58]. Despite this complexity, several molecular signatures have been suggested to reflect the aging process, also in the context of non-communicable diseases [36]. In particular, genomic instability [62-65], telomere attrition [66, 67], epigenetic alterations [68-74], mitochondrial dysfunction [77, 78], cellular senescence [79-82], steam cell exhaustion [85] and altered intercellular communication [86, 87] have been well investigated. In the last decades, telomere length has been proposed as a marker of biological aging due to its shortening in somatic cells, as a result of reducing activities of telomerase [546]. In vertebrates, telomeres are repetitive sequence of 5′-(TTAGGG)-3′ at the ends of each chromosome, with a function of preventing genomic instability [547]. In particular, telomere shortening has been associated with aging [89] and age-related diseases - cardiovascular diseases [35], cancer [34], and neurological disorders [90]. Several risk factors (e.g., physical inactivity, smoking cigarette, unhealthy diet etc.) might affect telomere length before the abovementioned diseases develop [548, 549]. Thus, the high variability and the responsiveness to external and internal stressors [91] have made telomere length a biomarker of interest in other context of epidemiological research [93]. Moreover, telomere shortening is regulated by epigenetic mechanisms and influenced by DNMTs and histone methyltransferases [550, 551]. For instance, an epigenome-wide association study (EWAS) has showed that DNA methylation of more than 800 CpG sites - within genes involved in circadian rhythm, coagulation, and wound healing - is associated with telomere length [552].

In the last decades, as done for DNA methylation and histone modification [220, 318, 368, 553], several studies have explored the association between diet and telomere length, as suggested by the protective role of antioxidants and the opposite effect of high-sugar and high-calorie products [554]. During pregnancy, several maternal factors - such as stress, smoking and exposure to pollutants – have been associated with shorter telomeres in cord blood [555-557] and placenta [558]. Similarly, maternal diet affects biological aging in children [559]. In fact, the first 1,000 days of life – from conception to two years of age – represents an important window in which several exposures might shape children's health. However, the evidence is currently limited and often controversial, with the lack of studies investigating the effect of maternal diet on telomere length of cell-free circulating fetal DNA (cfDNA) from amniotic fluid [559].

With respect to alcohol – that represents a main risk factor for human health with more than 3 million deaths in 2016 [560] – several studies have investigated the role of alcohol as confounder or mediator of the relationship between telomere length and age-related diseases. Recently, several studies have investigated the primary effect of alcohol consumption and related disorders on telomere length. However, findings are inconclusive due to differences in study design, characteristics of study population, methods used for measuring telomere length, and other factors taken into consideration. Moreover, the research on the effect of alcohol consumption on telomere length during pregnancy is currently lacking. In this scenario, it is also known that alcohol represents a risk factor for mothers and their children during pregnancy – especially in the first trimester – [561], increasing considerably the risk of miscarriage, premature birth and low birthweight [562]. Although molecular mechanisms underpinning the negative effects of alcohol consumption during pregnancy are not so clear, telomere shortening represents a plausibly involved factor. However, no studies have so far investigated the effect of maternal alcohol consumption on telomere length of newborns.

With this in mind, our study aimed to evaluate the relationship of maternal intake of nutrients in early pregnancy with telomere length of cfDNA from amniotic fluid, using data and samples from "Mamma & Bambino cohort". Next, we conducted a systematic review to discuss on the potential association between alcohol consumption, alcohol-related disorders, and telomere length. Moreover, we also conducted a pilot study to fill the gap about the relationship between maternal alcohol consumption during pregnancy and telomere length in cord blood at birth.

8.2 Methods

8.2.1 Study design

The "Mamma & Bambino" cohort is an ongoing Italian birth cohort designed to explore the effect of preconception, perinatal and early life exposure on maternal and infant health (further information can be found at http://www.birthcohorts.net). Full details on study design and protocol are reported in the paragraph 5.2.1. For the current analysis, we used data and samples from women who completed pregnancy and who provided an aliquot of amniotic fluid obtained through amniocentesis [563].

8.2.2 Data collection

For each woman, information on socio-demographic characteristics and lifestyles are collected through structured questionnaires. Full details on data collection and management are reported elsewhere [298-300, 563-566]. In particular, dietary information was assessed by a validated 95-item semi-quantitative FFQ, as described in the paragraph 5.2.3. For each item, information on

frequency of consumption and portions size are collected to calculate their daily dietary intake. Next, the intakes of calories, minerals (i.e., iron, calcium, magnesium, and zinc), fatty acids (i.e., saturated, monounsaturated, and polyunsaturated), and vitamins (i.e., A, B1, B6, C, D, and folate) are computed using the USDA Food Composition Database (http://ndb.nal.usda.gov/) adapted to typical Italian foods. Nutrient intakes are considered as continuous values or categorized according to the Recommended Dietary Allowance by the Food and Nutrition Board of the Institute of Medicine [430].

Regarding alcoholic drinks, standard portions are referred to a glass of wine, a bottle of beer or a shot of spirits, which approximately contain 10 g ethanol [567]. Although it is generally recommended to avoid drinking alcohol during pregnancy, a low proportion of pregnant women reports light-to-moderate alcohol consumption (1-7 standard drinks per week) [567].

8.2.3 DNA extraction and relative telomere length assessment

The "Mamma and Bambino" project also aims to uncover the effects of maternal exposures on several biomarkers of health and aging [298-300, 566]. To do that, biological samples from mothers and newborns are collected at recruitment (blood and amniotic fluid samples) and at delivery (cord blood whenever possible).

In the current analysis, we used an aliquot of 1ml amniotic fluid obtained from women who underwent amniocentesis, as previously reported [563]. In brief, after centrifugation at 12500 g, the cfDNA was extracted using the QIAamp Blood Kit (Qiagen, Milan Italy) on the QIAcube instrument (Qiagen, Milan, Italy). Quantity and quality of cfDNA were assessed using the dsDNA HS Assay Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) on the Qubit 3.0 Fluorometer and the NanoDrop 1000 spectrometer. Next, relative telomere length of cfDNA was evaluated real-time quantitative polymerase chain reaction (qPCR), using the Relative Human Telomere Length Quantification Assay Kit (ScienCell Research Laboratories, Carlsbad, CA, USA) on the QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, CA, USA). Different sets of primers were used: i) the telomere primer set amplified telomere sequences; ii) the single-copy reference primer set amplified a 100 bp-long region on human chromosome 17 and was used as reference for data normalization. Accordingly, each reaction contained 1 µl of DNA (5 ng/ µl), 10 µl of 2X GoldNStart TaqGreen qPCR master mix (ScienCell Research Laboratories, Carlsbad, CA, USA), 2 µl of primer solution (telomere or single copy reference), and 7 µl of nuclease-free water. The qPCR was conducted according to the following conditions: 95°C for 10 minutes; 32 cycles of 95°C for 20 seconds, 52°C for 20 seconds and 72°C for 45 seconds. All reactions were run in duplicate and relative telomere length was expressed as telomere/single copy

reference (T/S) ratio. The procedures described above were conducted according to the manufacturers' protocols, unless otherwise stated.

Moreover, in the pilot study, we used samples of maternal blood and cord blood to measure leukocyte telomere length. In brief, Leukocyte genomic DNA was extracted from 200 μl of maternal and cord blood samples using the DNeasy Blood & Tissue kit (Qiagen, Milan Italy), as described by the manufacturer's protocol. DNA purification was automated on the QIAcube instrument (Qiagen, Milan, Italy). Concentration and purity of DNA were assessed by NanoDrop 1000 spectrometer and by Qubit 3.0 Fluorometer using dsDNA HS Assay Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). Relative telomere length was measured using the Relative Human Telomere Length Quantification Assay Kit (ScienCell Research Laboratories, Carlsbad, CA, USA), as described above.

8.2.4 Estimation of statistical power

With respect to the pilot study, the primary hypothesis was that relative telomere length of leukocyte DNA from maternal and cord blood samples diverged between drinking and non-drinking pregnant women. In contrast, the null hypothesis was that maternal alcohol consumption was not associated with relative telomere length. However, the proportion of women who drank alcohol was very low (i.e., nearly 5%). Among them, we identified 5 non-smoking women who completed pregnancy at term (i.e., at 37 weeks of gestation) and who stated to drink alcohol during the first trimester. Thus, assuming an allocation ratio of 1:2, this pilot study had a statistical power of 80% for detecting a mean difference in relative telomere length of 1 point (standard deviation, SD = 0.5) with a two-sided significance level of 5%. Therefore, we next matched 10 non-smoking women with term pregnancy, who declared to not consume alcohol during the first trimester of pregnancy. The propensity score method was used to match the two groups for maternal age, gestational age at recruitment, pre-gestational body mass index and fetal sex.

8.2.5 Statistical analyses

All the statistical analyses were performed using SPSS (version 26). The characteristics of the study population were described using frequencies for qualitative variables, and median and IQR due to the skewness of quantitative variables. The correlations between maternal nutrient intakes and telomere length were tested using the Spearman's rank correlation test and adjusting for multiple comparisons with Bonferroni correction. For nutrients with a significant correlation ($p < 0.05$), we plotted their continuous value against relative telomere length. Next, we applied the Mann Whitney U test to compare relative telomere length between deficient and not deficient women. Finally, we tested the association between nutrient intake and relative telomere length, adjusting for potential

confounders (i.e., maternal age, smoking status, pre-gestational BMI, total daily energy intake, and supplement use).

For the pilot study, comparisons between drinking and non-drinking women were performed using the Student's t-test or the Chi-squared test. Relative telomere length was tested for normality using the Kolmogorov-Smirnov test, expressed as mean and SD, and compared using the Student's t-test. All tests were two-sided and performed at a significance level $\alpha = 0.05$.

8.2.6 Systematic review: search strategy

The systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [568]. The literature search – done on February 12, 2021 – was carried out on PubMed, Medline, and Web of Science databases using the following key words: ((Ethanol) OR (Alcohol*) OR (Drink*)) AND ((Telomere) OR (Telomere length) OR (Telomere Shortening)).

8.2.7 Systematic review: study selection

The systematic review included: (1) epidemiological studies of any design (2) evaluating the association of alcohol consumption and/or alcohol-related disorders (3) with telomere length. By contrast, narrative and systematic reviews, editorials, studies that did not report data about the association, and those that considered alcohol consumption only as a covariate were excluded. The literature search and study selection were performed independently by two investigators and disagreements were resolved by discussion with a third investigator.

8.2.8 Systematic review: data extraction

For each study, the following data were extracted: first author, year of publication, study design, study population, method for assessing alcohol consumption and/or alcohol-related disorders, sample used for DNA extraction, method for measuring TL, results on the association between alcohol consumption, alcohol-related disorders and TL, and additional findings if present.

8.3 Results

8.3.1 Characteristics of the study population

In the current analysis we included 174 women (median age = 38; IQR = 4) from the "Mamma & Bambino" cohort. Among them, 83.3% reported a high educational level, with 54.0% of them part-time or full-time employed. Moreover, nearly 80% of women were non-current smokers. The median pre-gestational BMI was 22.8 kg/m^2 (IQR = 4.6), with 21.3% of overweight or obese women before pregnancy. With respect to the median intake of nutrients considered in the current analysis, women had a total energy intake of 1662 kcal (IQR = 634) and their dietary deficiencies

varied from 17.8% for vitamin A to 98.3% for iron. About 35% of pregnant women took multivitamin or multi-mineral supplements.

8.3.2 The relationship between relative telomere intake and nutrient intakes

The Spearman's rank correlation test was used to evaluate the correlation between relative telomere length and nutrient intakes. Among the latter, only the intakes of magnesium, vitamin B1 and iron were positively but weakly correlated with relative telomere length, with p-values < 0.05. However, only the correlation with magnesium remained significant after adjusting for multiple comparison. We next assessed relative telomere length according to nutrient deficiencies. In particular, women with magnesium deficiency (i.e., 73% of the total pregnant women included) exhibited lower relative telomere length than those with adequate dietary intake (p = 0.005). Similarly, women with vitamin B1 deficiency (i.e., 53% of the pregnant women included) showed lower values than those with adequate intake (p = 0.040). By contrast, no significant difference was evident according to iron deficiency (p = 0.240).

8.3.3 The association of magnesium intake with relative telomere length

Interestingly, we performed a linear regression model to evaluate the association of maternal nutrient intakes with relative telomere length. However, only the intake of magnesium was positively associated with relative telomere length (β = 0.002; SE = 0.001; p = 0.024), after adjusting for maternal age, smoking, pre-gestational BMI, total energy intake, and supplement use. In line, as shown in **Figure 1**, magnesium deficiency was negatively associated with relative telomere length after adjusting for the same covariates (β = -0.467; SE = 0.176; p = 0.009). Instead, no significant relationships were evident for the intakes of vitamin B1 and iron.

8.3.4 Pilot study

In the "Mamma & Bambino" cohort, 5 women declared to never smoke but to habitually drink alcohol in the first trimester of pregnancy. In addition to the above, **Table 1** showed that we matched 10 non-smoking and non-drinking women, considering maternal age, gestational age at recruitment, pre-gestational BMI, and fetal sex. As shown in **Figure 2A,** the comparison of relative telomere length measured in leukocyte DNA from maternal blood samples did not demonstrate differences between drinkers and non-drinkers. However, we observed a significant difference when analyzing relative telomere length of leukocyte DNA from cord blood samples. Interestingly, newborns from drinking women exhibited shorter relative telomere length than their counterpart (**Figure 2B**).

8.3.5 Systematic review: selection of the studies

Figure 3 summarized the study selection process**.** After removing duplicates, a total of 327 articles were identified from the electronic databases searched. However, 307 were excluded after reading titles and abstracts. Among the remaining 20 articles, 6 studies were excluded for the following reasons: 1 study did not report information on alcohol consumption, 3 studies did not evaluate the association with telomere length, and 2 studies considered alcohol only as a covariate. Thus, the 14 remaining studies were included in this systematic review.

8.3.6 Systematic review: study characteristics

Of the 14 studies included, 5 were conducted in North America, 4 in Asia, 4 in Europe, and 1 in South America. 10 studies featured a cross-sectional design or performed cross-sectional analyses on prospective cohort, while 4 studies were prospective. Studies were classified according to alcohol consumption (n = 9 studies) or alcohol-related disorders (n = 5 studies). Among them, 12 studies used blood samples for evaluating telomere length, while 2 studies used samples of esophageal mucosa or oral epithelium. With respect to telomere length analysis, the majority of studies (n = 9) used qPCR, while the remaining used quantitative fluorescence in situ hybridization (Q-FISH) or southern blot analysis of terminal restriction fragment lengths.

8.3.7 Systematic review: Telomere length in patients with alcohol-related disorders

Due to heterogeneities, some studies compared telomere length between patients with alcohol use disorders or dependence and controls, while others tested the effect of drinking alcohol on telomere length in other groups of people. As reported by Aida and colleagues in 2011, the normalized telomere-to-centromere ratio, calculated from telomere length of DNA extracted from esophageal mucosa, appeared significantly lower in patients with alcohol dependence than in controls [569]. In the same year, Pavanello and colleagues supported this finding, reporting that relative telomere length was lower in alcohol abusers than in controls, independently of their smoking status. The authors also suggested that the number of drinks per year was associated with relative telomere length in the entire population and among alcohol abusers [570]. These findings were next confirmed in recent years. For instance, Yamaki and colleagues showed shorter telomere length in patients with alcohol-related disorders if compared with non-alcoholic controls. However, no difference was evident compared patients with alcohol-related disorders according to their cancer diagnosis [571]. Similarly, Martins de Carvalho and colleagues demonstrated that alcohol use disorders were associated with lower relative telomere length. Yet, drinking behaviors were not associated with relative telomere length in patient and healthy control groups [572]. Finally,

Tannous and colleagues reported that, although difference was not statistically significant, relative telomere length tended to be shorter in patients with alcohol use disorder [573].

8.3.8 Systematic review: Alcohol consumption and telomere length

In line with the above-mentioned evidence, Aida and colleagues also evaluated the association of alcohol consumption with telomere length in patients and controls classified as heavy, light and non-drinkers. Although DNA from oral mucosa of patients with cancer exhibited lower normalized telomere-to-centromere ratio than healthy controls, no relationship was evident with drinking behaviors in the latter group [574]. In general, most studies support the absence of relationship between alcohol consumption and telomere length also in blood samples [575-579]. However, Strandberg and colleagues suggested a cross-sectional association at the baseline but not at the last follow-up [580]. This was partially consistent with the study by Révész and colleagues, which showed a negative association between heavy drinking and relative telomere length among 2936 participants from the Netherlands Study of Depression and Anxiety [581]. However, the association was evident only at the baseline analysis and it was not significant after adjusting for other covariates [581]. The present systematic review also included a large cross-sectional study by Shin and Baik, which did not find an association between alcohol consumption and relative telomere length in the overall population. However, they observed an inverse association with heavy drinking among participants with mutant alleles of rs2074356 [582]. This is a SNP in an uncharacterized locus on chromosome 12, and its mutant allele seemed associated with increased risk of esophageal squamous cell cancer.

8.4 Discussion

Our analysis demonstrated a positive association between magnesium intake and relative telomere length, which resulted in telomere shortening in women with magnesium deficiency. In line with previous studies conducted in other settings, the positive effect observed for magnesium remained significant after adjusting for. In fact, in vitro and in vivo studies suggested that long-term exposure to magnesium deficiency led to telomere shortening [583, 584]. By contrast, a cross-sectional analysis conducted on the Sister Study showed a positive association between magnesium intake and telomere length of leukocyte DNA from women who did not use multivitamin supplements [585]. From a biological point of view, magnesium is an important cofactor for the catalytic activity of enzymes implicated in DNA replication and repair [586-589], and in RNA synthesis [586]. Magnesium deficiency is also often associated with oxidative stress [583] and pro-inflammatory status [590], which in turn might lead to telomere shortening.

With respect to other nutrients, the evidence instead remained scarce and inconclusive. The lack of any solid evidence in this field of research, therefore, encourages further efforts to understand the influence of maternal dietary factors on biological aging, as determined by telomere length. Furthermore, future studies investigating other biomarkers of aging could help resolve this question and to translate the answers into effective public health strategies.

With respect to alcohol, our review revealed two types of evidence. Alcohol-related disorders were associated with telomere shortening, while alcohol consumption did not appear to affect telomere length in absence of alcohol-related disorders. This was also confirmed when people were classified in light, moderate, or heavy drinkers [576]. However, if the association was non-linear, classifying alcohol consumption at different threshold could influence the result. Accordingly, future studies should test the shape of the association between alcohol consumption and telomere length, before drawing definitive findings.

Our systematic review also revealed the absence of studies during the gestational period. Yet, alcohol abuse has a negative impact on many targets of the SDGs, including those related to maternal and child health [561]. Drinking alcohol, especially in the first trimester of pregnancy, increases the risk of adverse outcomes [562]. Due to the lack of evidence, therefore, we designed a pilot study using data and samples from "Mamma & Bambino" cohort. Our analysis revealed that drinking alcohol in the first trimester of pregnancy might affect telomere length in DNA from cord blood. Indeed, newborns from drinkers showed shorter telomere length than those born from non-drinkers, according to the Developmental Origins of Health and Disease theory for which *in utero* exposures program the fetus for challenges that is likely to experience later in life [129]. Indeed, our preliminary findings support the current idea that biological aging at birth might reflect maternal and neonatal characteristics related to prenatal environmental adversity [594]. For these reasons, it should be necessary to study telomere shortening as a potential molecular mechanism underpinning the effects of maternal behaviors on the development of chronic disease later in life.

Our analysis had some limitations to be considered. Firstly, the limited sample size did not allow us to perform additional analyses for residual confounders. Secondly, the FFQ that we used for dietary assessment did not preclude measurement errors and inaccuracies [595]. Moreover, this tool did not consider any changes related to food cooking. To partially address these issues, the use of some biomarkers of validation (e.g., serum level of nutrients) could have made the evidence more solid. Thirdly, the method used for the assessment of telomere length (i.e., qPCR) had higher assay variability than terminal restriction fragment analysis [596]. [73]. Finally, although amniotic fluid is

considered a relatively pure fetal sample, a low proportion of cfDNA from placenta cannot be completely excluded [90].

Moreover, differences between studies – in terms of study design, study population, assessment of alcohol consumption, assay used for measuring telomere length – hindered the possibility for applying a meta-analytic approach. In conclusion, further studies should be encouraged to understand what maternal factors affect the aging process of their children.

Figure 1. The relationship of magnesium deficiency with relative telomere length

Table 1. Comparison of characteristics between drinking and non-drinking women

Characteristics	Drinkers (n=5)	Non-drinkers (n=10)	p-value
Age (years)[a]	38.1 (4.2)	37.9 (3.9)	0.934
Gestational age at sampling (weeks)[a]	16.1 (2.2)	16.2 (2.3)	0.937
Pre-pregnancy BMI (kg/m^2)[a]	24.2 (3.8)	24.0 (3.9)	0.926
Gestational age at delivery (weeks)[a]	38.9 (2.1)	39.1 (2.0)	0.860
Fetal sex (male/female)	3/2	6/4	1.000

This table is adapted from Maugeri et al., Int. J. Environ. Res. Public Health 2021

Figure 2. Comparison of relative telomere length between drinkers and non-drinkers in (A) maternal blood and (B) cord blood
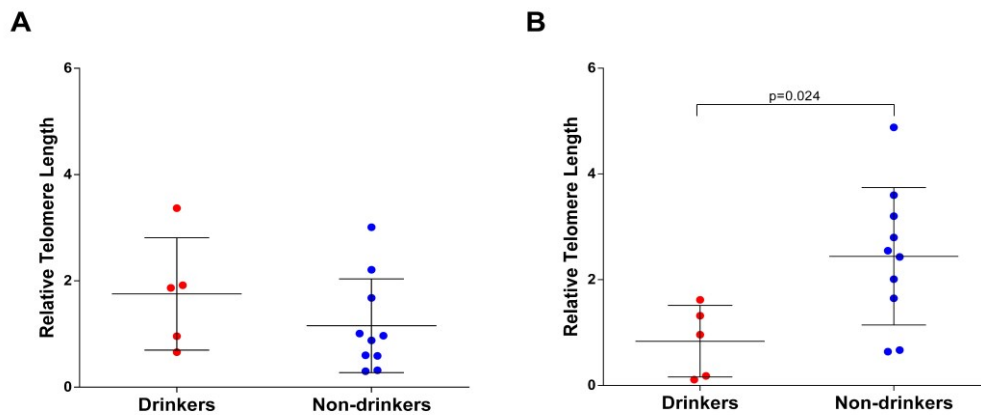
This figure is adapted from Maugeri et al., Int. J. Environ. Res. Public Health 2021

Figure 3. PRISMA flow diagram of study selection
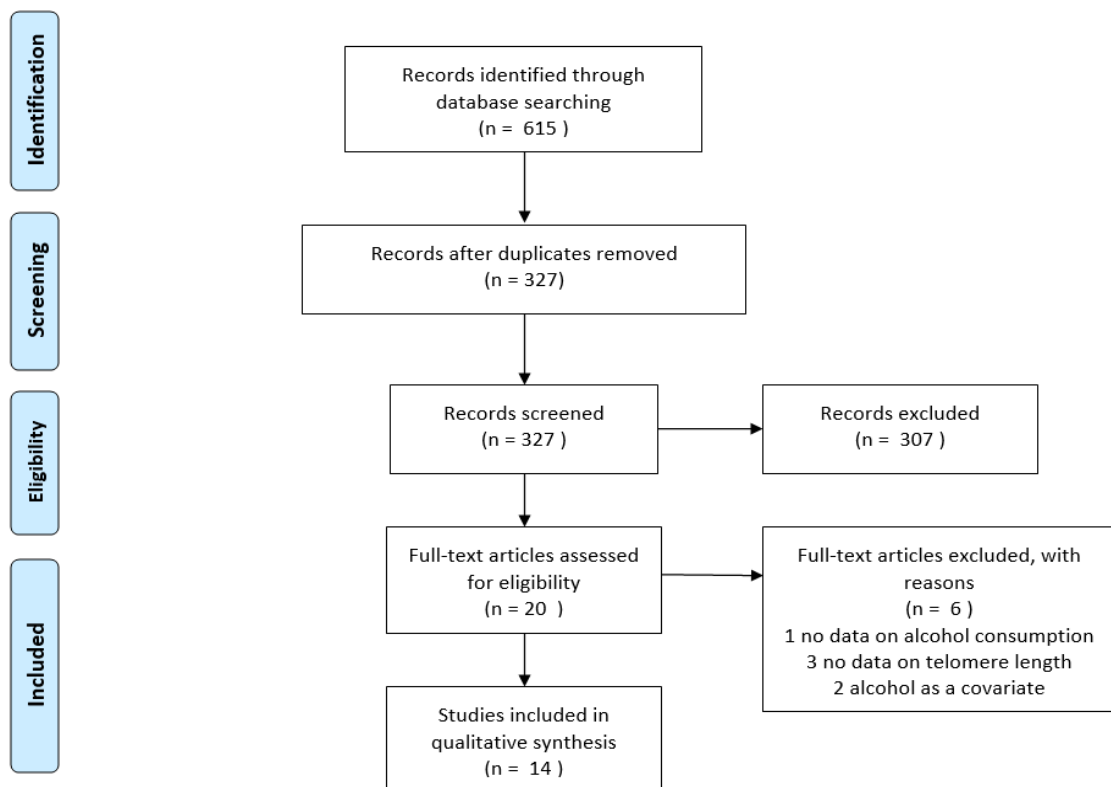
This figure is adapted from Maugeri et al., Int. J. Environ. Res. Public Health 2021

9. The relationship between telomere length and adverse pregnancy outcomes

9.1 Background

Gestational weight gain (GWG) – which depends on body composition, weight of the fetus, placenta, and amniotic fluid [476] - represents a natural response to host the growing fetus. The IOM has promoted recommendations to achieve optimal amount of GWG according to the pre-pregnancy BMI [476, 597-600]. However, during pregnancy more than half of women do not meet guidelines for weight gain [601-603]. The adherence to the recommendations is necessary to reduce the risk of adverse outcomes for mothers and their newborns [176, 331, 334-336]. In fact, both greater and lower weight gain contribute to short- and long-term health complications [597, 604-606]. For instance, excessive GWG is associated with maternal adverse outcomes, such as higher risk of high blood pressure [172], diabetes [173], cesarean section [174], postpartum weight retention [175] and obesity [176]. As regard newborns born from women with excessive weight gain, they are more likely to be large for gestational age [173, 333, 337] and to develop metabolic disorders during lifetime [340, 341]. By contrast, low GWG is associated with increasing risks of pre-term delivery [338]. Moreover, infant mortality in US is higher compared with other developed countries [607, 608], due to a high proportion of newborns with LBW and VLBW, particularly among blacks [609, 610]. In turn, LBW newborns are more likely to die during the neonatal period and who survive is more likely to have complications during adolescence [470]. For all these reasons, there is the need for uncovering molecular mechanisms associated with GWG to identify mothers who could benefit more from preventive strategies.

In this scenario, telomere length represents a promising biomarker for biological aging and age-related diseases. As stated before, telomeres are repeating DNA sequences at the ends of chromosomes that protect the chromosomes from DNA damage [88]. Telomeres progressively shorten with cell division and shorter telomeres are considered as a marker of the cumulative damage to which cells have been exposed [88]. In adults, shorter telomeres are associated with several diseases – such as diabetes [33], cancer [34] and cardiovascular disease [35]. Obesity might contribute to cumulative burden of oxidative stress, accelerating the telomere shortening process. During pregnancy, adverse exposures (e.g., maternal stress, smoking and higher levels of air pollution) are associated with shorter telomeres measured in cord blood [555-557] and placenta [558]. With respect to cell-free circulating fetal DNA (cfDNA), it has been proposed as a novel biomarker for prenatal diagnosis, and its relationship with several diseases has raised much interest

in investigating the role of cfDNA in different body fluids, including amniotic fluid that contains a greater amount of cell-free fetal- and pregnancy-related DNA than maternal serum [60–63]. Up to now, however, there are no study that have investigated the relationship between telomere length and adequate GWG. To do that, we used data and samples from the ongoing prospective "Mamma & Bambino" study for investigating the associations between telomere length – assessed on maternal leucocyte DNA (mlDNA) and cfDNA of amniotic fluid – and GWG.

9.2 Methods

9.2.1 Study design

The "Mamma & Bambino" cohort is an ongoing Italian birth cohort designed to explore the effect of preconception, perinatal and early life exposure on maternal and infant health (further information can be found at http://www.birthcohorts.net). Full details on study design and protocol are reported in the paragraph 5.2.1.

In the current analysis, we used data and samples from mothers who completed singleton pregnancy and with available data on GWG at delivery.

9.2.2 Assessment of Gestational Weight Gain

At recruitment, women were asked to report their height and pre-pregnancy weight to calculate pre-pregnancy BMI as kg/m^2. According to WHO criteria, women were classified as underweight, normal weight, overweight or obese based on their pre-pregnancy BMI, [429]. Firstly, maternal weight got at recruitment was calculated by subtracting the pre-pregnancy weight from the weight at recruitment. Next, maternal weight reached at delivery was collected from clinical records and total GWG was calculated by subtracting the pre-pregnancy weight from the weight at delivery. According to IOM guidelines [601], GWG was classified as reduced, adequate or excessive according to pre-pregnancy BMI.

9.2.3 Covariate Ascertainment

We considered several covariates - beyond anthropometric measures - that might affect GWG, telomere length, and their relationship. At recruitment, socio-economic information and lifestyles were collected through structured questionnaires [298-300, 563-566, 612]. Maternal age and gestational ages at recruitment and at delivery were considered because of their potential effect on sampling and telomere length. In addition, educational level and employment status were used as two proxy indicators of socio-economic status. Educational level was classified as low (i.e., primary education), medium (i.e. secondary education), or high (i.e. tertiary education). Women were categorized as employment or unemployment (that included also students and housewives).

Regarding lifestyles, we classified women according to smoking status, daily energy intake, and adherence to the MD. Specifically, dietary data were collected using a 95-item semi-quantitative FFQ, as described in the paragraph 5.2.3. Daily energy intake was calculated considering the table of food composition released by the USDA (http://ndb.nal.usda.gov/) and adapted to typical Italian foods. Adherence to MD was evaluated using the MDS, as described in detail elsewhere [304, 363].

9.2.4 DNA extraction

Biological samples are collected from mothers and newborns participating in the Mamma & Bambino cohort, at recruitment and at delivery. Sample collection and molecular analyses were fully described elsewhere. In the current study, we used the maternal blood collected at recruitment and an aliquot of amniotic fluid from women who underwent amniocentesis. Genomic mlDNA was extracted from 200 μl of maternal blood while the cfDNA was extracted from 1 ml of uncultured amniotic fluid after centrifugation at 12500 g to remove any remaining cells. DNA extraction was performed using the QIAamp Blood Kit (Qiagen, Milan Italy) on the QIAcube instrument (Qiagen, Milan, Italy), according to the manufacturer's protocol. Concentration and purity of DNA were evaluated by dsDNA HS Assay Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) on the Qubit 3.0 Fluorometer and by NanoDrop 1000 spectrometer.

9.2.5 Relative telomere length

Relative telomere length of mlDNA and cfDNA was assessed using the Relative Human Telomere Length Quantification Assay Kit (ScienCell Research Laboratories, Carlsbad, CA, USA), as previously described [51]. The qPCR was performed on a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, CA, USA), according to the manufacturer's protocol. Full details on the qPCR protocol are described in the paragraph 8.2.3. All reactions were run in duplicate and relative telomere length was expressed as the average of telomere/single copy reference (T/S) ratio.

9.2.6 Statistical analysis

Descriptive statistics was performed using frequencies, or median and IQR. The Kruskal–Wallis test was used for quantitative variables and the Chi-squared test for trend for categorical variables. Relative telomere length was also plotted against weight gain, both at recruitment and at delivery, to inspect linear or non-linear relationships. Next, we plotted relative telomere length by the tertile distribution of GWG, as well as by its classification in reduced, adequate, or excessive. Logistic regression model was performed using adequate GWG as dependent variables and the following covariates: relative telomere length, maternal age, gestational age at recruitment, educational level,

having children, pre-pregnancy BMI, total daily energy intake and gestational age at delivery. The adjusted association of relative telomere length with adequate GWG was reported as coefficient and its Standard error (SE). All tests were two-sided and performed at significance level = 0.05.

9.3 Results

9.3.1 Characteristics of study population

The current analysis included 270 women who completed singleton pregnancy, with complete information on GWG at delivery. Among them, 101 women reported adequate GWG, while 91 and 78 reported reduced and excessive weight gain, respectively. We observed strong relationships between maternal anthropometric measures and GWG (**Table 1**). For instance, women who gained adequate gestational weight were those with the lowest pre-pregnancy weight and BMI. By contrast, weight at delivery increased from reduced to excessive GWG categories. A similar trend was observed for education, with increased proportion of women with low or medium educational level from reduced to excessive GWG. Moreover, the proportion of women with at least one child was higher in those with adequate GWG than their counterparts. With respect to dietary habits, we did not find any association with adherence to MD, but total daily energy intake increased across GWG categories.

9.3.2 Relationships of telomere length with maternal characteristics

We used 252 maternal blood samples to analyse relative telomere length of mlDNA, which did not correlate with maternal age, pre-pregnancy BMI, total energy intake, MDS and gestational age at sampling and at delivery (p-values > 0.05). Moreover, relative telomere length of maternal DNA did not differ across categories of educational level, employment, smoking status, parity, and pre-pregnancy BMI (p-values > 0.05). Next, we used 150 samples of amniotic fluid from women who underwent amniocentesis to evaluate relative telomere length of cfDNA. Relative telomere length of cfDNA and mlDNA did not correlate with each other (p > 0.05). On the contrary, relative telomere length of cfDNA was negatively but weakly correlated with gestational age at sampling (Spearman coefficient = - 0.152; p = 0.046) and positively with total energy intake (Spearman coefficient = 0.157; p = 0.038). No correlations were evident with the other maternal characteristics, as well as with birth length and weight (p-values > 0.05). Moreover, relative telomere length of cfDNA did not differ across categories of educational level, employment, smoking status, parity, pre-pregnancy BMI, type of delivery and newborn gender (p-values > 0.05).

9.3.3 Relationships between gestational weight gain and telomere length

As shown in **Figure 1,** we next evaluated the relationship of relative telomere length in mlDNA and cfDNA with gestational weight gain. Thus, we classified pregnant women according to the tertile distribution of GWG: i) first tertile from -2 to 9 Kg; ii) second tertile from 10 to 13 Kg; iii) third tertile from 14 to 28 Kg. Relative telomere length of mlDNA seemed to weakly increase with GWG (**Figure 1A**). However, we did not find a significant difference according to the tertile distribution of GWG ($p = 0.559$; **Figure 1B**). By contrast, we showed a U-shaped relationship between GWG and relative telomere length of cfDNA (**Figure 1C**). The U-shaped relationship was confirmed by the comparison of relative telomere length across tertiles of GWG ($p = 0.016$; **Figure 1D**). Specifically, as shown in **Figure 1D**, women in the third tertile exhibited shorter relative telomere length than those in the second tertile ($p = 0.014$). Next, we compared relative telomere length across categories of GWG, considering reduced, adequate, or excessive weight gain during pregnancy. With respect to mlDNA, women with excessive GWG showed longer telomere length than those who gained weight adequately ($p = 0.017$; **Figure 2A**). By contrast, telomere length of cfDNA was lower in amniotic fluid from pregnant women with reduced or excessive GWG than those who gained weight adequately (**Figure 2B**). Yet, the difference was statistically significant for mothers with excessive GWG ($p = 0.044$) but not for women with reduced GWG ($p=0.117$; **Figure 2B**). Moreover, the relationship was already evident if considering weight gain at recruitment. Next, we compared relative telomere length of pregnant women who gained weight adequately with those who did not. Our results showed higher relative telomere length of cfDNA in mothers with adequate GWG ($p = 0.017$; **Figure 3**). Finally, we performed logistic regression model including other maternal characteristics (i.e., age, gestational age at sampling, educational level, parity, pre-pregnancy BMI, total daily energy intake, gestational age at delivery) that might affect telomere length and/or GWG. Notably, the association between cfDNA telomere length and adequate GWG remained significant, after adjusting for the abovementioned maternal characteristics ($\beta = 0.464$; SE $= 0.189$; $p = 0.014$).

9.4 Discussion

In our study, we showed for the first time a link between relative telomere length and GWG. We noted a U-shaped relationship when analysing cfDNA in amniotic fluid, with longer relative telomere length among women who gained weight adequately. In this context, a meta-analysis of nearly 120,000 subjects suggested an inverse association between obesity and telomere length [34]. This finding is in line with a collaborative cross-sectional meta-analysis of 87 observational studies and 146,114 individuals, showing a 3.99 bp decrease in telomere length for each unit increase in

BMI [613]. However, there is high degree of heterogeneity across studies – partially due to the effect of chronological age on telomere length – and an overall lack of evidence on pregnant women [34, 613]. Martens and colleagues observed that newborn telomere length decreased with increasing maternal pre-pregnancy BMI, both in cord blood and placental tissues [614]. Notably, Clemente and colleagues demonstrated that this effect seemed to persist in childhood, using data from the Human Early-Life Exposome (HELIX) study [615]. However, the role of telomerase in the association between increased BMI and shortened telomere length is not yet well investigated.

We add to this knowledge, suggesting the influence of maternal weight gain on telomere length of cfDNA from amniotic fluid. The observed difference was already evident in early pregnancy, considering GWG at a median gestational age of 16 weeks. Moreover, the relationship remained significant at delivery, after adjusting for potential confounders (i.e., age, gestational ages at recruitment and at delivery, educational level, previous pregnancies, pre-pregnancy BMI and total daily energy intake). This is in line with the DOHaD hypothesis, for which *in utero* environment programs the fetus for challenges that it is likely to experience after birth [129].

In line, there is the current need for developing non-invasive tests to understand fetal well-being. These tests should be based on maternal serum or urine, avoiding invasive tests such as amniocentesis. Yet, more than 80% of cfDNA fragments in the maternal serum are shorter and fragmented than cfDNA from amniotic fluid, which represent a relatively pure fetal sample. However, it will be interesting to evaluate if fetal DNA from maternal blood reflects the same difference observed in our study.

Our results also provide further motivation to study telomere length and telomerase activity as potential molecular mechanisms underpinning the effects of maternal lifestyles on the development of chronic disease later in life. Although mechanisms by which inadequate weight gain affects telomere length are not yet fully understood, it is plausible that they relies on a chronic inflammatory and oxidative state *in utero* [616].

Our study had some limitations. Firstly, we cannot completely exclude a potential reporting bias due to self-reported information on pre-pregnancy weight. However, previous studies showed how self-reported pre-pregnancy weight well correlated with that measured [489, 490]. Secondly, we did not account for weight trajectories throughout pregnancy. Yet, our analysis at the time of recruitment already showed an effect of maternal weight gain on telomere length of fetal DNA. Thirdly, we cannot completely exclude the presence of placental cfDNA in amniotic fluid samples. Moreover, we assessed relative telomere length by qPCR, which has higher assay variability than

terminal restriction fragment analysis [596]. Finally, the presence of residual confounders cannot be completely ruled out, such as that deriving from fatherly influence [617, 618].

In conclusion, we noted an early influence of GWG on telomere length, which could represent a molecular mechanism underpinning the effects of maternal behaviours on fetal well-being. However, further studies are needed to understand biological events involved in this relationship, and to include other factors influencing the uterine environment during pregnancy.

Table 1. Characteristics of women from the "Mamma & Bambino" cohort (n = 270) according to gestational weight gain categories

Characteristics	Overall (n=270)	Reduced GWG (n=91)	Adequate GWG (n=101)	Excessive GWG (n=78)	p-value
Age	37.0 (4.0)	37.0 (4.0)	38.0 (4.0)	37.0 (4.0)	0.699
Gestational age at sampling	16.0 (4.0)	16.0 (4.0)	16.0 (3.0)	16.0 (2.0)	0.953
Educational level (%)					
Low	17.8%	16.5%	16.8%	20.5%	
Medium	47.8%	40.7%	45.5%	59.0%	**0.038**
High	34.4%	42.8%	37.7%	20.5%	
Working (%)					
Employment	57.4%	54.9%	61.4%	55.1%	0.593
Unemployment	42.6%	45.1%	38.6%	44.9%	
Smokers (%)	20.5%	15.4%	20.0%	27.3%	0.216
Having children (% yes)	67.7%	64.3%	76.8%	59.7%	**0.041**
Total energy intake	1750 (620)	1667 (674)	1752 (545)	1858 (596)	**0.045**
MDS	4.0 (2.0)	4.0 (2.0)	4.0 (2.0)	4.0 (2.0)	0.102
Pre-pregnancy weight	61.0 (15.2)	62.0 (16.0)	59.0 (13.0)	64.5 (18.3)	**0.012**
Pre-pregnancy BMI	22.8 (5.1)	22.8 (4.8)	22.0 (3.8)	25.0 (5.7)	**0.002**
Pre-pregnancy BMI categories					
Underweight	6.7%	6.6%	6.9%	6.4%	
Normal weight	64.1%	68.1%	77.2%	42.3%	**<0.001**
Overweight	17.4%	9.9%	8.9%	37.2%	
Obese	11.9%	15.4%	7.0%	14.1%	
Weight at delivery	74.0 (15.0)	68.5 (11.5)	73.0 (12.7)	82.0 (15.2)	**<0.001**
Gestational age at delivery	39.0 (2.0)	38.0 (2)	39.0 (2)	39.0 (2.0)	0.383

This table is adapted from Maugeri et al., Biomedicines 2022

Figure 1. The relationship of gestational weight gain with relative telomere length
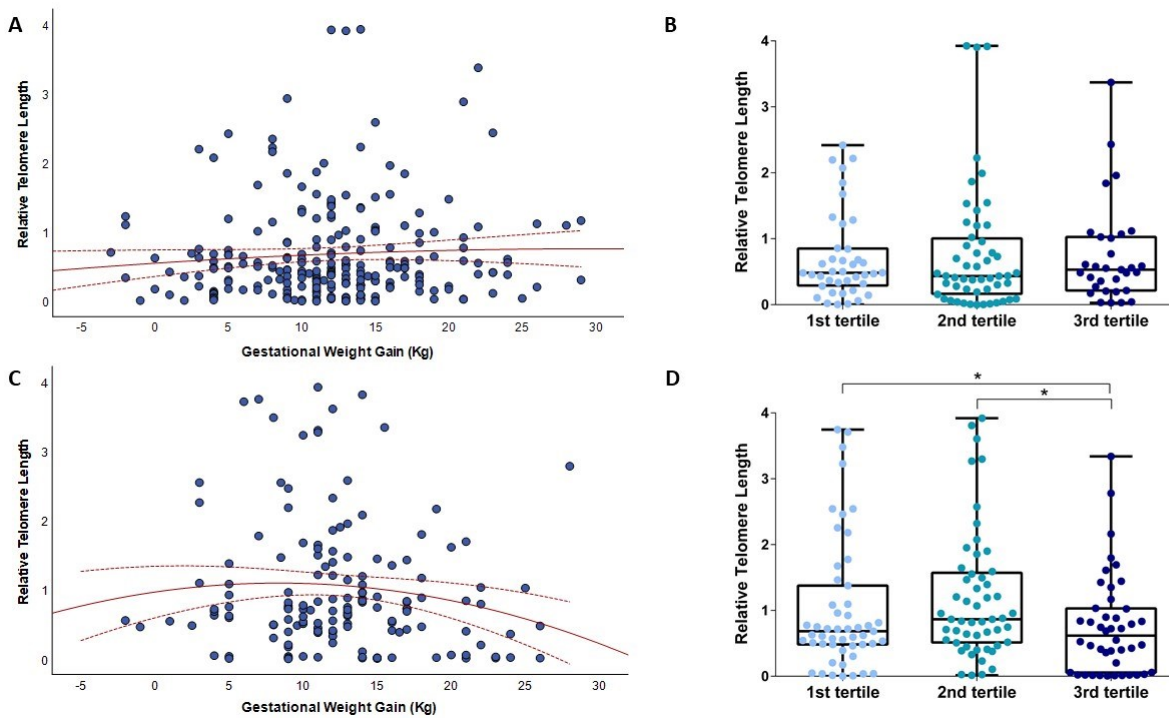
A) shows the relationship of gestational weight gain with maternal telomere length; (B) shows the box plots of maternal telomere length by the tertile distribution of GWG; (C) shows the relationship of gestational weight gain with telomere length of cell-free circulating DNA from amniotic fluid; (D) shows the box plots of telomere length of cell-free circulating DNA from amniotic fluid by the tertile distribution of GWG. * p-value <0.05 based on the Mann-Whitney or Kruskal–Wallis test.

This figure is adapted from Maugeri et al., Biomedicines 2022

Figure 2. The relationship between categories of gestational weight gain and relative telomere length
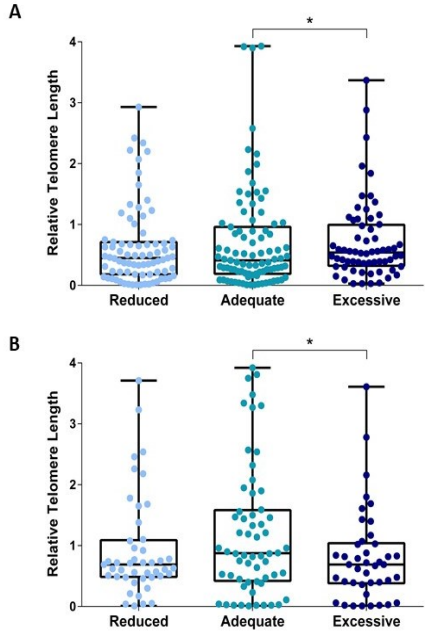
(A) shows the box plots of maternal telomere length according to GWG categories; (B) shows the box plots of telomere length of cell-free circulating DNA from amniotic fluid according to GWG categories. * p-value <0.05 based on the Mann-Whitney test.

This figure is adapted from Maugeri et al., Biomedicines 2022

Figure 3. The comparison of telomere length of cell-free circulating DNA from amniotic fluid between adequate and inadequate gestational weight gain.
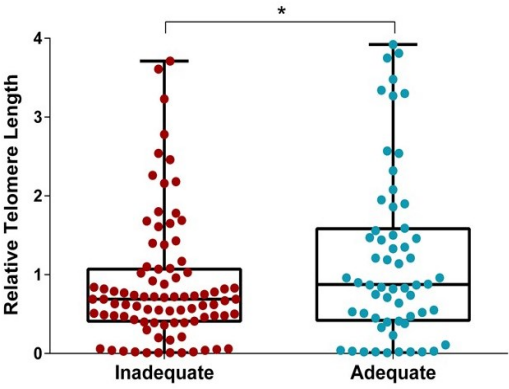
* p-value <0.05 based on the Mann-Whitney test

This figure is adapted from Maugeri et al., Biomedicines 2022

10. The main determinants of adherence to breastfeeding practice and their impact on DNA methylation signatures

10.1 Background

Breastfeeding represents one of the most effective ways to ensure children health [619]. Due to its importance, WHO recommends that: i) mothers should initiate breastfeeding within one hour of birth; ii) infants should be breastfed exclusively for the six months of life to achieve optimal growth and iii) breastfeeding should continue up to two years. However, worldwide, 7.6 million babies each year are never breastfed, with nearly two out of three newborns that are not exclusively breastfed for the recommended six months [620]. As defined by WHO, exclusive breastfeeding means "no other food or drink, not even water, except breast milk", while predominant breastfeeding requires breast milk as the primary source of nourishment allowing for supplementation with liquid (e.g., water and water-based drinks, fruit juice) [621, 622]. Breastmilk contains a huge amount of nutrients - such as proteins, fats, sugars, vitamins and minerals – ideal for infants [623]. The long-term benefits of breastfeeding can't be obtained with infant formula, that does not contain antibodies, hormones and growth factor [624]. Notably, breastfeeding promotes healthy growth and supports healthy brain development [625], as well as protects mothers against postpartum depression, ovarian and breast cancer, cardiovascular disease and diabetes [626]. Therefore, there is the growing need to evaluate the main factors involved in the breastfeeding practice to develop public health intervention for promoting exclusive breastfeeding among women. To identify mothers who could benefit from such public health interventions, however, it is necessary to identify maternal characteristics that might be associated with adherence to recommendations. Indeed, breastfeeding could be influenced by several psychological and physiological factors, which in turn are related to environmental, socioeconomic and cultural conditions [627]. As suggested by UNICEF, one in five babies are never breastfed in high-income countries, while almost all newborns are breastfed in low-income countries. Interestingly, in high-income countries, mothers from poorer households are less likely to breastfeed than the wealthier counterpart [625]. In line, the effects of maternal education and employment status on breastfeeding practice have been investigated by several studies [628-635]. Along with socio-economics determinants, breastfeeding could be affected by others factors, including maternal age [636], smoking [637-640] caesarean delivery [641-644] and complications [645, 646], parity [641, 643] and obesity [642, 647, 648].

Therefore, breastfeeding is associated with long-term health benefits, which might be mediated by epigenetics mechanism. Although DNA methylation has been extensively investigated in several diseases - including cardiovascular diseases, obesity, type-2 diabetes, and cancer [30-32] -, its role on the long-term effects of breastfeeding is not fully understood. Moreover, several lines of evidence support the notion that epigenetic effects of breast milk may not be restricted to DNA methylation, because breastmilk also contains microRNAs, which are in turn involved in gene expression regulation at the post-transcriptional level [649, 650]. However, only few studies have investigated the association between breastfeeding and DNA methylation in humans, and all of them with a small sample size. Furthermore, the evidence is mostly based on candidate gene studies and previous studies are conducted in single cohort [650-655]. With this in mind, there is a need to investigate the relationship between breastfeeding and DNA methylation in studies with larger sample size, using an EWAS approach.

For these reasons, our study firstly aimed to evaluate breastfeeding status and main maternal factors associated with adherence to WHO recommendations among women from the "Mamma & Bambino" study. Moreover, data from the HELIX project were used to analyse the association between any breastfeeding and child blood DNA methylation.

10.2 Methods

10.2.1 The "Mamma & Bambino cohort": study design

The "Mamma & Bambino" cohort is an ongoing Italian birth cohort designed to explore the effect of preconception, perinatal and early life exposure on maternal and infant health (further information can be found at http://www.birthcohorts.net). Full details on study design and protocol are reported in the paragraph 5.2.1.

In this study, we included all mothers who completed pregnancy with a full assessment of information at the 2-year follow-up. By contrast, mothers with plurality, pre-existing medical conditions and pregnancy complications were excluded.

10.2.2 The "Mamma & Bambino cohort": data collection

Information on mothers and their children are collected through face-to-face interviews at recruitment, as well as with planned telephone follow-ups at delivery, at 1 and 2 years. At recruitment, a structured questionnaire was administered by trained epidemiologists to collect information on socio-demographic factors and breastfeeding status. Educational level is categorized as low (≤8 years of school), medium (≤13 years of school) and high education level (greater than 13 years of school). Pregnant women are also classified as full-time employed, part-time employed and unemployed (including students and housewives). Women were also classified as no-smokers,

former and current smokers. Moreover, self-reported maternal pre-pregnancy weight and height were collected at the recruitment to calculate pre-pregnancy BMI, as weight in kilograms divided by height in meters squared and classified according to WHO criteria [429]. GWG was classified as adequate, reduced, or excessive according to IOM recommendations [656]. Dietary data were collected using a 95-item semi-quantitative FFQ, which was referred to the previous month [296, 297, 343, 363, 657]. The adherence to MD was evaluated using MDS as previously described [97, 302, 303, 363]. At birth, women were classified in those who had natural birth and those who had caesarean section. For the current analysis, we also used information regarding self-reported breastfeeding status, which were collected through telephone interviews at 1- and 2-year follow-up after birth. Specifically, we collected information on breastfeeding status (i.e., yes or no) at 1 and 2 years of life, date of starting and ending breastfeeding, type of breastfeeding (i.e., exclusive or predominant), and time of change from exclusive to predominant breastfeeding, if present. Thus, women were categorized into those who have breastfed and those who did not until the 2nd year of life of their child. Moreover, we defined women as those who have exclusively breastfed if their children have received only breast milk and no other liquids or solids for at least one month. During follow-up interviews, we also collect data on complementary feeding. Given that, we considered as outcomes the adherence to WHO recommendations on breastfeeding, which require (1) to initiate breastfeeding within the first hour of life, (2) to exclusively breastfeed for the first six months of life, and (3) to continue breastfeeding receiving complementary foods until 2 years of age.

10.2.3 The "Mamma & Bambino cohort": statistical analyses

Statistical analyses were performed using SPSS software version 26.0 (SPSS, Chicago, IL, USA). Maternal characteristics were described using frequency, or median and IQR. Prior to analysis, the normal distribution of continuous variables was checked using the Kolmogorov–Smirnov test. Accordingly, the Mann–Whitney U test was used to compare across groups the continuous variables underlying skewed distribution. Instead, binary and categorical variables were compared using the simple Chi-square test or the Chi-square test for trend, respectively. For categorical variables, the Cochran–Armitage test for linear trend was used to calculate the statistical power of our analyses. Since statistical power depends on sample size, group weights, and probabilities of exposure, we separately calculated it for each exposure variable according to sample distribution. Specifically, the statistical power ranged from 69% to 97%, assuming a difference of 15% between alternative group probabilities of exposure. We also performed logistic regression analyses to identify main factors associated with breastfeeding status in the general population, and with adherence to the WHO recommendation of exclusive breastfeeding for 6 months in women who

have breastfed. In both analyses, the logistic regression model simultaneously included educational level and employment status, which were associated with general breastfeeding status and/or with adherence to the WHO recommendation in univariate analyses. Both analyses were adjusted for maternal age and tested for interaction between educational level and employment status. Results were reported as OR and 95%CI. All statistical tests were two-sided, and p-values <0.05 were considered statistically significant.

10.2.4 The HELIX project: study design

In the context of my Erasmus experience at the Barcelona Institute for Global Health, ISGlobal (Barcelona, Spain), we used data from the HELIX project. HELIX project involves six existing prospective birth cohort studies in Europe [658]: the Born in Bradford (BiB) study in the United Kingdom [659], the Etude de cohorte généraliste, menée en France sur les Déterminants pré et post natals précoces du développement psychomoteur et de la santé de l'Enfant (EDEN) study in France [660], the INfancia y Medio Ambiente (INMA) cohort in Spain [661], the Kaunas cohort (KANC) in Lithuania [662], the Norwegian Mother and Child Cohort Study (MoBa) [663], and the RHEA Mother and Child Cohort study in Crete, Greece [664]. The HELIX 'early-life exposome' approach involves combining all environmental hazards that mothers and children are exposed to, and linking this to the health, growth, and development of children. Local ethical committees approved the studies that were conducted according to the Declaration of Helsinki. All participating women provided informed written consent. In this study, the population consisted of 788 mothers and their singleton children with information on breastfeeding and DNA methylation. Between December 2013 and February 2016, we obtained information about maternal diet and exposure to environmental contaminants during pregnancy, as well as information on child diet. Moreover, mothers and their singleton offspring were followed up until the children were aged 6 to 12 years. However, in the current analysis we included only European children based on self-reported data.

10.2.5 The HELIX project: exposure and DNA methylation assessment

We assessed breastfeeding status during the follow-up between 6 and 11 years old. Breastfeeding used in this study included: i) any breastfeeding, defined as never breastfed and ever breastfed babies, regardless of the introduction of formula feed and/or solid foods; ii) any breastfeeding duration, as total months that the mother breastfed the baby, considering upper limit of 12 months. Moreover, detailed information on maternal age at birth, gestational age, maternal BMI, maternal education, smoking status during pregnancy, parity, and type of delivery from each study participant was obtained by each cohort during the follow-up interview through a structured

questionnaire. Full details are described elsewhere [658]. In addition, DNA methylation levels in peripheral blood was measured at mean of 8 years. Full details are described elsewhere [658].

10.2.6 The HELIX project: statistical analysis

We performed statistical analysis using R studio. We checked the normal distribution of continuous variables using the Shapiro-test. Descriptive statistics were used to characterize the study population using frequency (%) or median and IQR. We explored the association between breastfeeding and the covariates - including cell type proportions - by using Mann-Whitney U for continuous variables and Chi-square test for categorical variables. Robust linear regression models were performed to test the association between breastfeeding and child blood DNA methylation, taking into account only complete cases and adjusting for batch effect and cohort. Q-Q plot was used to compare our samples vs. theorical distributions by plotting their quantiles against each other, while Volcano Plot allowed to see the most highly differentially methylated CpGs, by plotting the negative log of the p value on the y axis and the beta coefficients for each CpGs in the x axis. Two methods were applied to control multiple-testing: Bonferroni correction, that sets the significance cut-off at α/n, and FDR correction, defined as the proportion of false positives among all significant results (FDR < α).

10.3 Results

10.3.1 The "Mamma & Bambino cohort": characteristics of study population

The present analysis included 220 pregnant women (median = 37 years; median gestational age = 16 weeks) from the "Mamma & Bambino" cohort, with available information on breastfeeding status. In brief, 66.7% of women had at least one child. Moreover, 85.0% women exhibited a medium-high education level, while 58.2% were employed. With respect to lifestyles, 17.4% were current smokers and nearly 60.0% reported a medium adherence to MD. According to BMI (mean = 23.38; SD = 4.39), nearly 26% of women were overweight or obese, with 37.0% of pregnant women with adequate GWG. According to breastfeeding, 181 women declared to have breastfed. Specifically, 123 of them (68.0%) initiated breastfeeding within one hour of birth, out of which 81 (65.9%) have exclusively breastfed for the first 6 months. However, only 14 women (7.7%) who have breastfed continued until 2 years of age. (**Figure 1**).

10.3.2 The "Mamma & Bambino cohort": association between maternal characteristics and breastfeeding status

As shown in **Table 1**, according to the breastfeeding status, the proportion of women who have breastfed increased with increasing educational level (p = 0.001). Logistic regression analysis

confirmed that medium (OR = 3.171; 95% CI = 1.285-7.822; p = 0.012) and high educational level (OR = 4.549; 95% CI = 1.525- 13.570; p = 0.007) were positively associated with breastfeeding if compared with low educational level. By contrast, no association with employment status was evident (**Table 2**).

10.3.3 The "Mamma & Bambino cohort": association between maternal characteristics and adherence to breastfeeding recommendations

As shown in **Table 3,** we next compared maternal characteristics according to their adherence to the WHO recommendation of exclusive breastfeeding for the first six months. Interestingly, the proportion of women who have exclusively breastfed for six months increased with increasing educational level (p = 0.018). Moreover, the proportion of pregnant women who adhered to this recommendation was higher among those who were employed than those who were unemployed (p = 0.015). In line, medium–high educational level and being employed were positively associated with exclusive breastfeeding for the first six months. Specifically, the logistic regression analysis demonstrated that full-time employed women (OR = 2.158; 95% CI = 1.033–4.508; p = 0.041) and those who reported medium educational level (OR = 4.632; 95% CI = 1.227–17.484; p = 0.024) were more likely to adhere to the WHO recommendation than their less educated and unemployed counterparts. A borderline significant association of part-time employment and high educational level with adherence to the WHO recommendation was also evident **(Table 4).**

10.3.4 The HELIX project: study population

Overall, 788 European children were included in the present analysis. Each cohort made its contribution: UK (BiB) with 72 samples, France (EDEN) with 136 samples, Norway (MoBA) with 189 samples, Greece (RHEA) with 187 samples and Spain (INMA) with 204 samples. Among women, 84.1% of them have breastfed. Particularly, proportion of women who breastfed ranged from 94.7% (Norwegian women) to 61.8% (French women). About women characteristics, we observed that nearly 51% of women reported high educational level, and only 9% of women were smokers during pregnancy.

10.3.5 The HELIX project: major determinants of breastfeeding

We next evaluated major determinants of breastfeeding. Firstly, we tested the relationship between breastfeeding and quantitative variables, such as child and maternal age, maternal BMI, birth weight, gestational age, and cell types, using any breastfeeding (ever vs. never) as exposure. Particularly, we observed that child age (p = 0.01) and maternal BMI (p = 0.009) were higher among the ones who had never breastfed, while birth weight was higher among who had breastfed

(p = 0.02). With regard to cell proportions, B-cell and CD8T proportions were higher among who had breastfed (p = 0.003 and p = 0.02, respectively). By contrast, granulocytes proportion was lower among who had breastfed (p = 0.001). These results were similar when testing the association between breastfeeding duration and cell proportions. Particularly, Natural Killer (NK) cells and lymphocytes increased with increasing breastfeeding duration, instead of granulocytes that decreased with increasing breastfeeding duration. Next, we tested the relationship between breastfeeding and qualitative variables, such as sex, maternal education, parity, type of delivery and smoking, using any breastfeeding (yes or no) as exposure. Interestingly, the proportion of women who breastfed increased with increasing educational level (71.1% vs. 80.4% vs. 89.2%; p = 0.01), while proportion of women who breastfed was higher among no-smokers (86.1% vs. 61.3%; p<0.01).

10.3.6 The HELIX project: association between breastfeeding and DNA methylation

We first conducted a robust linear regression model using any breastfeeding (ever vs. never) as exposure and adjusting for all the covariates. We noted that the two distributions were similar, with almost all the points in the line y=x, and there was no strong indication of genome-wide inflation for breastfeeding (λ = 0.97). Moreover, only few CpGs were under the suggestive threshold that we used (p-value <1E-05). However, no CpGs passed multiple-testing (i.e Bonferroni correction and FDR). Next, we conducted a robust linear regression model using breastfeeding duration as exposure and adjusting for all the covariates. Similarly, we noted that the two distributions were similar, with almost all the points in the line y=x, and there was no strong indication of genome-wide inflation for breastfeeding (λ = 1.04). Moreover, only few CpGs were under the threshold that we used (p-value <1E-05). However, no CpGs passed multiple-testing (i.e Bonferroni correction and FDR).

10.4 Discussion

Worldwide, human breast milk is considered the optimal source for infant nutrition due to its short-term and long-term health benefits [619, 665] that might be mediated by molecular mechanisms [463]. Similarly, long-term breastfeeding has been associated with benefits for mothers [666-668]. In this scenario, we found that 82.3% of women have breastfed, regardless of breastfeeding type (i.e., exclusive or predominant). Interestingly, we noted that the proportion of women who have breastfed increased with increasing educational level. As suggested by logistic regression analysis, medium and high educational level were positively associated with breastfeeding after adjusting for maternal age and employment status. This pointed out the socioeconomic gaps in breastfeeding

attitudes between mothers with different educational level. According to previous studies [669, 670], women with high educational level were more likely to seek medical advice and exploit health services with regard to breastfeeding [671]. Interestingly, socioeconomic factors could also influence breastfeeding duration and its exclusivity. However, in our study, maternal characteristics did not seem associated with between exclusive or predominant breastfeeding. In a public health point of view, it is important that newborns are breastfed exclusively during the first six months of life. However, compliance with breastfeeding recommendations depends on several sociocultural factors [636]. In our study, nearly 64.0% of women declared to have exclusively breastfed for six months, and we noted that the proportion of women who adhered to breastfeeding recommendations increased with increasing educational level. Moreover, we demonstrated that full-time employed women were more likely to breastfeed. These findings could be explained by the positive effects that education has on breastfeeding knowledge in general, and on compliance with recommendations. With respect to employment status, it is important to consider the positive impact of Italian parental leave, which allows employed women to continue exclusive breastfeeding for the recommended period. Taking into account socioeconomic factors, few studies demonstrated that younger mothers with low educational level were more likely to interrupt breastfeeding before six months [672], as well as that the combined effects of age and educational level was so strong that others socioeconomic factors were no significant in the adjustment model [636]. With respect to employment status, healthcare advisors should inform women how best to continue breastfeeding when returning to work. Six studies evaluated the association between returning to work within 12-week post-birth and the cessation of breastfeeding before six months of life [628-633]. In particular, women who started working early after delivery were more likely to use infant formula, giving up exclusive breastfeeding [629]. Thus, time chosen for re-start working and types of job could explain differences in adherence to breastfeeding recommendations among mothers. Aside from socio-economics determinants, breastfeeding could be affected by others factors (maternal pre-pregnancy obesity, higher risk of non-initiation and shorter breastfeeding duration [673, 674], and smoking during lactation) [675, 676] However, in our study we did not find significant association between the above-mentioned factors and breastfeeding. In this scenario, breastfeeding programs should be implemented for all mothers, with specific interventions tailored toward less educated mothers. Particularly, public health strategies are needed to identify cultural factors that support infant feeding, in order to promote exclusive breastfeeding, which in turn affects maternal and neonatal health. Moreover, long-term effects might be mediated by epigenetic mechanisms, but evidence in humans is scarce and mostly based on candidate gene studies and on single cohort [650-655].

However, EWASs can offer new insights into DNA–environment interactions in determining child development and health [650]. The novelty of the analysis conducted in the HELIX cohorts was the multi-cohort study with larger sample size and the use of an EWAS approach to find DNA methylation changes associated with breastfeeding. Firstly, our results indicated that the proportion of Lymphocytes (CD4T and CD8T) were higher among who had breastfed, and increased with increasing breastfeeding duration, as well as the proportion of NK cells. By contrast, the proportion of Granulocytes were lower among who had breastfed and decreased with increasing breastfeeding duration. These cells are involved in several diseases and conditions, such as respiratory disorders [677, 678]. Thus, breastfeeding might protect for asthma, which in turn involves all the cells type above-mentioned [679]. However, further analysis is needed to better explain the twofold contribution of the reduction of granulocytes – that could explain the protective effect of breastfeeding on asthma – and, by contrast, of the increased proportion of lymphocytes and NK cell – that are involved in the asthmatic process. Furthermore, our list of top CpGs mapped to protein coding genes, but also to non-coding RNAs, such as microRNAs. Among the top associations, we identified that methylation of cg15118824 - located in the Human Leucocyte Antigen E (*HLA-E* gene) – decreased with increasing breastfeeding duration. In general, decreased methylation is associated with increased gene expression. *HLA-E* has been involved in the inhibitory effect on the cytotoxic activity of the NK cell, which in turn could link with the beneficial effects of breastfeeding on allergic diseases. None of the genes detected could be functionally linked to other beneficial effects of breastfeeding, such as obesity or neurodevelopment. However, further studies are needed to better understand the potential relationship and effect of breastfeeding on the methylation sites that we are detected. With respect to scientific literature, our top ten CpGs with lowest p-value were not reported among top CpGs in other studies. Thus, the current study was preliminary for more detailed analysis useful for a better interpretation of the results.

Both analyses had some limitations. With respect to the "Mamma & Bambino cohort", we cannot completely exclude potential associations in those tests with low statistical power, as well as potential association with factors that were previously related to breastfeeding. Moreover, data on breastfeeding were self-reported through telephone interviews, which did not preclude potential reporting errors. With respect to socio-demographic information on mothers, we collected the information at recruitment, without considering any changes occurred during the follow-ups at 1 and 2 years. Moreover, the proportion of infants who were breastfed until 2 years was low, not allowing us to determine what factors might be associated with prolonged breastfeeding with complementary feeding. With respect to the analysis conducted in the HELIX cohorts, the first

weakness was the definition of exposure, because the results are related to the binary categorisation, which includes highly heterogeneous individual regarding breastfeeding quality and type of foods given concurrently with breastmilk (for individuals that were not-exclusively breastfeed). Moreover, results are related to the duration of breastfeeding, which is likely prone to substantial measurement error in the self-reported months of duration. In addition, samples are restricted to peripheral blood. Further analyses in larger samples with more detailed definitions of breastfeeding will be useful to understand the biological basis of long-term associations between breastfeeding and healthy growth.

Table 1. Maternal characteristics according to breastfeeding practice

Characteristics	Overall (n=220)	Breastfeeding (n=181)	Not Breastfeeding (n=39)	p-value
Age (*years*)	37.0 (5.0)	37.0 (4.0)	38.0 (4.0)	0.656
Educational level				
Low	33 (15.0%)	20 (11.0%)	13 (33.3%)	**0.001**
Medium	106 (48.2%)	89 (49.2%)	17 (43.6%)	
High	81 (36.8%)	72 (39.8%)	9 (23.1%)	
Employment status				
Full-time employed	88 (40.0%)	77 (42.5%)	11 (28.2%)	0.240
Part-time employed	36 (16.4%)	29 (16.0%)	7 (17.9%)	
Unemployed	96 (43.6%)	75 (41.4%)	21 (53.8%)	
Smoking status				
Current	38 (17.4%)	30 (16.8%)	8 (20.5%)	0.601
Former	52 (23.9%)	45 (25.1%)	7 (17.9%) %	
Never	128 (58.7%)	104 (58.1%)	24 (61.5%)	
Pre-gestational BMI	22.7 (4.5)	22.6 (4.5)	23.2 (4.9)	0.768
Gestational weight gain				
Reduced	86 (39.3%)	72 (40.0%)	14 (35.9%)	0.291
Adequate	81 (37.0%)	69 (38.3%)	12 (30.8%)	
Excessive	52 (23.7%)	39 (21.7%)	13 (33.3%)	
Parity				
Yes	138 (66.7%)	115 (68.5%)	23 (59.0%)	0.258
No	69 (33.3%)	53 (31.5%)	16 (41.0%)	
Adherence to Mediterranean diet				
Low	76 (34.7%)	61 (33.9%)	15 (38.5%)	0.638
Medium	131 (59.8%)	110 (61.1%)	21 (53.8%)	
High	12 (5.5%)	9 (5.0%)	3 (7.7%)	
Type of delivery				
Natural	121 (57.3%)	100 (57.8%)	21 (55.3%)	0.774
Caesarean	90 (42.7%)	73 (42.2%)	17 (44.7%)	

This table is adapted from Magnano San Lio et al., Medicina 2021

Table 2. Association between educational level and employment status with breastfeeding

Characteristics	OR	95% CI	p-value
Educational level			
Low	*ref*		
Medium	3.171	1.285-7.822	**0.012**
High	4.549	1.525- 13.570	**0.007**
Employment status			
Unemployed	*ref*		
Part-time employed	0.922	0.339-2.508	0.874
Full-time employed	1.206	0.489-2.977	0.684

This table is adapted from Magnano San Lio et al., Medicina 2021

Table 3. Characteristics of women who have breastfed, according to the WHO recommendation of 6-month exclusive breastfeeding.

Characteristics	Adherent (n=81)	Non-adherent (n=100)	p-value
Age (*years*)	37.0 (4.0)	38.0 (4.0)	0.293
Educational level			
Low	3 (3.7%)	17 (17.0%)	**0.018**
Medium	43 (53.1%)	46 (46.0%)	
High	35 (43.2%)	37 (37.0%)	
Employment status			
Full-time employed	41 (50.6%)	36 (36.0%)	**0.015**
Part-time employed	16 (19.8%)	13 (13.0%)	
Unemployed	24 (29.6%)	51 (51.0%)	
Smoking status			
Current	13 (16.3%)	17 (16.3%)	0.983
Former	20 (25.0%)	25 (25.3%)	
Never	47 (58.8%)	57 (57.6%)	
Pre-gestational BMI	22.3 (4.2)	22.7 (4.8)	0.236
Gestational weight gain			
Reduced	37 (46.3%)	35 (35.0%)	0.304
Adequate	27 (33.8%)	42 (42.0%)	
Excessive	16 (20.0%)	23 (23.0%)	
Parity			
Yes	48 (66.7%)	67 (69.8%)	0.666
No	24 (33.3%)	29 (30.2%)	
Adherence to Mediterranean diet			
Low	25 (30.9%)	36 (36.4%)	0.730
Medium	52 (64.2%)	58 (58.6%)	
High	4 (4.9%)	5 (5.1%)	
Type of delivery			
Natural	45 (58.4%)	55 (57.3%)	0.879
Caesarean	32 (41.6%)	41 (42.7%)	

This table is adapted from Magnano San Lio et al., Medicina 2021

Table 4. Association between educational level and employment status with adherence to the WHO recommendation of 6-month exclusive breastfeeding

Characteristics	OR	95% CI	*p-value*
Educational level			
Low	*ref*		
Medium	4.632	1.227-17.484	**0.024**
High	3.727	0.925-15.009	0.064
Employment status			
Unemployed	*ref*		
Part-time employed	2.423	0.978-5.999	0.056
Full-time employed	2.158	1.033-4.508	**0.041**

This table is adapted from Magnano San Lio et al., Medicina 2021

Figure 1. Distribution of women according to breastfeeding status and WHO recommendations
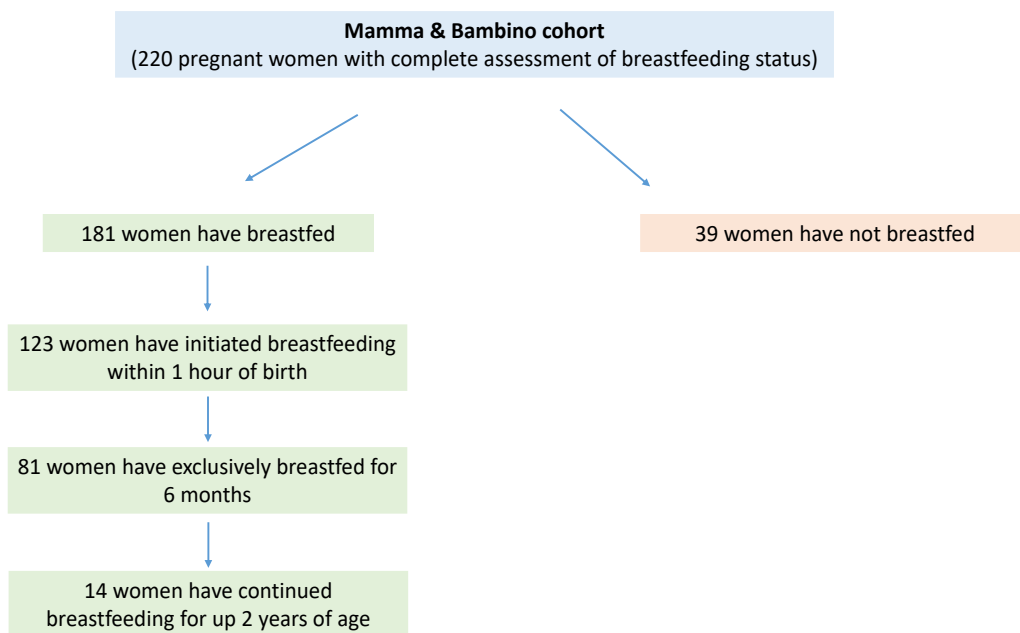
This figure is adapted from Magnano San Lio et al., Medicina 2021

11. The effects of antioxidant and inflammatory potential of diet on the risk of HPV infection and cervical cancer

11.1 Background

Bioactive food components, as well as the adherence to specific dietary patterns, might be associated with the risk of female cancer. Cervical cancer represents the fourth most common cancer in women after breast, colorectal and lung cancers [680]. The persistency of high-risk human papillomavirus (HPV) infection is a necessary but not a sufficient event for the progression of cervical intraepithelial neoplasia (CIN) to cervical cancer [681] [612, 682-685]. Chronic inflammation and oxidative stress might be involved in the etiology of cervical cancer, activating HPV-associated carcinogenesis and sustain tumor progression [686-688]. In fact, a chronic inflammatory process leads to the production of reactive oxygen species (ROS) and the release of pro-inflammatory factors, such as IL-1 and IL-6, tumor necrosis factor-α, and interferon gamma [682]. Moreover, chronic inflammation seems also associated with a decreased level of antioxidants in HPV-infected cells [689], with a decreasing antioxidant activity against ROS at cellular level, and hence an increasing oxidative DNA damage [686, 687]. Although oxidative stress is considered a mechanism underpinning the host cellular response to viral infection, it has also been demonstrated that some viruses could thrive in an oxidative environment [690]. With respect to HPV, the virus induces oxidative stress and DNA damage, leading to cancer progression [687, 691]. The interaction between chronic inflammation and oxidative stress can partially explain the biological aspects underpinning the effect of various factors implicated in cervical cancer development – as smoking status, dietary deficiencies, sedentary lifestyle, and use of contraceptives [268, 683, 686, 687, 692, 693]. Among these, diet have been estimated to contribute to 20–60% of cancers [694]. The adherence to a healthy dietary pattern protects against several non-communicable diseases, including cervical cancer [268, 276-278, 362, 695]. In this scenario, several studies have investigated the antioxidant and anti-inflammatory impact of specific foods and nutrients against HPV infection and cervical cancer progression (i.e., polyphenols, vitamins A, C, and E, folates, carotenoids, and minerals) [264, 268, 686, 696, 697]. Notably, several lines of evidence have proposed an inverse relationship between antioxidant plasma concentrations and the risk of HPV infection [698-704]. However, findings are currently inconclusive [252].

The combined effect of foods and nutrients with antioxidant and anti-inflammatory activity remains to be clarified. On one hand, only one study reported the relationship between the adherence to a proinflammatory diets and an increased risk of cervical cancer, warranting further studies to

confirm their findings [705].On the other hand, instead, there is a lack of supporting evidence about the effect dietary antioxidant intake on the risk of cervical cancer. In a previous study on women with normal cervical epithelium, we showed a negative association between dietary antioxidant intake and the risk of HPV infection [657].

Here, we first aimed to evaluate the associations of the Composite Dietary Antioxidant Index (CDAI) and the Dietary Inflammatory Index (DII) with the prevalence of cervical cancer among Italian women. Next, we have assessed dietary intake of antioxidants and CDAI among women with normal cervical cytology from Catania, Italy. Moreover, we aimed to evaluate differences in dietary antioxidant intake and CDAI between hrHPV positive and hrHPV negative women to test the association between cumulative antioxidant intake and hrHPV infection

11.2 Methods

11.2.1 Study design

In the present cross-sectional analysis, we used data from a previously published study on women who referred to the Cervical Cancer Screening Unit of the Azienda Sanitaria Provinciale (ASP) in Catania, Italy [102, 268]. Full details have been described elsewhere [102, 268]. The study has been approved by the ethics committee of the involved Institution (CE Catania 2; Prot. N. 227/BE and 275/BE) and conducted in line with the Declaration of Helsinki. In brief, during a three-year period, the study recruited women with an abnormal Papanicolaou (PAP) test prior to undergoing treatment. Women were tested for high-risk HPV infection using the Digene HC2 HPV DNA Test (Qiagen, Milan, Italy), and then classified as: i) women with normal cervical epithelium or CIN1 (Controls) and ii) women with a diagnosis of CIN2 or more severe lesions (CIN2+) according to histological diagnosis. All women who completed the interview and subjected to HPV DNA test and histological examination were included in the first analysis to evaluate the associations of the CDAI and the DII – with the prevalence of cervical cancer among Italian women. Next, we have assessed dietary intake of antioxidants and CDAI among the subgroup of women with normal cervical and complete assessment of dietary antioxidant intake, sociodemographic and behavioral information. Each woman was tested for hrHPV using the Digene HC2 HPV DNA Test (Qiagen, Milan, Italy), and categorized as hrHPV positive or hrHPV negative.

11.2.2 Data collection

At recruitment, women were interviewed to collect sociodemographic information and behavioral factors. With respect to social characteristics, women were classified as having a low (primary education) or medium-high (secondary and tertiary education) educational level, and as being employed or unemployed. Regarding behaviors, women were asked to report their height and

weight to calculate their BMI as defined by the WHO [293]. Women were also classified according to their smoking status as current or non-current smokers (i.e., including both former and never smokers). Finally, women were asked to indicate if they used oral contraceptives and supplements (i.e., including multivitamin and/or multi-mineral supplements). However, no information about the regimen and dose of specific antioxidant administration were available.

11.2.3 Dietary assessment

Dietary assessment and the semi-quantitative FFQ used in this study were fully described elsewhere [97, 268, 297, 343, 363]. In brief, the FFQ was used to calculate daily intake of foods and nutrients with antioxidant and anti- or pro-inflammatory properties during the month before the recruitment. In particular, nutrient composition of each food was determined using the U.S. Department of Agriculture (USDA) Food Composition Database.

Prior to further analyses, dietary intakes were adjusted for total energy intake using the residual method [706]. Based on energy-adjusted nutrient intakes, we computed two dietary indexes which reflected the cumulative dietary antioxidant intake and the inflammatory potential of diet. In particular, we adapted the CDAI - proposed by Wright and colleagues [707] - and the DII developed by Shivappa and colleagues [708]. The CDAI was calculated as the sum of z-scores of the dietary intakes of zinc, selenium, magnesium, vitamin A, vitamin C, vitamin E, β-carotene, and flavonoids. With respect to the DII, we first calculated the z-score for each dietary factor. Next, z-scores were converted into percentile-scores, with a symmetrical distribution (i.e., mean 0 and range from -1 to 1). The DII was then calculated as the sum of the products of percentile-scores of dietary factors and their respective inflammatory effect scores. Inflammatory effect scores were previously derived from a systematic review of studies investigating the inflammatory potential of 45 dietary factors based on six inflammatory markers (i.e. C-reactive protein, IL-1β, IL-4, IL-6, IL-10, and tumor necrosis factor-alpha) [708]. In the current study, we used the following 33 of the 45 dietary factors considered in the original DII: alcohol, vitamin B12, vitamin B6, β-carotene, coffee, carbohydrates, cholesterol, calories, total fats, fiber, folic acid, iron, magnesium, MUFA, niacin, proteins, PUFA, riboflavin, saturated fats, selenium, thiamin, trans fats, vitamin A, vitamin C, vitamin D, vitamin E, zinc, black/green tea, flavan-3-ol, flavones, flavonols, flavonones, and anthocyanidins. Finally, women were classified according to the tertile distribution of each dietary index, so that the cumulative dietary antioxidant intake and the pro-inflammatory potential increased from the 1st to the 3rd tertile.

11.2.4 Statistical analyses

All statistical analyses were performed on Stata (version 16.0) and SPSS software (version 22.0). Prior to analysis, the Kolmogorov-Smirnov test was applied to test the normality of quantitative variables. Descriptive statistics were used to characterize the study population, using percentages for qualitative variables and median with IQR for quantitative variables. Mann-Whitney U test or the Kruskal–Wallis test were used for comparisons of quantitative variables, and the Chi-square test for qualitative variables. For instance, continuous variables underlying non-normal distribution were compared using the Mann-Whitney U test for comparisons between hrHPV positive and negative women or using the Kruskal–Wallis test for comparisons across CDAI categories.

The Bonferroni method was applied to adjust for multiple comparisons. Different regression models were used for the analysis. Specifically, logistic regression models were applied to evaluate the association of dietary indexes with histological diagnosis of CIN2 or more severe lesions. For the DII index, results were reported as OR and 95% CI of women in the 2nd or 3rd tertile compared with those in the 1st tertile. For the CDAI index, the reference group was constituted by women in the 3rd tertile. Model 1 was adjusted for age and HPV status, while Model 2 further adjusted for several confounders (i.e., educational level, BMI, smoking status, parity, use of oral contraceptives and supplements.

Moreover, linear regression models were used to evaluate the association of social and behavioral factors with CDAI. Next, logistic regression models were used to investigate the associations of dietary antioxidant intake with hrHPV status. The models included the standardized dietary intake of antioxidants (i.e., either separately or simultaneously) or the CDAI (i.e., both as continuous and as categorical) as independent variables, and were adjusted for age, smoking status, BMI, parity, educational level, marital status, and use of supplements and oral contraceptives. All statistical tests were two-sided, and p value < 0.05 was considered as statistically significant.

11.3 Results

11.3.1 Study population

Overall, the present study recruited 539 women with a mean age of 40.2 years (SD = 10.0 years). All women satisfied the inclusion criteria for the analysis of the association of antioxidant and inflammatory potential of diet with cervical cancer. **Figure 1** shows the study population according to high-risk HPV status and histological diagnosis. Specifically, this study included 237 HPV-negative women with normal cervical epithelium and 302 HPV-positive women. Among the latter, 175 women showed normal cervical epithelium or a diagnosis of CIN1, while the remaining 127 were diagnosed with CIN2 or more serious lesions.

11.3.2 Intake of foods and nutrients with antioxidant and anti-inflammatory potential

As shown in **Figure 2** and **Table 1**, CIN2+ women reported lower intakes of calories and other foods with antioxidant and anti-inflammatory potential (i.e., coffee, black and green tea, carbohydrates, fiber, PUFA, vitamins A, B6, C, and E, β-carotene, niacin, magnesium, and flavonoids). However, none of the abovementioned foods and nutrients was significantly associated with histological diagnosis after adjusting for multiple comparison.

11.3.3 Composite dietary antioxidant index

Next, we used the CDAI to investigate the synergistic effect of dietary antioxidants, classifying women into tertiles. Women with higher CDAI were older ($p < 0.001$) and heavier ($p < 0.001$). Moreover, women with higher CDAI were less educated ($p = 0.001$), more likely to have children ($p < 0.001$), and less prone to use oral contraceptives than their counterpart (1st tertile). We also observed a lower proportion of HPV-positive among women with higher CDAI ($p < 0.001$) (**Table 2**).

11.3.4 Dietary inflammatory index

Similarly, we computed the DII and categorized women into tertiles. Women with higher DII were younger ($p < 0.001$) and thinner ($p < 0.001$). Moreover, women in the 3rd tertile were more educated ($p < 0.001$), less likely to have children ($p < 0.001$) and more likely to be smokers ($p < 0.001$) than those in the 1st tertile. Women with higher DII were also more prone to use oral contraceptives ($p < 0.001$) and less prone to use supplements ($p = 0.020$) than their counterpart. With respect to HPV status, we noted more HPV-positive women among those with higher DII ($p < 0.001$) **(Table 2).**

11.3.5 The associations of dietary antioxidant and inflammatory indexes with cervical cancer

In line with findings presented above, we next evaluated the associations of CDAI and DII with CIN2+ status. As shown in **Figure 3A,** the proportion of women with CIN2 (or more severe lesions) decreased from the 1st tertile to the 3rd tertile of CDAI. However, this difference was not statistically significant ($p = 0.070$). In fact, as shown in **Table 3,** we did not demonstrate an association between CDAI and CIN2+ status also considering the effect of covariates. In contrast, **Figure 3B** shows that the proportion of women with CIN2 or more severe lesions significantly increased from the 1st tertile to the 3rd tertile of DII ($p < 0.001$). Accordingly, prevalence of CIN2 or more severe lesions increased with increasing diet's inflammatory potential. In fact, women with medium (2nd tertile) or high DII (3rd tertile) had higher odds than those with low DII (1st tertile) (OR = 2.02; 95%CI = 1.05–3.88; $p = 0.035$ and OR = 2.51; 95%CI = 1.33–4.73; $p = 0.004$,

respectively) after adjusting for age and HPV status. Notably, women in the 2nd and the 3rd tertiles exhibited higher odds of CIN2+ than those in the 1st tertile (OR = 2.15; 95%CI =1.11–4.17; p = 0.024 and OR = 3.14; 95%CI = 1.50–6.56; p = 0.002, respectively) after adjusting for age, HPV status, educational level, BMI, smoking status, parity, use of oral contraceptives and supplements (**Table 3**).

11.3.6 Dietary intake of antioxidants according to HPV status

Next, we aimed to assess dietary intake of antioxidants and CDAI among women with normal cervical cytology, evaluating differences in dietary antioxidant intake and CDAI between hrHPV positive and hrHPV negative women. Thus, for this analysis we included 251 women with a median age = 46.5 years (IQR = 19.0). The characteristics of women according to their hrHPV diagnosis were fully reported in a previous study [268]. In brief, 33.5% of women (n = 84) were hrHPV positive and, if compared to their hrHPV negative counterpart, they were younger (p < 0.001), more likely to be smokers (p = 0.003), less likely to be obese (p < 0.004) and to have children (p < 0.001).

Table 4 shows total energy intake and dietary intake of antioxidants according to hrHPV diagnosis. In general, hrHPV positive women reported a lower total energy intake than their hrHPV negative counterpart. This was consistent with a lower intake of zinc, manganese, vitamins A and C among hrHPV positive women. In particular, we noted a negative association between dietary intake of zinc and hrHPV positive status (OR = 0.49; 95%CI = 0.27-0.89; p = 0.018, for one-unit increase in the standardized intake) after adjusting for potential confounders (i.e., age, smoking status, BMI, parity, educational level, marital status, and use of supplements and oral contraceptives). Notably, this negative relationship remained significant when considering the effect of all antioxidants simultaneously (OR = 0.46; 95%CI = 0.27-0.80; p = 0.006, for one-unit increase in the standardized intake).

11.3.7 Population characteristics according to the Composite Dietary Antioxidant Index

Next, we computed the CDAI and classified each woman according to the tertile distribution to evaluate the synergistic effect of dietary antioxidants. As reported in **Table 5**, women with higher CDAI were older and more likely to live in couple, to be less educate, and to have children, but less likely to be normal weight and to use oral contraceptives than those with lower CDAI. However, only age remained positive associated with CDAI when all these factors were evaluated simultaneously ($\beta = 0.34$; SE = 0.04; p < 0.001).

11.3.8 Association of Composite Dietary Antioxidant Index with HPV status

Our results suggested that CDAI was lower among hrHPV positive women than their negative counterpart (**Figure 4A**). In line, the proportion of hrHPV positive women decreased with increasing CDAI (**Figure 4B**). Interestingly, we noted that the odds of being hrHPV positive decreased by 8% for each one-unit increase in CDAI (OR = 0.92; 95%CI = 0.86-0.98; p = 0.020), after adjusting for age, smoking status, BMI, parity, educational level, marital status, and use of supplements and oral contraceptives. Similarly, women with high CDAI had lower odds of being hrHPV positive than those with low CDAI (OR = 0.39; 95%CI = 0.18–0.85; p = 0.018), after adjusting for covariates.

11.4 Discussion

Several studies have already demonstrated how dietary habits might affect the risk of cancer in women [709, 710]. Bioactive foods and nutrients with antioxidant and anti-inflammatory potential might reduce the risk of HPV infection and cervical cancer progression. In a previous study, we demonstrated the twofold effect of diet on the risk of hrHPV infection. On one hand, the risk increased among women who highly adhered to a western dietary pattern; on the other hand, it decreased among those who adhered to the Mediterranean diet [268]. However, epidemiological studies using specific dietary indexes are still scarce.

Firstly, our cross-sectional study evaluated dietary intakes of foods and nutrients with antioxidant, anti- or pro-inflammatory potential among women with abnormal PAP test results. In this population, we previously demonstrated the association between the adherence to an healthy dietary pattern and the lower risk of CIN2 or more severe lesions [268]. Here, we reported that women with CIN2 or more severe lesions had a lower intake of calories, as well as of other dietary factors with antioxidant and anti-inflammatory potential than their counterpart with negative histological diagnosis or CIN1. Thus, we hypothesized that the cumulative antioxidant intake and the inflammatory potential of diet might be associated with the odds of having CIN2+ lesions.

With respect to the CDAI, the proportion of HPV positive women decreased with increasing antioxidant score [657]. In fact, dietary antioxidant intake might counteract oxidative stress and DNA damage induced by HPV persistence [687, 691], producing a positive environment for viral clearance [252, 689]. Yet, despite antioxidants also prevent cervical cancer progression [252], we did not find an association between CDAI and CIN2+ status after adjusting for HPV status and other covariates. Further research are needed to understand if antioxidants have a protective role in the entire carcinogenesis process or, on the contrary, only in the first phase [657].

Moreover, we also evaluated the DII, a cumulative score based on the anti- and pro-inflammatory potential of foods and nutrients [708]. Our results demonstrated that the proportion of HPV positive women increased with increasing dietary inflammatory potential. Interestingly, women with higher DII – and hence women with a pro-inflammatory diet – were more likely to have CIN2 or more severe lesions than those with lower index. The association between DII and CIN2+ status remained significant after adjusting for HPV status and other covariates. This was consistent with a previous study [705], that is the first attempt in this field of research. Thus, dietary inflammatory potential might modulate the risk of cervical cancer independent of HPV infection and other risk factors (e.g., age, cigarette smoking, parity, use of oral contraceptives).

Additionally, we evaluated whether dietary antioxidant intake was associated with hrHPV status among women with normal cervical cytology. We did not find differences in dietary intake of carotenoids between hrHPV positive and negative women. However, we showed a lower dietary intake of zinc, manganese, vitamin A and C among women with hrHPV infection. Among these, when all antioxidants were considered simultaneously, dietary zinc intake remained negative associated with the risk of hrHPV positive status. This finding supports the notion that zinc is involved in the immunomodulatory pathways [711-715]. To our knowledge, our study is also the first evaluating the influence of a combination of dietary antioxidants on hrHPV status using the CDAI. Notably, women with higher dietary antioxidant intake had lower odds of being infected by hrHPV than those who reported a lower intake. However, further research is needed to understand to what extent dietary intake of antioxidants could prevent HPV infection and promote its clearance. Moreover, further studies are encouraged to understand molecular mechanisms underpinning the protective or detrimental effect of dietary factors against cervical cancer, also considering genetic susceptibility and epigenetic markers [612, 720].

The current analyses had some limitations. First, the cross-sectional design did not allow us to evaluate the causality of observed associations. With respect to dietary assessment, we used a FFQ that might be affected by measurement errors and inaccuracies. To partially manage reporting bias, we excluded women with the lowest and highest values of total energy intake, and we also adjusted food and nutrient intakes for total energy intake prior to further analyses. Although the FFQ used in this study allowed us to obtain the intake of 33 of the 45 dietary factors included in the DII, the residual effect of other foods and nutrients with anti- or pro-inflammatory potential was not evaluated. Moreover, we cannot rule out the possible impact of unmeasured factors, which might be associated with dietary habits or cervical cancer risk.

In conclusion, further large-scale prospective studies are encouraged to evaluate additional factors and inflammatory markers in the serum of patients. Similarly, further research is needed to understand whether antioxidants exert their effect on hrHPV infection, rather than on its persistence.

Table 1. Comparison of daily dietary intakes between controls and cases

Foods and Nutrients	Controls	CIN2+	p-value
Proteins (g)	71.0 (64.9)	65.5 (50.6)	0.381
Alcohol (mg)	3.2 (5.1)	2.2 (4.8)	0.059
Vitamin B12 (µg)	8.6 (10.1)	8.4 (8.0)	0.851
Vitamin B6 (mg)	1.9 (0.7)	1.8 (0.6)	0.030
β-Carotene (mg)	1.7 (1.9)	1.3 (1.3)	0.044
Coffee (g)	10.2 (6.0)	8.9 (5.9)	0.033
Carbohydrates (g)	304.9 (288.1)	225.4 (198.0)	0.004
Cholesterol (g)	0.2 (0.2)	0.2 (0.1)	0.972
Calories (kcal)	2016 (546)	1859 (442)	0.003
Total fats (g)	64.1 (42.0)	62.0 (36.0)	0.610
Fiber (g)	10.2 (13.2)	7.0 (8.4)	0.010
Folic Acid (µg)	188.5 (111.7)	188.5 (115.3)	0.999
Iron (mg)	13.4 (5.0)	12.7 (4.7)	0.146
Magnesium (mg)	297.5 (84.2)	271.9 (68.1)	0.002
MUFA (g)	49.6 (17.3)	45.3 (16.4)	0.014
Niacin (mg)	41.5 (20.3)	35.1 (18.3)	0.002
PUFA (g)	14.6 (5.8)	13.0 (4.8)	0.005
Riboflavin (mg)	4.7 (3.7)	4.2 (2.8)	0.180
Saturated fats (g)	3.7 (1.4)	3.5 (1.4)	0.125
Selenium (µg)	59.9 (51.6)	57.9 (42.0)	0.680
Thiamin (mg)	1.5 (0.5)	1.4 (0.4)	0.076
Trans fats (g)	1.2 (1.5)	1.1 (1.3)	0.625
Vitamin A (IU)	1084.5 (553.1)	961.7 (454.2)	0.023
Vitamin C (mg)	122.5 (90.7)	105.67.6	0.049
Vitamin D (µg)	4.8 (3.5)	4.5 (5.3)	0.581
Vitamin E (mg)	6.7 (5.4)	5.4 (3.7)	0.012
Zinc (mg)	9.0 (2.8)	8.6 (3.0)	0.124
Black/green tea (g)	6.3 (12.6)	3.4 (7.8)	0.017
Flavan-3-ol (mg)	75.3 (144.4)	42.5 (89.8)	0.016
Flavones (mg)	1.0 (1.1)	0.7 (0.9)	0.016
Flavonols (mg)	34.5 (52.7)	20.6 (33.8)	0.005
Flavonones (mg)	36.5 (73.9)	22.1 (56.9)	0.045
Anthocyanidins (mg)	54.3 (118.3)	36.8 (99.4)	0.132

The table is adapted from Maugeri et al., Public Health Nutrition 2021

Table 2. Comparison of population's characteristics across tertiles of Dietary Inflammatory Index and Composite Dietary Antioxidant Index

Characteristics	Dietary Inflammatory Index (DII)				Composite Dietary Antioxidant Index (CDAI)			
	1st tertile	2nd tertile	3rd tertile	p-value	1st tertile	2nd tertile	3rd tertile	p-value
Age, years	51.0 (7)	40.0 (5)	29.0 (6)	<0.001	30.0 (8.0)	40.0 (9.0)	50.0 (10.0)	<0.001
Smokers	31.1%	40.2%	51.1%	0.001	46.6%	41.7%	34.1%	0.052
BMI, kg/m2	23.5 (4.8)	22.8 (4.9)	20.8 (4.3)	<0.001	21.1 (4.1)	23.0 (5.0)	23.4 (5.2)	<0.001
BMI categories								
Underweight	1.1%	7.3%	15.7%	<0.001	14.0%	7.3%	2.8%	<0.001
Normal weight	58.1%	64.6%	69.7%		70.9%	61.8%	59.6%	
Overweight	30.2%	18.5%	9.6%		11.2%	20.8%	26.4%	
Obese	10.6%	9.6%	5.1%		3.9%	10.1%	11.2%	
Employed	46.7%	45.8%	46.9%	0.976	46.4%	49.4%	43.6%	0.537
Having children	92.8%	78.8%	35.2%	<0.001	42.5%	72.8%	91.6%	<0.001
Low educational level	50.0%	40.8%	25.8%	<0.001	28.5%	40.6%	47.5%	0.001
Oral contraceptive use	2.8%	10.1%	15.6%	<0.001	12.8%	11.7%	3.9%	0.007
Supplement use	15.6%	14.5%	6.7%	0.020	9.5%	11.7%	15.6%	0.199
High-risk HPV infection	38.3%	59.2%	70.9%	<0.001	69.8%	56.7%	41.9%	<0.001

The table is adapted from Maugeri et al., Public Health Nutrition 2021

Table 3. Association of Dietary Inflammatory Index and Composite Dietary Antioxidant Index with CIN2 or more severe lesions

Regression models	Dietary index group	OR (95%CI)	p-value
Dietary Inflammatory Index			
Model 1	1st tertile	*Ref*	
	2nd tertile	2.02 (1.05-3.88)	0.035
	3rd tertile	2.51 (1.33-4.73)	0.004
Model 2	1st tertile	*Ref*	
	2nd tertile	2.15 (1.11-4.17)	0.024
	3rd tertile	3.14 (1.50-6.56)	0.002
Composite Dietary Antioxidant Index			
Model 1	3rd tertile	*Ref*	
	2nd tertile	1.18 (0.70-2.01)	0.532
	1st tertile	1.12 (0.62-2.00)	0.711
Model 2	3rd tertile	*Ref*	
	2nd tertile	1.20 (0.69-2.09)	0.523
	1st tertile	1.17 (0.61-2.23)	0.637

The table is adapted from Maugeri et al., Public Health Nutrition 2021

Table 4. Dietary intake of antioxidants according to high-risk HPV status

| Dietary intake	HPV negative (n=167)	HPV positive (n=84)	p-value
Total energy intake, kcal	2080 (703)	1747 (722)	**<0.001**
Zinc, mg	9.21 (2.93)	7.60 (3.80)	**<0.001**
Selenium, µg	319.14 (485.53)	311.63 (461.46)	0.272
Manganese, mg	314.64 (93.03)	266.33 (101.19)	**<0.001**
Vitamin A, IU	1097.59 (538.14)	827.32 (586.10)	**0.002**
Vitamin C, mg	116.71 (107.55)	82.21 (84.34)	**0.001**
Vitamin E, mg	37.97 (23.44)	34.08 (20.49)	0.158
Carotenoids, µg	9267.17 (7369.62)	7749.60 (6973.24)	0.052
Flavonoids, µg	1624.20 (6850.78)	819.81 (4964.17)	0.163

The table is adapted from Barchitta et al., Nutrients 2020

Table 5. Characteristics of the study population according to tertile distribution of Composite Dietary Antioxidant Index

Characteristics	Composite Dietary Antioxidant Index			p-value
	1st tertile (n=73)	2nd tertile (n=89)	3rd tertile (n=89)	
Age, years	31.0 (8.0)	41.0 (7.0)	52.0 (9.0)	**<0.001**
Current smokers (%)	40.3%	38.2%	25.8%	0.102
BMI, Kg/m2	20.8 (4.2)	23.4 (4.8)	23.7 (5.4)	**<0.001**
BMI categories (%)				
Underweight	12.3%	6.8%	1.1%	
Normal weight	74.0%	61.4%	55.7%	**<0.001**
Overweight	6.8%	18.2%	31.8%	
Obese	6.8%	13.6%	11.4%	
Living in couple (%)	39.7	61.8%	74.2%	**<0.001**
Employed (%)	46.6%	47.2%	40.4%	0.613
Low educational level (%)	26.0%	39.3%	47.2%	**0.022**
Having children (%)	50.7%	78.7%	91.0%	**<0.001**
Use of oral contraceptive (%)	16.4%	9.0%	4.5%	**0.036**
Use of multivitamin supplements (%)	8.2%	12.4%	16.9%	0.258

The table was adapted from Barchitta et al., Nutrients 2020

Figure 1. Description of the study population according to high-risk HPV status and histological diagnosis. Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.
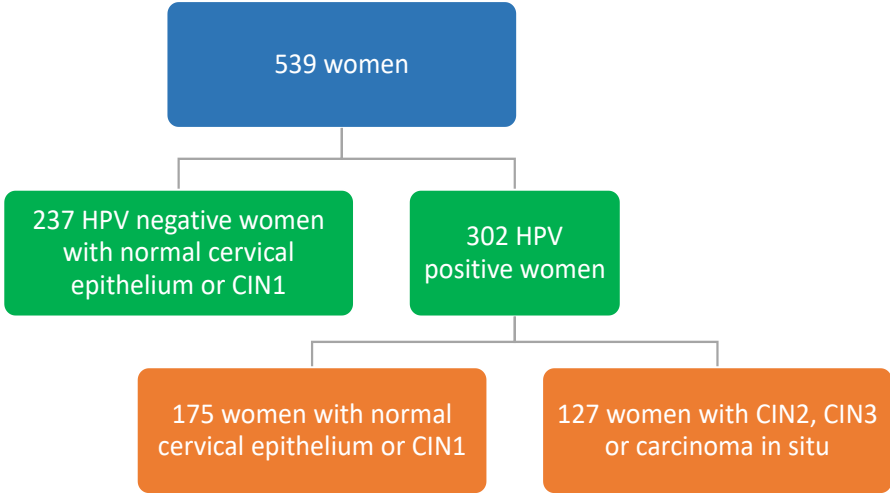
The figure is adapted from Maugeri et al., Public Health Nutrition 2021

Figure 2. Radar plot illustrating dietary intakes between cases and controls
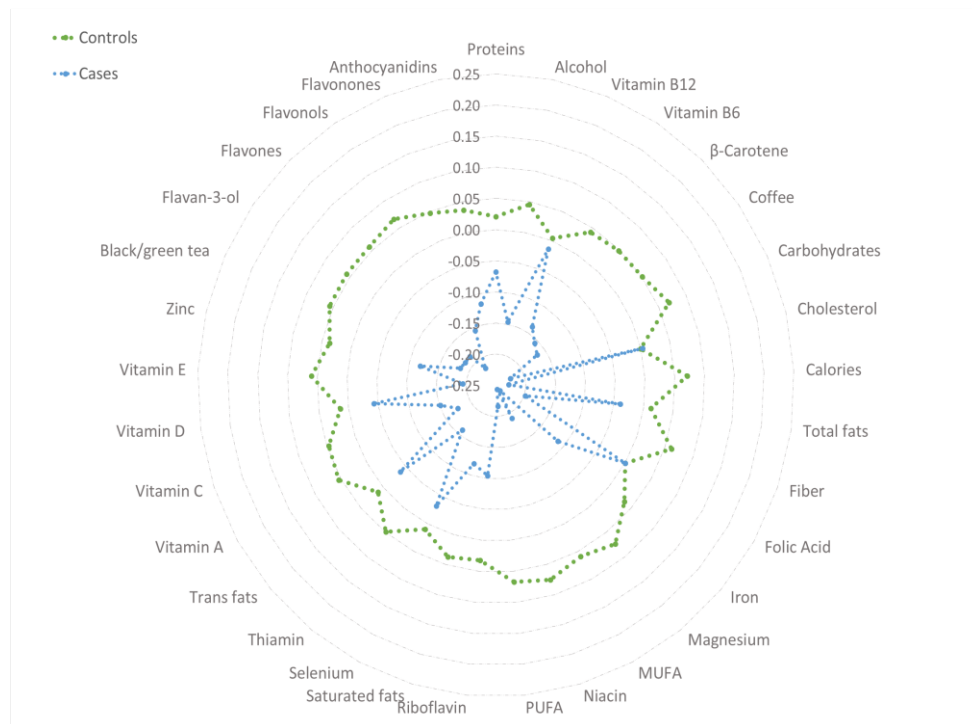
This plot shows the z-scores of dietary factors and their comparison between cases (CIN2+ women; blue line) and controls (women with diagnosis of normal cervical epithelium or CIN1; green line)

The figure is adapted from Maugeri et al., Public Health Nutrition 2021

Figure 3. Proportions of cases and controls across tertiles of Dietary Inflammatory Index (A) and Composite Dietary Antioxidant Index (B)
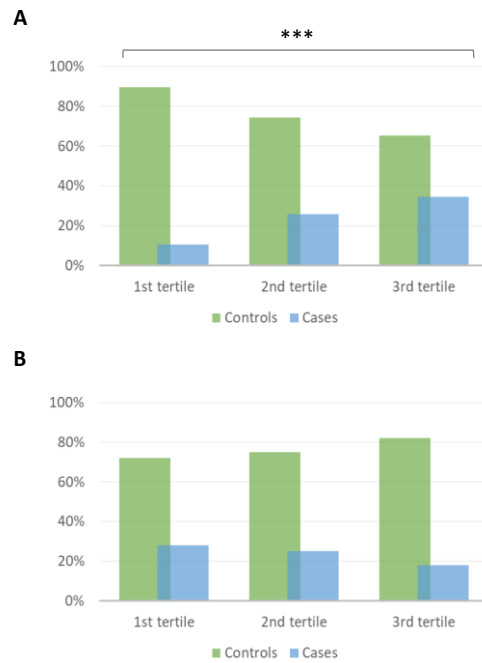
The bars represent the proportion women with CIN2 or more severe lesions (cases; blue bars) and those with diagnosis of normal cervical epithelium or CIN1 (controls; green bars). *** p-value<0.001 based on the Chi-Squared test

The figure is adapted from Maugeri et al., Public Health Nutrition 2021

Figure 4. Association between the Composite Dietary Antioxidant Index and high-risk HPV status
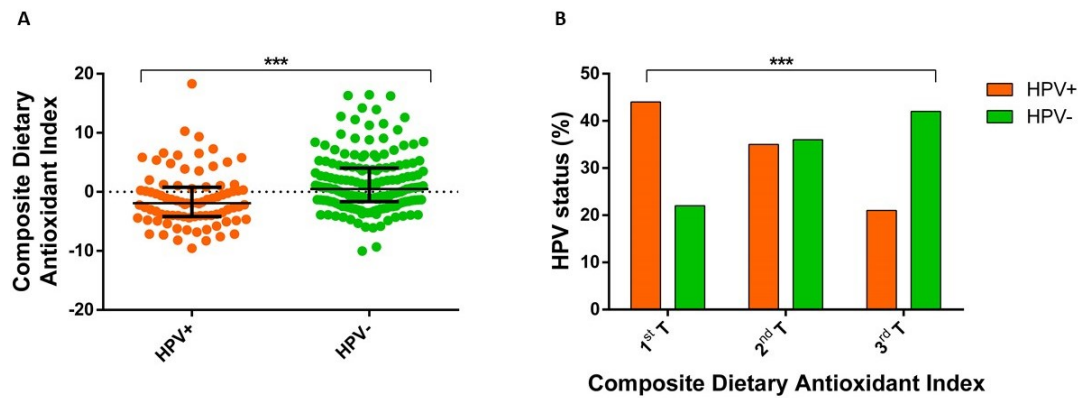
(A) Comparison of Composite Dietary Antioxidant Index between high-risk HPV positive and negative women using the Mann-Whitney U test (***p<0.001); (B) Distribution of women by high-risk HPV status and tertiles of Composite Dietary Antioxidant Index (***p<0.001 based on the Chi-Squared test)

The figure is adapted from Barchitta et al., Nutrients 2020

12. Dietary interventions effective for improving the quality of life of breast cancer survivors

12.1 Background

The promotion of healthy dietary habits might be crucial for sustaining the survival and the quality of life (QoL) of women diagnosed with cancer. Among female cancers, breast cancer is the most common one [234], accounting for 2.09 million cases and 627.000 deaths in 2018 [721]. Since breast cancer and its treatments are also associated with adverse effects [722, 723], the survivors are an important target population for developing and promoting preventive strategies [246].

As suggested by previous evidence, the promotion of healthy diet – rich in vegetables, fruits and whole grains regularly - might reduce risk of recurrence [724], stress and breast/arm symptoms [725]. Specifically, breast cancer patients showed reduced QoL than their healthy counterpart, with dietary problems such as dyspepsia, nausea and anorexia [726]. Changes in health-related habits were evident among breast cancer survivors [727], even if it was not clear the relationship between diet and QoL. For instance, one study did not demonstrate correlations between healthy eating index score and QoL [728]. By contrast, another study showed a negative correlation with depression, assessed by the Center for Epidemiologic Studies Depression scale (CES-D) score [729]. Others reported better global health status [730, 731] and social, emotional, cognitive and role functioning [731], lower risk of dyspnea [732], but higher risk of insomnia [732] among breast cancer survivors with healthy dietary habits. For these reasons, 9 out of 10 women asked for a personalized dietary counseling after a breast cancer diagnosis [726]. However, dietary interventions did not modify dietary habits, with no significant changes in fruit/vegetable servings per day or take-away and fast-food frequency per week [733].

Beyond dietary habits, guidelines for cancer survivors also recommend to achieve and maintain a healthy weight and to practice physical activity, in order to improve health status and QoL of breast cancer survivors [734-738]. Moreover, a multidisciplinary rehabilitation program – with occupational and physical counseling – supported return to work by reducing fatigue and increasing working ability and QoL [739]. A trend analysis showed a positive association between the number of recommendations and QoL in breast cancer survivors [246], which, however, was limited to those with stage II or III cancer [734]. In line, increasing adherence to the guidelines was positively associated with several QoL subscales [726]. Yet, recent population-based studies [740-742] in the United States and Australia have reported that up to 70% of cancer survivors ignore these recommendations.

Here, we first conducted a cross-sectional study to evaluate the association of adherence to MD, physical activity, and weight status with QoL of Italian breast cancer survivors. Next, we performed a systematic review of experimental studies to summarize whether dietary interventions – alone or in combination with physical activity recommendations – might significantly improve QoL among women with a breast cancer diagnosis.

12.2 Methods

12.2.1 Cross-sectional study: study population

From 2013 to 2014, we conducted a cross-sectional study on Italian women with breast cancer (stage I-III breast) and on those who completed radiotherapy or chemotherapy treatment at least 6 months prior to the recruitment. Among 162 invited women, 42% (n = 68) completed the assessment of behavioral and dietary data, anthropometric measures, and QoL. The recruitment phase was supported by A.N.D.O.S. Onlus (Associazione Nazionale Donne Operate al Seno). The study protocol was approved by the ethical committees of the involved institutions, all women signed an informed consent, and the study was conducted according to the Declaration of Helsinki.

A structured questionnaire designed *ad-hoc* was administered to collect information on age, on adherence to MD and physical activity, and on self-reported anthropometric measures, that were used to calculate and categorize BMI according to the WHO criteria [293]. Physical activity level was assessed using the long form of the IPAQ-L [294], and categorized as low (no moderate and vigorous activities), moderate (1–149 min/ week moderate or 1–74 min/ week vigorous or 1–149 min/ week moderate + vigorous), or high (≥150 min/week moderate or ≥75 min/ week vigorous or ≥150 min/ week moderate + vigorous) according to the American Heart Association recommendations [743].

12.2.2 Cross-sectional study: dietary assessment

The adherence to the MD was assessed using the Mediterranean Diet Assessment Tool proposed by Martinez-Gonzalez and colleagues [744-747]. The tool was developed in a Spanish case-control study of myocardial infarction [748], and includes 14 items. Based on the overall score, the adherence to MD was categorized as i) low (≤5 positive items); ii) medium (6-9 positive items) or iii) high (≥10 positive items).

12.2.3 Cross-sectional study: assessment of Quality of Life

The assessment of functional status and global QoL was performed using the European Organization for the Research and Treatment of Cancer Quality-of-Life (EORTC) Questionnaire–Core 30 (QLQ-C30) [749]. In particular, the QLQ-C30 includes the global health status/QoL, the

functional, and the symptom scales [749]. Moreover, QoL assessment was accompanied by the administration of the Quality of Life Questionnaire Breast Cancer Module 23 (QLQ-BR23) [750, 751]. The QLQ-BR23 is composed by 23 items organized in functional and symptom scales. The raw scores of the 4-point or 7-point scales were transformed to a 0 to 100 scale based on the EORTC scoring manual, with higher score reflecting better QoL in functioning and global health status/quality of life and a worst QoL in symptoms [750, 751].

12.2.4 Cross-sectional study: statistical analyses

All statistical analyses were performed using SPSS software (version 21.0, SPSS, Chicago, IL). The Kolmogorov-Smirnov test was used to test the normality for continuous variables, reported as mean and SD. Moreover, the Student t-test was used for comparison between two groups, while the one-way ANOVA for comparison between more than two groups. All statistical tests were two-sided, and p-values < 0.05 were considered statistically significant. We also reported statistically significant results after Bonferroni correction (p-values ≤ 0.003 for QLQ-C30 and ≤ 0.006 for QLQ-BR23).

12.2.5 Systematic review: literature search and study selection

From the inception to May 2019, two researchers independently conducted a literature search in the PubMed-Medline and Web of Science databases. The MeSH terms used for the research were: "Breast Cancer" AND "Quality of Life" AND ("Diet" OR "Exercise"). We included: (1) experimental studies (2) on women with a history of stage I-III breast cancer diagnosis, (3) focusing on the effect of dietary interventions (alone or in combination with physical activity recommendations) on QoL. The preferred reporting items for literature search (PRISMA) guidelines were followed [752].

12.2.6 Systematic review: data extraction

From all the selected articles, two researchers independently extracted the following information: first author's last name, year of publication, study design, country, ethnicity, number of participants, and method for QoL assessment, and the main findings of the effects of dietary intervention, alone or in combination with other recommendations, on QoL and secondary outcomes (changes in weight status, dietary habits, physical activity).

12.2.7 Systematic review: risk of bias assessment

For randomized controlled trials included in the systematic review, two researchers also evaluated the risk of bias using the Cochrane's Collaboration tool [537]. For each study, a score ('low risk of bias', 'unclear risk of bias' or 'high risk of bias') was assigned to the following items: random

sequence generation; concealment of the allocation sequence; blinding of outcome assessment; incomplete outcome data; selective outcome reporting; and other biases. Disagreements were resolved by discussion with a third Author.

12.3 Results

12.3.1 Cross-sectional study: study population and Quality of Life

In the current cross-sectional analysis, we included 68 stage I-III breast cancer women, aged 36-68 years. We first compared QoL of our study population with reference values from the EORTC Quality of Life Group's Cross-Cultural Analysis Project [753]. With respect to the QLQ-C30 module, study participants exhibited worst scores for emotional ($p = 0.015$) and cognitive functioning ($p = 0.023$), financial impact ($p = 0.002$) and insomnia ($p < 0.001$) scales than the EORTC reference values. By contrast, they showed better scores for loss of appetite ($p = 0.003$). However, only insomnia, financial impact and loss appetite remained significantly different from reference values after Bonferroni correction (p-values ≤ 0.003). Interestingly, regarding QLQ-BR23 module, study participants reported worst scores for all scales than those reported by the EORTC project also after adjusting for multiple comparisons (p-values ≤ 0.006).

12.3.2 Cross-sectional study: Mediterranean Diet and Quality of Life

Firstly, in our cross-sectional study we evaluated the association of adherence to MD and its typical food groups with QoL. The comparison between women who met MD criteria with who did not demonstrated the beneficial effects of several food groups. In particular, women who consumed less than 1 serving of red meat per day exhibited better scores for dyspnea ($p = 0.035$) and financial difficulties ($p = 0.008$). Women who drank less than 2 servings of carbonated beverages per day showed better scores for dyspnea ($p = 0.004$) and insomnia ($p = 0.016$). Moreover, women who drank 7 or more glasses of wine per week exhibited better score for sexual enjoyment ($p = 0.025$); women who consumed 2 or more dishes seasoned with sofrito per week showed better score for sexual functioning ($p = 0.035$). By contrast, we also noted the negative effects of other food groups on QoL: women who used olive oil as main culinary fat reported worst scores for sexual functioning ($p = 0.044$) and enjoyment ($p = 0.008$). Moreover, women who consumed 3 or more fish servings per week reported worst scores for emotional ($p = 0.049$) and cognitive functioning ($p = 0.034$), financial difficulties ($p = 0.034$), side effects ($p = 0.019$), and breast symptoms ($p = 0.008$). Additionally, women who consumed less than 3 servings of commercial sweets or pastries per week showed worst scores for loss of appetite ($p = 0.043$), body image ($p = 0.027$), and arm symptoms ($p = 0.045$); women who consumed 3 or more servings of nuts per week reported worst score for role

functioning (p = 0.004). However, no differences were evident after correction for multiple comparisons. Similarly, the comparison across categories of adherence to MD showed that it did not affect overall QoL and its subscales **(Figure 1**).

12.3.3 Cross-sectional study: physical activity and Quality of Life

Next, we observed that 24.7% of women reported low physical activity, while 50.5% and 24.7% showed moderate or high physical activity, respectively. As shown in **Figure 2**, we observed better QLQ-C30 scores for emotional (p = 0.028) and cognitive (p = 0.016) functioning, loss of appetite (p = 0.008), and diarrhea (p = 0.001) among moderately physically active women than those who performed less or more physical activity. These findings resulted in better global health status in women who performed moderate physical activity (p = 0.035). However, only diarrhea subscale remained significantly better among moderately physically active women after Bonferroni correction (p ≤ 0.003). With respect to the QLQ-BR23 module, no differences were evident.

12.3.4 Cross-sectional study: weight status and Quality of Life

According to BMI (mean = 26.4 kg/m2; SD = 4.8 kg/m2), we classified women in underweight (0.7%), normal weight (44.9%), overweight (33.1%), and obese (21.3%). In particular, we noted that several QoL sub-scores (i.e., physical and role functioning, fatigue, nausea, pain, dyspnea, insomnia, appetite loss, constipation, diarrhea, and financial difficulties) decreased from underweight to obese women (p-values < 0.05). As reported in **Figure 3,** the comparison between underweight/normal weight and overweight/obese women demonstrated worst scores for physical (p = 0.001) and role functioning (p = 0.004), fatigue (p = 0.025), pain (p = 0.009), dyspnea (p = 0.003) in the latter group. However, only physical functioning and dyspnea remained significantly better among underweight/normal weight women after Bonferroni correction (p-values ≤ 0.003). Similar results were obtained with respect to the QLQ-BR23 module, no differences by weight status were evident.

12.3.5 Systematic review: principal findings

The detailed steps of the study selection are given as a PRISMA flow diagram in **Figure 4.** The current systematic review finally included 9 experimental studies from Europe (n = 4), America (n = 4) and Asia (n = 1), and different tools for assessing QoL in breast cancer survivors were reported. With respect to study design, we included 6 randomized controlled trials, 2 single-arm trials, and 1 randomized cross-over pilot study. Duration of intervention ranged from 2 weeks to 12 months. In general, all the studies demonstrated significant improvements in overall QoL and/or its subscales after the interventions. However, differences in study design, interventions and tools used for QoL

assessment does not allow to provide an overall estimate. Moreover, only one study evaluated the effect of an intervention based exclusively on dietary recommendations, demonstrating that short-term fasting followed by normo-caloric diet counteracted the reduction of QoL in the first half of chemotherapy [754].

The remaining studies, instead, proposed various combined interventions to promote healthy diet and regular physical activity among breast cancer survivors, including stage-matched telephone counselling [755], automated prompts [756], physical and nutritional interventions in hydrothermal centers [757], and weight loss programs [758, 759]. Befort and colleagues conducted a single-arm trial on obese stage I-III breast cancer survivors that were instructed to follow dietary (i.e., consuming ≥5 fruit and vegetable servings per day, prepackaged frozen entrees and shakes) and physical activity (i.e., 225 minutes per week of moderate intensity activity) recommendations [760]. This intervention significantly improved several QoL domains, such as mood, body image, and sexuality, and determined improvements in weight, waist circumference, daily energy intake, fruit and vegetables consumption, and physical activity level [760]. The randomized controlled trial by Demark-Wahnefried and colleagues confirmed the efficacy of diet and physical activity recommendations for improving QoL among overweight/obese breast cancer survivors [761].

The randomized controlled trial by Kim and colleagues evaluated the effects of a telephone counseling with personal prescription for regular exercise and balanced diet based on guidelines. They reported that the intervention group showed significantly improvement in emotional functioning, fatigue, and depression than the control arm, as well as in motivational readiness for exercise and diet [755]. Similarly, Kwiatkowski and colleagues conducted a randomized controlled trial to evaluate the effect of a two-week intervention in hydrothermal centers [757], consisting of daily group supervised physical training, dietary education, physiotherapy, and psychological support. The intervention significantly improved QoL after 6 and 12 months, with greater improvements in mental and physical sub-scores [757]. In line with these findings, the randomized controlled trial by Morey and colleagues was based on a 12-month diet and exercise intervention via telephone counseling and tailored mailed materials [756]. The authors demonstrated a significant improvement in physical activity, dietary behaviors, and overall QoL in the intervention arm. Moreover, after 12 months, the mean function scores declined less rapidly in the intervention group compared with controls group [756]. The randomized controlled trial by Travier and colleagues aimed to promote weight loss evaluating the effect of a 12-week intervention based on dietary and physical activity recommendations [759]. Participants who completed the intervention reported significant improvements in QoL and its sub-scores, and beneficial effects on weight loss

and cardiorespiratory fitness [759]. Similarly, two studies aimed to investigated beneficial effects of interventions based on aerobic exercise and dietary counselling [762, 763]. Ghavami and colleagues reported that a 24-week intervention significantly improved symptom relief, functional and global health status [763]. In line, Swisher and colleagues observed significant improvements in overall physical wellbeing, as well as in breast-cancer specific subscales and total score. The intervention also reduced body fat and sedentary time [762].

With respect to risk of-bias assessment in randomized controlled trials, we identified low risk of selection bias due to random sequence generation and allocation concealment. In 4 studies, we noted an unclear risk of detection bias due to insufficient information on the blinding of outcome assessment. For the other domains (i.e., attrition, reporting and other bias), low risk was evident.

12.4 Discussion

Our cross-sectional analysis aimed to investigate the potential effect of MD on QoL of stage I-III breast cancer patients who completed radiotherapy/chemotherapy treatment at least 6 months prior to the recruitment. Our results showed that low consumption of red meat and carbonated beverages, daily consumption of wine, and high consumption of dishes seasoned with sofrito were associated with better scores for several QoL subscales. This was partially in line with previous studies reporting better scores in some QoL subscales among women who adhered to healthy dietary patterns [730, 732]. Other studies, however, reported a positive effect of healthy diet on depressive symptoms [729], but no direct correlation with overall QoL was evident [764]. Accordingly, in our study population, we did not demonstrate association between adherence to MD and QoL. Indeed, MD is also characterized by some typical products – for instance olive oil, fish, and nuts - that seemed to have a negative effect on several QoL subscales. The low sample size did not allow us to adjust for potential confounders (e.g., age and other social and demographic factors) nor evaluate a mediating effect.

Moreover, worst scores for physical and role functioning, fatigue, pain, dyspnea were observed among overweight or obese women compared with their normal weight counterpart. Regarding exercise, the most positive effect on QoL was among women who performed moderate physical activity, with better scores for global health status, emotional and cognitive functioning, loss of appetite, and diarrhea. Several observational studies already demonstrated benefits from more engagement in physical activity on QoL of breast cancer survivors [735-737]. Interestingly, a meta-analysis of thirty-three randomized controlled trials stated that QoL was significantly improved in exercise intervention group. Besides, exercise was also associated with positive outcomes in BMI, and in the serum concentration of insulin [765].

While benefits of physical activity are already well known, findings about a potential effect of dietary intervention are still inconclusive. Thus, we also conducted a systematic review of experimental studies investigating the effect of dietary interventions - alone or in combination with other recommendations - on QoL of breast cancer survivors. Although all the studies demonstrated significant improvements in QoL after the interventions, only a trial evaluated the exclusive effect of dietary intervention [754]. Beyond that, differences in study design and in tools used for QoL assessment did not allow to provide an overall estimate. Thus, QoL increased with increasing number of lifestyle recommendations. However, more efforts are needed to understand the exclusive effect of diet and dietary interventions on the QoL of breast cancer survivors.

Figure 1. Comparison of quality of life according to adherence to Mediterranean Diet.
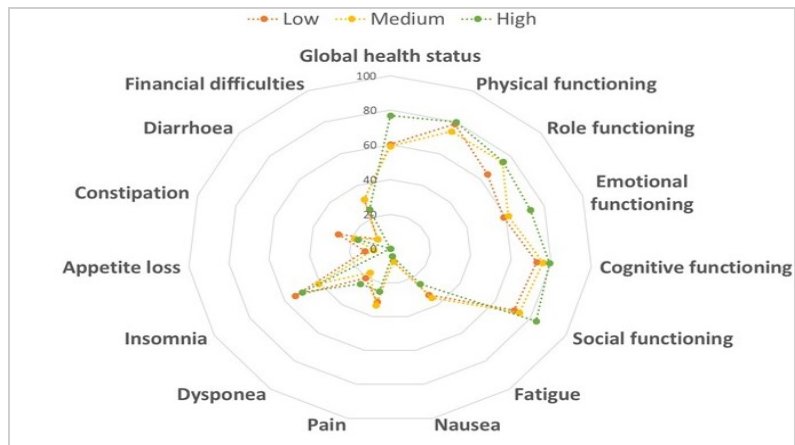
Data were compared using the one-way ANOVA.

This figure is adapted from Barchitta et al., Cancer 2020

Figure 2. Comparison of quality of life according to physical activity level
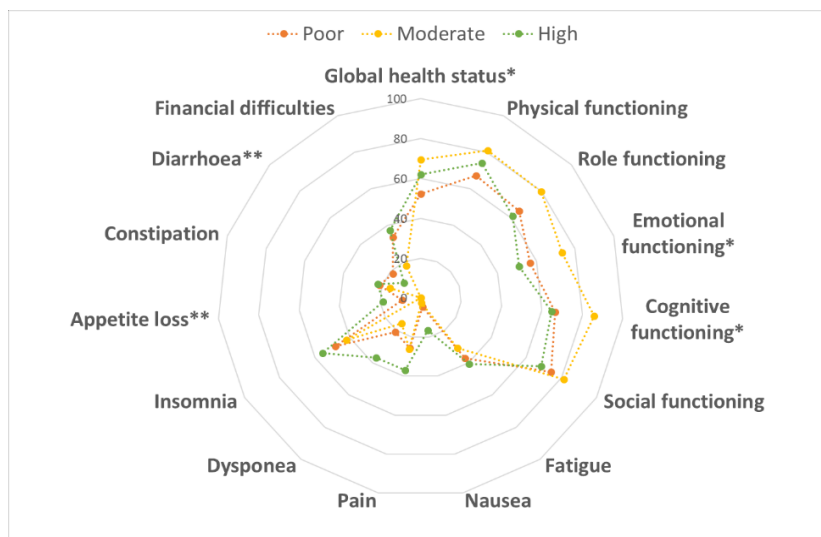
Data were compared using the one-way ANOVA. * $p<0.05$; ** $p<0.01$

This figure is adapted from Barchitta et al., Cancer 2020

Figure 3. Comparison of quality of life between overweight/obese and underweight/normal weight women
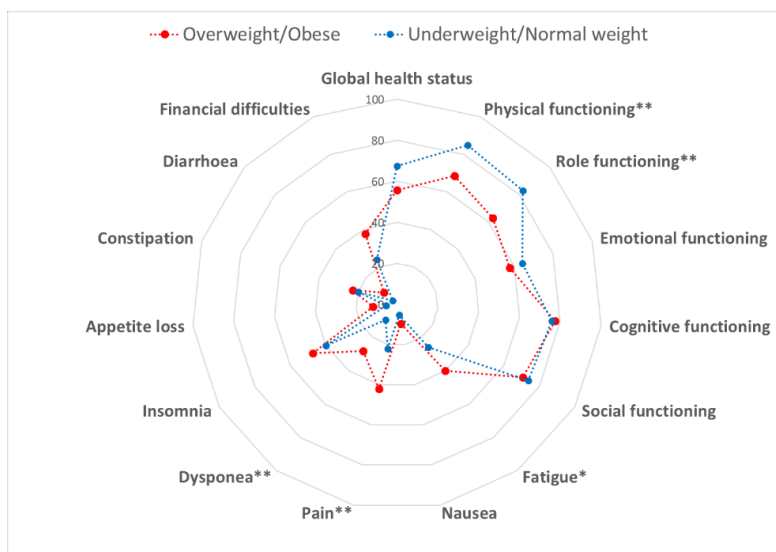
Data were compared using the Student t-test. * p<0.05; ** p<0.01

This figure is adapted from Barchitta et al., Cancer 2020

Figure 4. PRISMA flow diagram of study selection
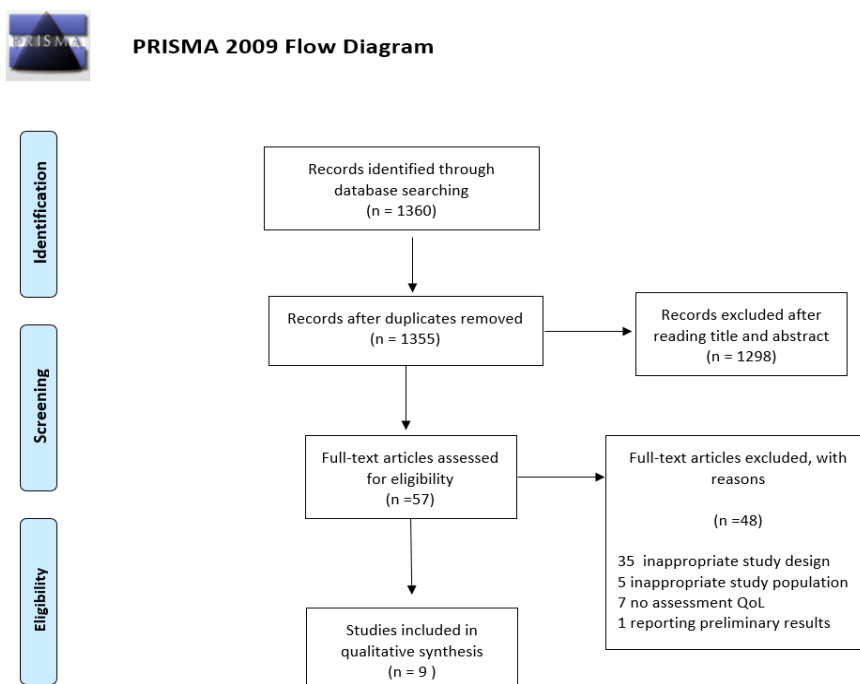
This figure is adapted from Barchitta et al., Cancer 2020

13. Discussion and Conclusions

Women's health represents an important target for Public Health strategies due to several biological and socio-cultural reasons. During the reproductive period - especially in pregnancy - environmental factors and lifestyles can affect physiological and pathological conditions both in women and in future generations. The set of individual features that characterize each woman makes them susceptible to environmental stressors. Indeed, the interplay between health status, physiology, genetics, and epigenetics, as well as changes in these individual components caused by previous exposures, can influence the effects of current or future exposures.

For this reason, understanding the role of determinants of health and its interaction with the exposome is become a Public Health priority. Several studies already demonstrated how the determinants of health might act on some behaviors, such as the adherence to healthy dietary patterns, which in turn can reduce the risk of many non-communicable diseases [276-278]. With the purpose of overcoming the approach of assessing the association between single nutrients or food groups and the occurrence of disease [280-283], an increasing number of studies has focused on summing mix of foods considered to be important for health [284]. In this scenario, greater adherence to MD — estimated a priori using dietary scores based on the characteristic components of the traditional diet of the Mediterranean area—has been found to be associated with a reduced risk of cardiovascular and cancer mortality, as well as of Parkinson's and Alzheimer's incidence [286, 287]. Due to its positive effect on health status, we aimed to identify social and behavioral factors associated with adherence to MD in different cohorts of women during their reproductive age. Specifically, we first performed a cross-sectional study on women, without history of severe diseases, to assess the major determinants of adherence to MD in general, and the consumption of its specific food components. Our findings indicated that more engagement in physical activity is a positive determinant of the ideal consumption of vegetables and legumes, resulting in a high adherence to MD. By contrast, the coexistence of unhealthy dietary choices with sedentary behaviors exacerbates their deleterious effects on health. People who perform more physical activity tend to be healthier than their sedentary counterparts [305], probably also due to their variegated diet and social contacts [306]. Instead, it has been demonstrated that sedentary individuals consume more fast-food products and fewer fruits and vegetables [289, 290]. Although a direct relationship between physical activity and food choices has not been yet elucidated, it has been well established that a lack of exercise and an unhealthy diet tend to coexist among individuals [307]. Similarly, we found that current smoking was a negative determinant of the ideal consumption of meat products, which means that people who smoked tobacco reported a higher

intake of red and processed meats. In this context, a meta-analysis of 51 studies showed an association between smoking status and a higher intake of energy, total and saturated fat, and cholesterol, while a lower intake of vitamins and fiber than non-smokers [309]. The positive aspect, however, is that changes in one of these behaviors could lead to an overall improvement of lifestyle [766-768]. Additionally, healthy dietary habits are more common among high-educated individuals since they are more aware about benefits and risks of their food choices [311]. In our study, after adjusting for other factors, having a medium or high educational level was the only positive determinant of an ideal consumption of alcohol. Moreover, living in couple was associated with an ideal consumption of cereals, while having children was the only positive determinant of an ideal consumption of fruits.

In general, we focused on women since they have a crucial role in food choice by providing meals for their families and making up most of the workforce in food-related jobs, health care and education. The role of women is even more prominent during pregnancy, because maternal diet – as well as other behaviors – plays a critical role in determining maternal and newborns health. Recent evidence highlighted the influence of both social determinants and lifestyles on the adherence to different dietary patterns [398-404], suggesting the need of robust evidence for both Public Health professionals and for the scientific community. Thus, we evaluated the relationships between *a posteriori* dietary patterns, social determinants, and lifestyles in a cohort of pregnant women from the Mediterranean area. In our cohort, we found that young women with low educational level adhered more to the "western" dietary pattern, which was characterized by high intake of red meat, junk foods and alcoholic drinks. This partially supports previous evidence that healthy diet is more common among old women with higher educational level [398, 399], though we demonstrated no association of socio-demographic characteristics with the adherence to a "prudent" dietary pattern rich in vegetables. Moreover, we also pointed out the positive association between the adherence to the western dietary pattern and smoking status, even adjusting for socio-demographic factors. Generally, non-smokers follow dietary recommendations better than smokers, while who smoke tend to have unhealthier dietary habits than former- or non-smokers [404]. A possible explanation is that women who smoke have an attenuation of the senses of smell and taste, and an unhealthier lifestyle compared to former and non-smokers [457, 458].

Since the high adherence to western dietary patterns is associated with adverse maternal and infant outcomes - including GDM [453], PTB [195] and reduced birth length [195] - our results raise the need for strategies to promote of healthy dietary habits among women of reproductive age, with a particular focus on young women with low educational level.

It therefore remains important to better identify the determinants of health involved not only in food choices, but in women's choices in general. Since pregnancy is a crucial stage of life for the health of women and their children, we investigated the main determinants of some women's choices – i.e., breastfeeding practice and vaccination choices – in the "Mamma & Bambino" cohort.

With respect to breastfeeding, its short-term and long-term health benefits on newborns [619, 665], which in turn might be mediated by molecular mechanisms [463], have been already clarified. However, women do not always adhere to WHO recommendation, according to which women should i) initiate breastfeeding within one hour of birth; ii) exclusively breastfeed for the first six months; and iii) continue breastfeeding until two years of age. In a subsample of 220 pregnant women, we firstly noted that the proportion of women who have breastfed increased with increasing educational level. This is probably because more educated women are more likely to seek medical advice and exploit health-related services. This pointed out the widening socioeconomic gaps in breastfeeding attitudes between mothers with different educational level [669, 670]. Interestingly, different socioeconomic factors might also influence the duration of breastfeeding and its exclusivity. In a public health perspective, it is important that newborns are breastfed exclusively during their first six months of life. However, compliance with WHO recommendations depends on several sociocultural factors [636]. In our study, we noted that the proportion of women who adhered was higher among those with medium-high educational level and those who were employed. In line, we demonstrated that full time employed women and those with medium educational level were more likely to breastfeed than their counterparts. These findings could be explained by the positive effects of education on breastfeeding knowledge in general, and on compliance with recommendations. Moreover, with respect to employment status, the Italian parental leave allows full-time and part-time employed women to continue exclusive breastfeeding for the recommended period. Aside from socio-demographic determinants, breastfeeding could be affected by other factors. For instance, studies conducted in developed countries stated an association between maternal pre-pregnancy obesity and higher risk of non-initiation and shorter breastfeeding duration [673, 674]. Similarly, smoking was also associated with higher rates of early cessation, probably due to nicotine effects on milk volume and sleeping patterns [675, 676]. However, in our study, we did not find a significant association between the above-mentioned factors and breastfeeding. In this scenario, socioeconomic factors should be considered through public health strategies for improving maternal knowledge about health benefits of exclusive breastfeeding. In general, breastfeeding programs should be implemented for all mothers, with specific interventions tailored toward less educated mothers.

Additionally, since long-term effects of breastfeeding on human health might be mediated by epigenetic mechanisms, further research is needed to investigate the association between breastfeeding and DNA methylation in humans. For this reason, the study conducted on the HELIX cohorts may constitute a starting point for evaluating the role of epigenetic mechanisms on breastfeeding in the "Mamma & Bambino" cohort.

In a specular way, we conducted an analysis on the main determinants of vaccination choices among pregnant women from the "Mamma & Bambino" cohort. Our study underlined the need for improving women knowledge about recommendations for vaccination. For instance, in our study none of women reported to be informed about the recommended vaccines during the pregnancy, suggesting the need to increase healthcare providers' availability and to improve women's level of knowledge during the maternal counselling. Moreover, common determinants of non-vaccination also include the belief of vaccines unsafety, due to the facts that their long-term effects are unknown or that risks exceed benefits. Rates of low coverage are more determined by the complacency rather than socio-demographic and/or socio-economic factors [461]. However, social determinants can vary among countries of different income levels, supporting the idea that their effects on vaccination choice should be considered for designing appropriate interventions [427]. Although we proposed a positive association between age and no-adherence to vaccine programs, further research is encouraged to investigate other determinants involved in vaccination choice.

Among the appropriate public health strategies developed for women health, the WHO and the FAO proposed several dietary recommendations for the prevention of adverse pregnancy outcomes [156].

Particularly, during the pre-conceptional and gestational periods, inadequate intake of micronutrients might affect the risk of adverse pregnancy outcomes [184]. Our hypothesis is that folate deficiency leads to PTB [769] and SGA [442, 444-452, 770-778], but further studies are necessary to better investigate the potential protective role of adequate folate intake and/or folic acid supplementation. In fact, magnitude of folate deficiency varies between and within countries, with higher prevalence in those without folic acid fortification of cereal-grain products [434, 435]. In our study on pregnant women, the proportion of women who did not meet current recommendation was in line with high prevalence of deficient women showed by previous studies in Italy [102, 268, 296, 297, 436]. Since MD is characterized by high intakes of green leafy vegetables, dark green vegetables, legumes, and some fruits [437], which in turn are rich in folates, our results confirmed that women with dietary restrictions and low adherence to MD were more likely to report inadequate folate intake. We also reported higher odds of inadequate folate intake

among current smokers, underlying the prevalence of unhealthy diet among smokers [291, 292, 308], with greater intake of saturated fat and cholesterol, and lower intake of vitamins and fiber [309]. In this context, also the prevalence of folic acid supplementation remains often inadequate in several countries [395, 438]. In our study, the proportions of insufficient users or non-users were not significantly different according to dietary folate status. However, among women with inadequate folate intake, those with low educational level were more likely to not use folic acid supplements than their more educated counterpart. Among the main determinants of health, younger age [395], low income [441], educational level [438], and employment status [396] might affect the use of folic acid supplements. Our findings supported the need for increasing the prevalence of folic acid supplementation through the identification of people at the highest risk for folate deficiency. This would also allow to develop preventive strategies to reduce the higher proportions of SGA and LGA births among women with inadequate folate intake, as well as the higher proportion of SGA births among women who did not take supplement before pregnancy and those who did not take at all. However, although we proposed a protective effect of folic acid supplements against the risk of SGA, previous studies were heterogeneous. The majority of them demonstrated that supplement use before and during pregnancy reduced the risk of SGA [442-448], while others showed an opposite [449, 450] or null effect [451, 452]. Thus, further research is encouraged not only to identity the social determinants affecting the prevalence of folate and folic acid deficiency, but also to better investigate the role of folic acid supplement on the reduced burden of adverse pregnancy outcomes.

It has become clear that the first thousand days of life, from conception to the end of the second year, represent a critical period for the development of obesity. In early pregnancy, excessive GWG is a risk factor for increased birth weight, which in turn has been associated with higher risk of obesity in childhood and adulthood [467, 468]. Beyond birth weight, the amount of GWG may affect both maternal and new-born health. In mothers, excessive GWG has been associated with an increased risk of hypertension [172], diabetes [173], cesarean section [174], postpartum weight retention [175] and obesity [176]. In newborns, the most common outcomes of inadequate GWG are neonatal and infant mortality, PTB and fetal growth retardation [470]. Our hypothesis was that dietary habits during the early phase of pregnancy - specifically those referred as "healthy" - might improve adequate GWG according to pre-gestational BMI. Specifically, we first demonstrated that the adherence to the prudent dietary pattern was negatively associated with pre-pregnancy BMI, with ≈1.3 point reduced pre-pregnancy BMI for women with high adherence to this pattern. We also reported a dual opposite effect of the prudent dietary pattern on GWG across BMI categories: while the adherence to this pattern was positively associated with GWG among underweight, it was

negatively associated among overweight and obese. This is in line with the previous evidence from the prospective Norwegian Mother and Child Cohort Study [475]. These results might be attributable to the healthy effect of fruits and vegetables on women's health and fetal growth, as demonstrated by previous studies on US pregnant women [477] [478]. Thus, the adherence to a prudent dietary pattern might provide a balanced intake of energy, macro- and micronutrients [477] and, therefore, promote an adequate GWG independent of pre-gestational BMI. Moreover, women who adhered to a prudent diet were more likely to have a healthy lifestyle, which in turn contributed to adequate GWG independent of pre-pregnancy BMI [480]. With respect to the dual effect across BMI categories, it can be partially explained by dietary reporting errors [481] and/or in different composition of GWG [482] - which consists of gains in total body water, fat-free mass and fat mass - across BMI categories. Thus, our study demonstrated that adherence to a prudent dietary pattern ameliorated pre-pregnancy BMI and GWG across BMI categories, which in turn have been previously associated with increased birth weight [38]. Of note, our study also confirmed that birthweight was higher in infants born from mothers with excessive GWG, compared to those with adequate or reduced GWG.

With respect to the western dietary pattern, we demonstrated that it did not affect pre-gestational BMI but increased GWG, reporting a ≈13.7 Kg increased GWG among obese women with high adherence compared to women with low adherence. In conclusion, the promotion of healthy dietary habits, even during the periconceptional period, represents a potential strategy to maintain an adequate weight independent of pre-gestational BMI.

Aside from pregnancy, women nutrition plays a key role in determining the risk for several diseases in other stages of life [694]. It is now well established that the adherence to a dietary pattern rich in healthy foods – characterized by fruit, vegetables, whole grains, and fish – protects against several non-communicable diseases, including cervical cancer [268, 276-278, 362, 695]. In this scenario, several studies have been conducted to investigate the antioxidant and anti-inflammatory effect of specific foods and nutrients against HPV infection and cervical cancer progression [264, 268, 686, 696, 697]. However, the combined effect of foods and nutrients with antioxidant and anti-inflammatory activity remains to be clarified. Our cross-sectional study - conducted on women with abnormal PAP test and tested for high-risk HPV infection – demonstrated that the risk of CIN2+ was higher among women with medium or high DII than in those with low DII, after adjusting for covariates. In contrast, increasing CDAI was negatively associated with the prevalence of HPV positive women but not with the prevalence of cervical cancer. In general, dietary antioxidant intake might counteract oxidative stress and DNA damage induced by HPV persistence [687, 691],

producing a cellular environment that could help viral clearance [252, 689]. Yet, despite it has been suggested that antioxidants also prevent cervical cancer progression [252], we did not find an association between CDAI and CIN2+ status after adjusting for HPV status and other covariates. In this context, further research is needed to understand if antioxidants exercise their protective role in the entire carcinogenesis process or, on the contrary, if they intervene only in the first phase [657]. By contrast, our results on the associated DII and CIN2+ were consistent with the study by Sreeja and colleagues [705], which represented the first attempt in this field of research. This was in line with previous studies showing how higher DII was associated with the risk of other cancers [779-781]. Although other studies did not consider the relationship with cervical cancer, our results are in line with the notion that healthy nutrients and dietary patterns might reduce the risk of cervical cancer. By contrast, the consumption of high-calorie, high-fat, and processed foods might increase the risk [268]. Moreover, our analyses conducted in a subgroup of women with normal cervical cytology found a negative association between dietary intake of zinc and hrHPV positive status when all antioxidants were considered simultaneously. With respect to cumulative dietary antioxidant intake, we demonstrated that women with high CDAI had lower odds of being hrHPV positive than those with low CDAI. To our knowledge, this is the first study demonstrating that a diet based on the combined intake of nutrients with antioxidant properties might reduce the risk of hrHPV infection. Thus, our findings, if confirmed by large-scale prospective research, might inform novel preventive strategies against HPV infection based on dietary recommendations, especially in regions where vaccination is not implemented yet or vaccination coverage levels are not sufficient for prevention of infections. However, further studies are needed to understand molecular mechanisms underpinning the protective or detrimental effect of dietary factors against cervical cancer, also considering genetic susceptibility and epigenetic markers [612, 720].

In this context, the American Cancer Society (ACS) guidelines for cancer survivors recommend consuming healthy foods, as well as to maintain a healthy weight to engage in regular physical activity. With respect to diet, recommendations for cancer survivors regards the frequent consumption vegetables, fruits and whole grains regularly [694], and previous evidence showed that the promotion of healthy diet might reduce risk of recurrence [724], stress and breast/arm symptoms [725]. Among female cancers, breast cancer survivors exhibit an increased risk for secondary tumors, cardiovascular disease, diabetes [782-784], and reduced QoL [785, 786]. Thus, breast cancer survivors represent an important target population for promoting prevention strategies [246]. With this in mind, we evaluated the association of adherence to MD, physical activity, and weight status with QoL of Italian breast cancer survivors – who have completed treatments at least 6

months prior. We showed that low consumption of red meat and carbonated beverages, daily consumption of wine, and high consumption of dishes seasoned with sofrito had beneficial effects on several QoL subscales. By contrast, using olive oil as main culinary fat, low consumption of commercial sweets, and high consumption of nuts were associated with negative effects. Overall, these findings resulted in a null effect of adherence to MD on QoL. Furthermore, we observed better QoL sub-scores among women who performed moderate physical activity (i.e., diarrhea) and those who were underweight/normal weight (i.e., physical functioning and dyspnea) if compared with their counterparts. Next, our systematic review on dietary interventions for improving QoL of breast cancer patients demonstrated significant improvements in overall QoL and/or its subscales after the interventions. However, differences in study design, interventions and tools used for QoL assessment did not allow to provide an overall estimate. Thus, our study confirmed that more efforts are needed to understand the exclusive effect of diet and dietary interventions on QoL of breast cancer survivors. Instead, evidence of benefits from physical activity and weight management is already well established. Our results therefore raise the need for understanding whether tackling sedentary habits represents the best strategy to improve QoL instead of promoting intensive exercise. To fill these gaps, further research on the effects of physical activity and healthy diet on QoL among breast cancer survivors should be based on more homogenous methods, larger population-based studies and further randomized controlled trials which might allow us to evaluate the interactions of healthy behaviors and to improve the robustness of current evidence.

Given the need to promote women health through novel preventive strategies, the present thesis aims to investigate the effects of environmental exposures and lifestyles on genetic, epigenetic, and aging biomarkers in women during their reproductive age. With this in mind, increasing interest concerns the effect of genetic variants affecting vitamin D metabolism and functions during pregnancy. Vitamin D activity is mediated by the vitamin D receptor (VDR), a nuclear receptor that acts as a high-affinity ligand-activated transcription factor [521]. Since some studies proposed the potential association between VDR polymorphisms and the risk of adverse pregnancy outcomes (i.e., PTB, LBW and SGA births) [157, 161, 526-534], we aimed to evaluate their effects even considering dietary intake of vitamin D. In line with a previous study [527], for the *FokI* polymorphism, we showed that gestational duration and birth weight decreased with increasing number of mutated allele (A). Compared to the GG and GA, mothers who carried the AA genotype exhibited higher PTB risk, independent of socio-demographic characteristics, lifestyle, vitamin D intake, use of vitamin D supplements, type of delivery and parity. The meta-analysis – conducted for corroborating our results – confirmed this association under the recessive model, also pointing

out the protective effect of BsmI polymorphism against the risk of PTB. Thus, our results suggest the association between some maternal VDR polymorphisms with neonatal anthropometric measures and the risk of PTB, even if the mechanistic link between VDR expression and foetal outcome is still unclear. Although several studies suggest that vitamin D system - including VDR, its ligands and the metabolizing enzymes - plays a key role in innate immunity and implantation [540-544], the functional effects of VDR and its allelic variants in pregnancy are not yet clarified. In a personalized medicine perspective, it should be necessary to identify groups of women at risk of adverse outcomes and that could benefit from specific interventions. For these reasons, additional research - including observational prospective studies and clinical trials - is recommended to develop and validate effective preventive strategies against adverse outcomes, also taking into account the potential role of vitamin D and its related factors.

Not only biomarkers of susceptibility, but also biomarkers of aging could define a panel of measures for women health during lifetime. Despite its enormous complexity, a slight number of molecular mechanisms underpin the aging process, which represents an important risk factor for many non-communicable diseases [36]. Age-related changes in DNA methylation patterns, as those included in the so-called epigenetic clock, are among the best-studied aging biomarkers. Along with epigenetic mechanisms, also telomere length has been proposed as a marker of biological aging. In this context, we presented results from a systematic review of epidemiological studies to investigate the potential association between alcohol consumption, alcohol-related disorders, and telomere length. Our analyses showed that people with alcohol-related disorders exhibited shorter telomere length, but also that alcohol consumption per se did not appear to affect telomere length in absence of alcohol abuse or dependence. However, due to the lack of evidence during the periconceptional period, we also conducted a pilot study in the "Mamma & Bambino" cohort, comparing five non-smoking but drinking women with ten non-smoking and non-drinking women. Interestingly, we detected a significant difference when analysing relative telomere length of leukocyte DNA of cord blood samples from newborns. In particular, newborns from drinking women exhibited shorter relative telomere length than those born from non-drinking women. This was in line with the DOHaD theory that *in utero* exposures program the fetus for challenges that is likely to experience later in life [129]. Indeed, our preliminary findings support the current hypothesis that biological aging of the offspring at birth might reflect maternal and neonatal characteristics related to prenatal environmental adversity [594]. This provides further motivations to study telomere shortening as a potential molecular mechanism underpinning the effects of maternal behaviors on the development of chronic disease later in life. However, molecular mechanisms underpinning the effects of

maternal alcohol consumption on biological aging of the offspring are not yet well understood. Although it is plausible that our findings rely on an alcohol-related state of inflammation and oxidative stress *in utero* [616], further studies are necessary – if possible, by including more mother-child pairs from different birth cohorts – to shed light into this relationship.

To better understand the high variability and the responsiveness to environmental exposures and lifestyles [91], our research was also focused on the relationship of nutrient and dietary patterns with telomere length. As summarized by Freitas-Simoes and colleagues, for instance, the intake of antioxidants and the consumption of plant-derived foods help protect against telomere shortening, while the intake of saturated fats and the consumption of high-sugar and high-calorie products seem to be associated with shorter telomere length [554]. In addition, a recent systematic review by Habibi and colleagues has sought to unravel how the diet of pregnant women affects telomere length in their offspring [559]. Due to controversial and limited evidence, we investigated the effect of maternal dietary factors on telomere length of cfDNA from amniotic fluid, showing a positive association between maternal intake of magnesium and telomere length. Our result is consistent with previous studies [583, 584], even if it is the first evidence of an early effect of dietary intakes of mothers on biological aging of their offspring. From a biological point of view, magnesium is an important cofactor for the catalytic activity of enzymes implicated in DNA replication and repair [586-589], and in RNA synthesis [586]. Magnesium deficiency is also often associated with oxidative stress [583] and pro-inflammatory status [590], which in turn might lead to telomere shortening. The lack of any solid evidence in this field of research, therefore, encourages further efforts to understand the influence of maternal dietary factors on biological aging, as determined by telomere length.

In this scenario, telomere length represents a potential molecular mechanism also associated with maternal GWG during pregnancy. Our hypothesis was that measuring telomere length of mlDNA and cfDNA in early pregnancy could be helpful for identifying women who adequately or inadequately gain weight at delivery. Notably, relative telomere length of cfDNA was able to discriminate women who gained weight adequately, also after adjusting for other maternal characteristics.

Moreover, according to the DOHaD theory, the effect of maternal weight gain on telomere length of cfDNA was already evident in early pregnancy. Due to its relevance, further experimental studies are needed to understand molecular mechanisms underpinning this relationship and to take into account other factors influencing the uterine environment during pregnancy.

Since diet, smoking, alcohol consumption, physical activity, and metabolic status can potentially modify epigenetic signatures [224], we also evaluated their effects on DNA methylation in a cancer-free population. Although the molecular mechanisms underpinning the relationship between exposome and several adverse outcomes are currently not clarified, Epigenetics offers a partial explanation of this concern [787]. In fact, the transgenerational nature of epigenetic events suggests the pathways of transmission of the associated risk from mother to child [42]. For this reason, recent studies have proposed a remarkable link between epigenetic and environmental exposure, suggesting how nutrients, pollutants and other environmental factors can influence the turnover of epigenetic marks [318]. With respect to dietary patterns – towards which the research is moving – our study found that intake of "healthy" foods - such as wholemeal bread, cereals, fish, fruit, raw and cooked vegetables, legumes, and soup - positively correlated with LINE-1 methylation, which in turn was negatively associated with chromosomal instability and aberrant genome function. These findings partially confirmed previous studies reporting that higher intake of vegetables and/or fruits decreased the risk of LINE-1 hypomethylation [297, 322], probably due to the wide variety of nutrients and bioactive compounds provided by fruits and vegetables. Moreover, our analysis described how women with high adherence to the prudent dietary pattern showed increased LINE-1 methylation level than those with low adherence. Although our study suggested LINE-1 methylation as a molecular mechanism underpinning the protective effect of healthy diet, further outcome-driven research and mechanistic studies should be encouraged to evaluate the causal relationship between dietary intake and DNA methylation.

The importance of the adherence to a healthy dietary pattern is also linked to the consequences that an unhealthy lifestyle could have on human health, such as obesity. Indeed, overweight and obesity account for an important burden for public health [329], because raised BMI is often associated with an increased risk of cardiovascular diseases, diabetes, musculoskeletal disorders, and some cancers [330]. It is also noteworthy that raised BMI could have adverse consequences on women of childbearing age and especially during pregnancy [176, 331-336] and at delivery [173, 333, 335, 337]. In this complex scenario, it would be interesting to uncover molecular mechanisms associated with raised BMI and obesity. Among them, epigenetic mechanisms surely attracted the attention of many researchers, due to their potential role in development of obesity from the early stages of life [323]. For instance, previous studies already suggested the involvement of DNA methylation, aberrant miRNA expression, histone modification and nucleosome release in obesity and associated comorbidities [324-326]. In addition to what has been demonstrated [323], our study on healthy women showed a negative relationship between BMI and LINE-1 methylation, which resulted in

lower methylation level among obese women. These findings – adding to the current knowledge on the relationship between obesity and DNA methylation – sustained the hypothesis that measuring obesity-related DNA methylation markers could be helpful to understand the molecular effects of inadequate weight gain. Moreover, it could be also useful for identifying people at higher risk of obesity or those who respond well to weight loss programs. However, at present, these are just interesting perspectives that merit further investigation through longitudinal and well-structured studies.

Summarizing what addressed in the above-mentioned studies, an integrated approach is necessary to understand the relationship between exposome, molecular mechanisms and human health. Nowadays, strategies aiming to reduce health disparities should also include the promotion of healthy behaviors (e.g., diet, smoking habits and physical activity), which are considered as risk factors for several health conditions and diseases [365]. Social disadvantages and unhealthy behaviors – occurring either *in utero* or during lifetime – may induce sustainable biological changes involved in individual non-communicable risk profile [366, 367]. Despite recent strides in this field of research, molecular mechanisms involved are still not fully understood. For this reason, uncovering the epigenetic mechanisms underpinning this relationship might offer a plausible explanation.

Previous studies suggested some social disparities in epigenetic markers, including DNA methylation levels. However, the social status alone cannot explain these inequalities that might be mediated by behavioral factors. For this reason, we tested the mediating effect of behaviors (i.e., adherence to MD, smoking status, physical activity, and weight status) in the relationship between socioeconomic status and LINE-1 methylation level. Our findings confirm previous evidence that SES is a determinant of health, also through biological changes such as epigenetic mechanisms. Notably, we propose that behaviors – especially the adherence to MD – might mediate this association, leaving room for public health interventions aimed at promoting healthy dietary habits in social disadvantaged people. However, further prospective studies should be recommended to confirm this evidence taking into account additional social factors and behaviors.

In conclusion, we reported results from different cohorts of women of childbearing age to study the relationship between individual characteristics, environmental exposure during the lifetime, behaviors and molecular mechanisms involved in different physiological and pathological conditions. The integrated approach that we applied would be important for developing personalized strategies to prevent, diagnose and treat diseases and to improve the quality of life of women in all the phases of their life. The novelty of our study lies on this innovative approach for

the epidemiological research, but further efforts are needed. Indeed, some of the relationships observed in healthy women should be confirmed – or at least investigated – in other cohorts. For this reason, our promising results lay the foundation for future studies to identify subgroups of women that need tailored interventions of Public Health.

14. References

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