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Epigenetics of nutrition in aging

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Part two: Development of a new epigenetic biomarker of aging and its application to samples collected within the NU-AGE project

Introduction

In many countries worldwide, the population is aging rapidly and the proportion of people aged 65 and over is continuously increasing, especially in Europe (Eurostat; https://ec.europa.eu/eurostat/web/products-datasets/-/tps00028). This demographic shift towards higher proportions of older adults is associated with societal and economic consequences (Rae et al. 2010). Despite the gains in life expectancy, the period of life spent in good health (*i.e.* healthspan) is not keeping pace, with high prevalence levels of chronic diseases, disabilities and multimorbidity. Aging is the most important risk factor for many non-communicable and chronic diseases, such as cancer, diabetes, cardiovascular diseases or neurodegenerative disorders. According to recent conceptualizations, gathered under the term Geroscience, aging and age-related diseases share common mechanisms (Kennedy et al. 2014; Franceschi et al. 2018). A small number of hallmarks or pillars, which are basic molecular and cellular processes, underlies the biology of aging in a highly interconnected way (Kennedy et al. 2014; López-Otín et al. 2013) (**Figure 1**).

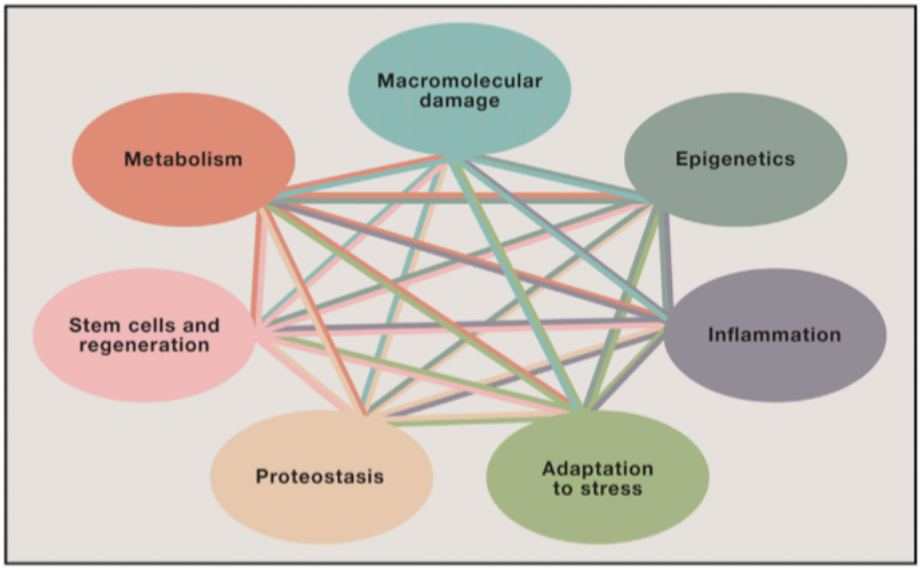


Figure 1: The seven pillars of aging. Adapted from Kennedy *et al*. 2014.

Age-related diseases rely on the same pillars and can be considered as an acceleration of the aging processes, *i.e.* as deviations from the trajectories of healthy aging. These deviations lead to a discrepancy between the chronological age of an individual and his/her effective biological age. Within the framework of Geroscience, there is growing interest in biomarkers of biological age, which could be able to capture this discrepancy. There is currently no consensus on how to best measure on individual's biological age and until now a valid and unique aging biomarker has not yet been identified. The American Federation for Aging

Research has proposed some criteria for the development of aging biomarkers: it must predict the rate of aging; it must monitor a basic process underlying the aging process; it must be able to be tested repeatedly without harming the person; and it must be something that works both in humans and in laboratory animals (Butler et al. 2004). In the last decade, there has been tremendous interest in epigenetic biomarkers of aging. As their name imply, these biomarkers rely on epigenetics, which are one of the seven pillars of the aging process.

Epigenetics refers to heritable and reversible changes in chromatin structure and gene function that are not related to changes in the DNA sequence itself. DNA methylation, which is one of the best studied and most mechanistically understood epigenetic mechanism, involves the addition of a methyl group to a cytosine in a cytosine-guanine dinucleotide (named CpG site). DNA methylation is affected by both genetic and environmental factors and is subject to profound rearrangements during aging (Jung & Pfeifer 2015). Four main different types of age-related changes coexist: global hypomethylation (Bollati et al. 2009; Luo et al. 2014), differential methylation of specific genomic loci (Bell et al. 2012; Bell et al. 2016; Rakyan et al. 2010; Christensen et al. 2009), increase in inter-individual divergence between patterns of DNA methylation (Fraga et al. 2005; Maegawa et al. 2017; Tan et al. 2016; Mendelsohn & Larrick 2017) and increase in the rate of epimutations (Gentilini et al. 2015). DNA methylation role in aging process, its implication in cellular senescence and in the development of various diseases has been extensively investigated (Calvanese et al. 2009; Pal & Tyler 2016).

During the last years, DNA methylation-based biomarkers, designated under the term "epigenetic clocks", have been put forward as accurate aging biomarkers. Epigenetic clocks are algorithms based on the combination of DNA methylation levels at some specific CpG sites, that can predict the age of a DNA source. They are nowadays considered as among the most robust predictors of chronological age in humans (Horvath & Raj 2018; Field et al. 2018; Jylhävä et al. 2017; BLUEPRINT consortium 2016). Two of these clocks, Horvath's (based on 353 CpG sites) and Hannum's (based on 71 CpG sites) calculators, developed in large samples cohorts covering the entire adult life span, show high correlations with age and small deviations from chronological age (Horvath 2013; Hannum et al. 2013). Besides these two models, researchers have also developed several other age-prediction models (Levine et al. 2018; Lu, Quach, et al. 2019; Horvath, Oshima, et al. 2018; Bocklandt et al. 2011; Weidner et al. 2014). These clocks are mostly derived by linear regression algorithms that train against the chronological age of the sample and ultimately select a set of CpG sites. The weights associated with each CpG site are determined and the product of the methylation level at each site with

the learned coefficient is summed, leading to an estimated epigenetic age, also called DNAm age (**Figure 2**).

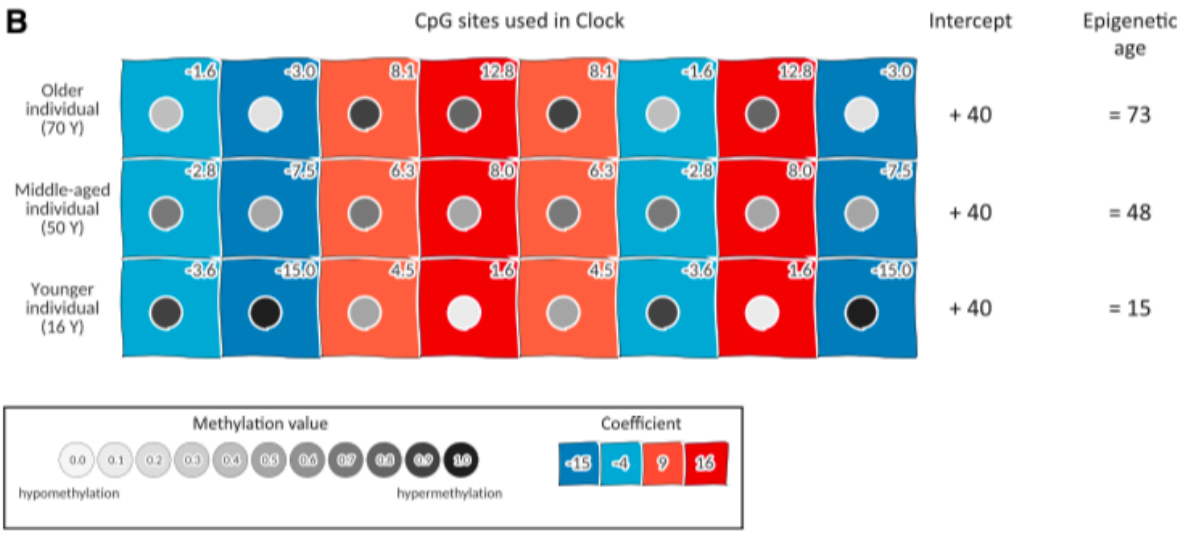


Figure 2: DNA methylation clock: how it works. Adapted from Field *et al*. 2018.

Epigenetic clocks are not only able to predict chronological age with high accuracy, but evidence also suggests that they can be informative of the biological age of an individual. Thus, a positive or negative deviation of the biological age to chronological age according to these models reflects the presence of an accelerated or decelerated aging phenomenon, respectively. Horvath's and Hannum's clocks are able to predict all-cause mortality independently of classic risk factors (Marioni, Shah, McRae, Chen, et al. 2015; Chen et al. 2016; Dugué et al. 2018; Zheng et al. 2016; Christiansen et al. 2016; Perna et al. 2016; Fransquet et al. 2019; Marioni et al. 2016) and they also can be informative on other age-related outcomes such as frailty or cognitive and physical functioning (Ryan et al. 2019a; Simpkin et al. 2017; Sillanpää et al. 2018; Gale et al. 2018; Breitling et al. 2016; Gale et al. 2018; Marioni, Shah, McRae, Ritchie, et al. 2015). A newly developed epigenetic biomarker of aging, known as PhenoAge, outperformed the previous models for mortality prediction and is so far the best predictor of age-related decline (Levine et al. 2018). This model, based on 513 CpG sites, was not calibrated on chronological age but on "phenotypic age", derived from a set of different bio-clinical parameters (such as albumin, creatinine or C-reactive protein levels), and was better able to predict mortality (Levine et al. 2018). Apart from mortality and physical functioning, many other phenotypes and age-related conditions have been linked to epigenetic age acceleration, in blood or in specific tissues (**Table 1**). It was demonstrated that Horvath's clock is able to

capture the age acceleration phenomenon associated with progeroid syndromes, such as Down syndrome (Horvath, Garagnani, et al. 2015) or Werner syndrome (Maierhofer et al. 2017). Importantly, the epigenetic clocks are also able to detect age-deceleration effects in models of healthy aging and longevity, such as centenarians and their offspring (Horvath, Pirazzini, et al. 2015a).

Phenotypes	References
Dementia	(Levine, Lu, et al. 2015) (Zhang et al. 2017) (Lu et al. 2017)
Parkinson's disease	(Horvath & Ritz 2015)
Menopause	(Levine et al. 2016)
Psychiatric disorders - Post-traumatic stress disorders - Bipolar disorders - Major depressive disorders	(Wolf et al. 2016) (Wolf et al. 2017) (Fries et al. 2017) (Han et al. 2018)
Cardio-vascular events	(Perna et al. 2016) (Roetker et al. 2018) (Levine et al. 2018)
Development of cancer	(Levine, Hosgood, et al. 2015) (Zheng et al. 2016) (Perna et al. 2016) (Durso et al. 2017) (Ambatipudi et al. 2017) (Dugué et al. 2018) (Kresovich et al. 2019)
Huntington disease	(Horvath, Langfelder, et al. 2016)
Obesity	(Horvath et al. 2014) (Grant et al. 2017) (Nevalainen et al. 2017) (Ryan et al. 2019b) (Li et al. 2019)
Chronic viral infections - HIV infection - CMV infection	(Horvath & Levine 2015) (Kananen et al. 2015) (Gross et al. 2016) (Horvath, Stein, et al. 2018)

Table 1: Phenotypes associated with the epigenetic clocks.

In view of the demographic remodeling that our societies face and its consequences, there is an important need to identify factors that are able to influence health in old age and to develop and validate interventions that could slow down or counteract the process of aging and its associated pathologies. Fundamental aging processes, as defined within the framework of Geroscience perspective, can be targeted by genetic or pharmacologic interventions, but another possible strategy to impact on healthspan is to intervene on lifestyle factors, such as diet or physical activity. Nutritional interventions seem to be one of the most promising approaches to promote healthy aging, and growing amount of data indicates that they can be associated with major health benefits (Longo et al. 2015; Dato et al. 2016; Wahl et al. 2016; Shlisky et al. 2017; Heiss et al. 2017; Xia et al. 2017). In order to monitor the impact of interventions aiming at increase healthy lifespan, accurate biological measures of age are needed. In this case, biomarkers could serve as surrogate endpoints, by showing that the intervention is affecting the underlying fundamental aging processes, before sufficient clinical events, such as age-related disease diagnosis or death, have accumulated (Justice et al. 2018). Biomarkers could also help for the early selection of patients at higher risk of accelerated aging and for the personalized evaluation of the effectiveness of the intervention.

Regarding the epigenetic clocks, it is clear that they can be influenced by lifestyle factors and environmental exposures, such as education (Ryan et al. 2019a; Fiorito et al. 2019; Quach et al. 2017; Zhao et al. 2019), exposition to stress, violence or socio-economic disadvantages (Sumner et al. 2019; Austin et al. 2018; Jovanovic et al. 2017; Lawn et al. 2018; Fiorito et al. 2017; Wolf et al. 2018; Zannas et al. 2015), but also exposition to pollution (Li et al. 2018; Curtis et al. 2019; White et al. 2019; Ward-Caviness et al. 2016), smoking (Zhao et al. 2019; Levine et al. 2018), and alcohol dependence (Rosen et al. 2018; Luo et al. 2019). Dietary habits usually associated with healthy eating, such as fish, poultry or fruit/vegetables intakes, have been associated with decreased age acceleration effects according to Horvath's clock or Levine's model, although the observed effects were weak (Quach et al. 2017; Levine et al. 2018). A recently published paper examined the effects of a vitaminic supplementation on epigenetic age estimated with Horvath's model (Sae-Lee et al. 2018), however, data on a possible rejuvenation of epigenetic age with a dedicated nutritional intervention are still scarce.

The objectives of the work presented here are dual. In the first part, we evaluate the impact of a one-year Mediterranean-like diet intervention, delivered within the framework of the NU-AGE project, on epigenetic age acceleration measures calculated with Horvath's model. In the second part of the work, we present the development a new epigenetic biomarker of

aging. This in-house developed predictor is based on a limited number of CpG sites assessed by the Agena EpiTYPER® system and is therefore potentially cost-effective compared to Horvath's clock. We validate the new epigenetic biomarker of age in cohorts of individuals characterized by accelerated or decelerated aging and we use it to evaluate the impact of the nutritional intervention of the NU-AGE project.

Part one

Impact of Mediterranean-like diet on epigenetic biomarkers of aging

1.1 *Introduction*

Mediterranean diet, which is considered by UNESCO as a heritage of humanity, is a well-balanced mix of nutrients, antioxidants and anti-inflammatory molecules. This diet is characterized by a high intake of plant-based foods (vegetables, fruits, unrefined cereals, whole grain, legumes, nuts, seeds) and olive oil; a low-to-moderate intake of dairy products, fish and poultry; and a low intake of red meat and alcohol (Willett et al. 1995; Martucci et al. 2017). Mediterranean diet could represent a powerful tool for promoting healthy aging. Indeed, this diet has been extensively investigated in relation to several health outcomes and it has demonstrated favorable effects on mortality, cardiovascular risk and blood pressure, cancer, cognitive status, frailty, as well as on inflammation (Estruch et al. 2013; Estruch et al. 2006; Mitjavila et al. 2013; Ostan et al. 2015; Martínez-González et al. 2015; Kojima et al. 2018; Trichopoulou et al. 2003; Trichopoulou et al. 2015; Psaltopoulou et al. 2013; Singh et al. 2014). Some studies have suggested that Mediterranean diet prevents telomere shortening, a well-established biomarker of age, but results are not consistent among different studies (Davinelli et al. 2019).

The role of Mediterranean diet in promoting healthy aging has been recently investigated in the framework of the European project NU-AGE (‘‘New dietary strategies addressing the specific needs of elderly population for an healthy aging in Europe’’), a large multidisciplinary consortium with 30 partners across Europe (Santoro et al. 2014; Berendsen et al. 2014). The aim of NU-AGE project was to investigate how an intervention based on Mediterranean diet, specifically tailored according to the nutritional needs of people over 65 years of age, can impact on age-related diseases and functional decline, possibly counteracting inflammaging and its outcomes (Franceschi et al. 2000). Probands were enrolled in five European countries (Italy, Poland, France, Netherlands and United Kingdom) and a one-year Mediterranean-like diet was administered to the intervention subgroup. A comprehensive clinical and molecular characterization of participants was performed at baseline and after the one-year intervention, and results achieved so far in the framework of this study have demonstrated a beneficial effect of the Mediterranean-like diet on global cognition and episodic memory (Marseglia et al. 2018), osteoporosis (Jennings et al. 2018), immune function (Maijo et al. 2018), on cardiovascular health (Jennings et al. 2019), on the rate of bone loss in individuals with osteoporosis (Jennings et al. 2018), as well as on the proteasomal proteolysis (Athanasopoulou et al. 2018). The NU-AGE study design (different countries with different dietary traditions and habits) and the large number of collected data allowed to evaluate the impact of relevant variables usually poorly investigated (age, sex, ethnicity/genetics, as well as

individual characteristics) on different parameters at baseline and after the intervention (Konz et al. 2018; Marseglia et al. 2018; Ostan et al. 2018; Pujos-Guillot et al. 2018; Santoro et al. 2018; Santoro et al. 2019; Jennings et al. 2019). Importantly, the design of NU-AGE project offers the possibility to evaluate the effects of the intervention taking into account the background (in terms of lifestyle habits, environmental conditions and genetic profile) of the enrolled subjects, which were recruited also in non-Mediterranean countries (Poland, Netherlands and United Kingdom). The effects of a Mediterranean diet intervention on non-Mediterranean countries are not granted, because its transferability requires specific changes in dietary habits (Martínez-González et al. 2017) and because genetic and environmental factors, that can be country-specific, can hamper/enhance its effects (Mayr et al. 2018).

Here, we decided to study the impact of the nutritional intervention on the epigenetic biomarkers of aging, focusing on subjects enrolled in a Mediterranean country (Italy) and in a non-Mediterranean country (Poland).

1.2 *Methods*

1.2.1 *NU-AGE study*

NU-AGE was a one-year, multicenter, randomized, single-blind, controlled trial (registered with *clinicaltrials.gov*, NCT01754012) with two parallel groups (*i.e.* dietary intervention and control) carried out during April 2012–January 2015 in five European centers in Italy (Bologna), Poland (Warsaw), France (Clermont-Ferrand), Netherlands (Wageningen), and the United Kingdom (Norwich). Volunteers from the community aged 65–79 years, free of major overt chronic diseases for at least two years (*i.e.* cancer, severe organ disease), living independently, and free of dementia, were recruited to participate in the baseline assessment (Santoro et al. 2014; Berendsen et al. 2014). At enrollment, exclusion criteria included severe chronic diseases, type 1 and insulin-treated type 2 diabetes, chronic use of corticosteroids, recent (previous 2 months) use of antibiotics, recent (previous 3 months) change in habitual medication use, malnutrition (as diagnosed by Body Mass Index (BMI) <18.5 kg/m^2 or >10% weight loss within the previous 6 months), food allergy/intolerance requiring special diets, or frailty (as assessed by the presence of at least three out of five criteria: unintentional weight loss, self-reported exhaustion, weakness (grip strength), slow walking speed, and low physical activity) (Fried et al. 2001). Of the 2668 participants, 1512 were screened for inclusion and 1296 were eligible to participate in the NU-AGE trial. Participants were randomly assigned (1:1 allocation ratio) to the control or intervention groups, after stratification by sex, age (65-72 or >72-79 years), frailty status (pre-frail or non-frail), and BMI (<25 or ≥25 kg/m^2).

Randomization was done by computer-generated allocation. All participants provided written informed consent before participating, in accordance with the Declaration of Helsinki, and the study protocol was approved by ethics committees in each country.

Participants assigned to the intervention group received individually tailored standardized dietary advice, delivered by a trained dietician or research nutritionist. Dietary advice was administered 9 times during the year and supported by mail or e-mail. The NU-AGE dietary guidelines were based on nutrient reference values and food-based dietary recommendations for older adults from each of the 5 countries where the intervention took place, the modified MyPyramid for Older Adults and nutrient requirements from the European Commission and the Institute of Medicine. To help compliance, participants received commercially available foods, such as olive oil, whole-grain pasta, margarine rich in mono- and polyunsaturated fatty acids, and low-fat, low-salt cheese. They also received vitamin D_3 supplements (10 μg per day).

Adherence to study protocol was evaluated using the 7-day food records and calculated by the NU-AGE index scoring system, specifically constructed to this purpose (Jennings et al. 2018; Berendsen et al. 2018). The NU-AGE index is a continuous scale in which 16 dietary components were included. For the adequacy components whole grains, fruits, vegetables, legumes, low-fat dairy, low-fat cheese, fish, low-fat meat and poultry, nuts, olive oil, fluids, and vitamin D_3 supplements, a score ranging from 0 to 10 could be obtained for greater intakes of these components. For moderation components (alcohol, sodium and sweets), participants with lower intakes received 10 points ranging to 0 points for participants with greater intakes. Here, we used the NU-AGE index transformed in percentage and scaled 0 to 100 (with 0 equal to no adherence and 100 to fully adherent).

Height was measured with a stadiometer to the nearest 0.1 cm, while weight was measured to the nearest 0.1 kg with a calibrated scale. BMI was calculated as follows: weight [kg]/height[m]^2.

From the whole NU-AGE cohort, a sub-group of 120 subjects was randomly selected from the Italian and Polish sub-cohorts to be analyzed by specific -omics at baseline (T0) and after dietary intervention (T1). Thus, genome-wide DNA methylation analysis was performed in a subgroup of 120 subjects (60 from the Italian cohort and 60 from the Polish one) from the intervention group, both at T0 and T1, for a total of 240 samples. Exact chronological age of

the subjects (in years) at T0 was calculated as follows: [(T0 date) – (Date of birth)] / 365. Exact chronological age at T1 was calculated as follows: [(T1 date) – (Date of birth)] / 365.

1.2.2 *DNA methylation analysis*

Samples were analyzed for genome-wide DNA methylation patterns using the Illumina® Infinium HumanMethylation450 BeadChip array (Illumina Inc., CA, USA), which interrogates more than 450 000 methylation sites quantitatively across the genome at single-nucleotide resolution. Genomic DNA was extracted from 250 μL of whole blood (drawn on ethylenediamine tetra acetic acid (EDTA) tubes), using the QIAamp 96 DNA Blood Kit (QIAGEN, Hilden, Germany). DNA quantification was performed using the Quant-iT™ dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), with a robotic liquid handling system, Hamilton MicroLab Star (Hamilton, Reno, NEV, USA), allowing quantification of a 96-well format. One microgram of DNA was bisulfite converted, using the EZ DNA Methylation Kit (Zymo Research Corporation, Orange, CA, USA) according to manufacturer's instructions. After bisulfite conversion, DNA was whole-genome amplified, enzymatically fragmented and hybridized to Illumina® Infinium HumanMethylation450 BeadChips (Illumina Inc., CA, USA), according to manufacturer's protocols. Samples from the different groups (Italy and Poland, T0 and T1) were accurately randomized across the experimental sessions. Arrays were scanned using the HiScan instrument (Illumina Inc., CA, USA). Raw fluorescence intensities were extracted using '*minfi*' Bioconductor package (Aryee et al. 2014) and normalization was performed using the *preprocessQuantile* function (Touleimat & Tost 2012).

1.2.3 *Evaluation of DNA methylation age and of epigenetic age acceleration*

Normalized DNA methylation data were uploaded into the DNA methylation age calculator, developed by Steve Horvath and freely available at the website: https://dnamage.genetics.ucla.edu, to calculate DNAm age. DNAm age is calculated using the weighted average of DNA methylation levels at 353 CpG sites, as described by Steve Horvath (Horvath 2013). The 'advanced blood analysis' option was selected in the online calculator, allowing the calculation of three measures of epigenetic age acceleration (AA) that were further considered here. These measures have been previously described by Horvath and colleagues and have been applied to date in several publications. The first measure is considered as the universal measure of epigenetic AA and is denoted AgeAccel. It corresponds to the residual that results from regressing DNAm age on chronological age. The second measure of epigenetic

AA is referred as Intrinsic Epigenetic Age Acceleration (IEAA), denoted as AAHOAdjCellCounts in the online software. IEAA is defined as the residual resulting from regressing DNAm age on chronological age and seven measures of immune blood cells count estimates: naive CD8+ T cells, exhausted CD8+ T cells, plasma B cells, CD4+ T cells, natural killer cells, monocytes and granulocytes. IEAA is independent of changes in blood cell composition that occur with time and is considered as a measure of 'pure' epigenetic aging effects in blood cells. Finally, the third measure considered is referred as Extrinsic Epigenetic Age Acceleration (EEAA), known as BioAge4HAStaticAdjAge in the online software. EEAA is based on a weighted average of the epigenetic age measure with Hannum's clock (Hannum et al. 2013) and three blood cell types that are known to change with age: naive cytotoxic T lymphocytes (CD45RA+CCR7+), exhausted cytotoxic T lymphocytes (CD45RA-CD28-) and plasma B cells. EEAA is defined as the residual formed from regressing the resulting weighted epigenetic age on chronological age. This measure is dependent on age-related changes in blood cell composition and can be considered as a measure of aging in immune system.

1.2.4 *Estimating blood cell counts based on DNA methylation levels*

Blood cell counts used in the measures of IEAA and EEAA were estimated based on DNA methylation data using the epigenetic clock online software. Blood cell proportions of CD8+ T cells, CD4+ T cells, natural killer cells, B cells and granulocytes are based on Houseman's estimation method (Houseman et al. 2012). An advanced analysis option of the epigenetic clock software is used to estimate the percentage of naïve and exhausted CD8+ T cells.

1.2.5 *Genotyping*

Genomic DNA was extracted from 250 μL of whole blood (drawn on EDTA tubes), using the QIAamp 96 DNA Blood Kit (QIAGEN, Hilden, Germany). Two hundred nanograms of genomic DNA were genotyped for 713 014 genetic markers by the Illumina® OmniExpress BeadChip (Illumina Inc., CA, USA), according to manufacturer's protocol. After quality control, 118 samples were retained. Quantitative trait association analysis and estimation of Single Nucleotide Polymorphisms (SNPs) allele frequencies were performed using PLINK toolset.

1.2.6 *Statistical analysis*

The effects of the nutritional intervention on the three above-mentioned measures of epigenetic AA (AgeAccel, IEAA and EEAA) were analyzed with a Student's paired-sample t-test. For each epigenetic AA measure, Benjamini-Hochberg procedure was applied to correct for multiple tests, considering a total of 6 tests. Pearson correlations between measures of epigenetic age and chronological age or scores of adherence to Mediterranean diet were calculated. All statistical analyses and graphics were produced using R v3.3.2.

1.3 Results

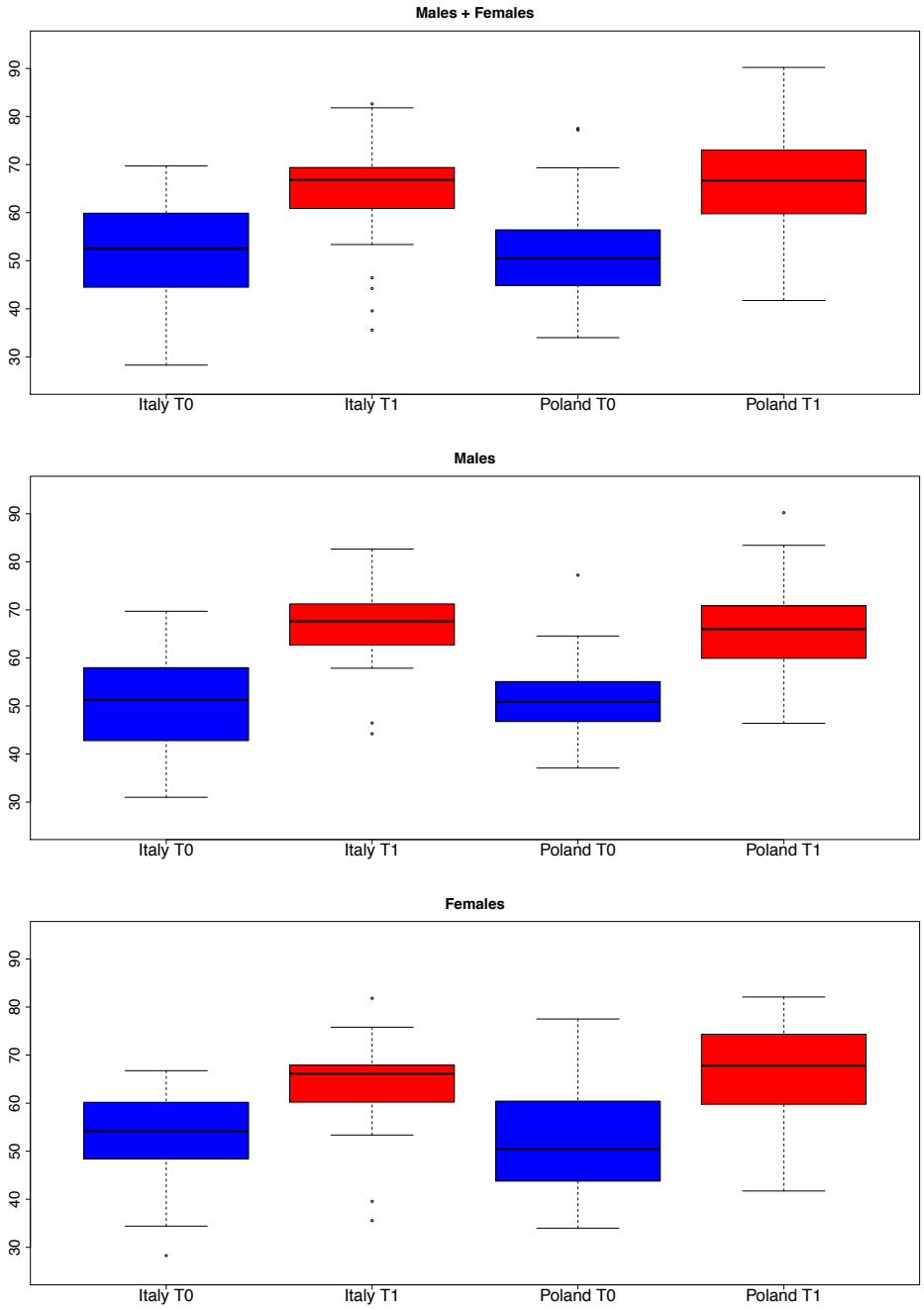
1.3.1 *Subjects*

Genome-wide DNA methylation profiles were analyzed by the Illumina® Infinium HumanMethylation450 Beadchip (Illumina Inc., CA, USA) in whole blood of 120 European subjects, with chronological age ranging from 65 to 79 years old. Sixty patients were recruited in Italy and the other half was recruited in Poland. Characteristics of enrolled subjects are summarized in **Table 1.1**. Baseline characteristics were similar between the two groups in terms of chronological age and adherence to Mediterranean diet (Student's t-test p-value > 0.05) (**Figure 1.1**). Body Mass Index (BMI) tended to be higher in Polish subjects compared to Italian ones, and this difference was statistically significant when considering only males (Student's t-test p-value = 0.027) (**Figure 1.2**). After one year of nutritional intervention (T1), adherence to Mediterranean diet significantly increased in both Italian and Polish participants, and a significant decrease in BMI was observed in Italian males (paired Student's t-test p-value = 0.008) (**Figures 1.1 and 1.2**).

Country			**Italy**		**Poland**	
Subjects (n)			60		60	
Males / Females (n)			27 / 33		24 / 36	
Time			**T0**	**T1**	**T0**	**T1**
Mean chronological age	(years), mean ± SD	**Males + Females**	72.23 ± 3.82	73.28 ± 3.81	71.08 ± 4.10	72.10 ± 4.09
Mean BMI	kg/m^2, mean ± SD		26.99 ± 3.60	26.67 ± 3.59	28.07 ± 3.37	28.02 ± 3.24
Adherence to NU-AGE diet	(according to NU-AGE diet score), mean ± SD		51.86 ± 9.78	64.84 ± 8.84	51.62 ± 9.52	66.69 ± 10.09
Mean chronological age	(years), mean ± SD	**Males**	72.41 ± 3.91	73.48 ± 3.91	71.55 ± 4.27	72.58 ± 4.25
Mean BMI	kg/m^2, mean ± SD		26.30 ± 2.88	25.79 ± 2.76	28.20 ± 3.06	28.19 ± 2.81
Adherence to NU-AGE diet	(according to NU-AGE diet score), mean ± SD		50.63 ± 10.43	66.48 ± 8.45	51.30 ± 8.39	66.74 ± 10.28
Mean chronological age	(years), mean ± SD	**Females**	72.07 ± 3.80	73.12 ± 3.79	70.76 ± 4.01	71.78 ± 4.00
Mean BMI	kg/m^2, mean ± SD		27.55 ± 4.05	27.39 ± 4.05	27.98 ± 3.61	27.91 ± 3.53
Adherence to NU-AGE diet	(according to NU-AGE diet score), mean ± SD		52.87 ± 9.25	63.49 ± 9.06	51.84 ± 10.31	66.66 ± 10.10

Table 1.1: Characteristics of the study population at baseline (T0) and after one year of Mediterranean-like diet (T1).

BMI: Body Mass Index. SD: Standard Deviation.



<u>Figure 1.1</u>: Adherence to Mediterranean-like diet increases from T0 to T1.

Boxplots of NU-AGE diet score at T0 and at T1, in Italian and Polish subjects, considering all the subjects (upper panel), only males (middle panel) and only females (lower panel).

There was no difference between the groups at baseline. After one-year of nutritional intervention, adherence significantly increased in all groups.

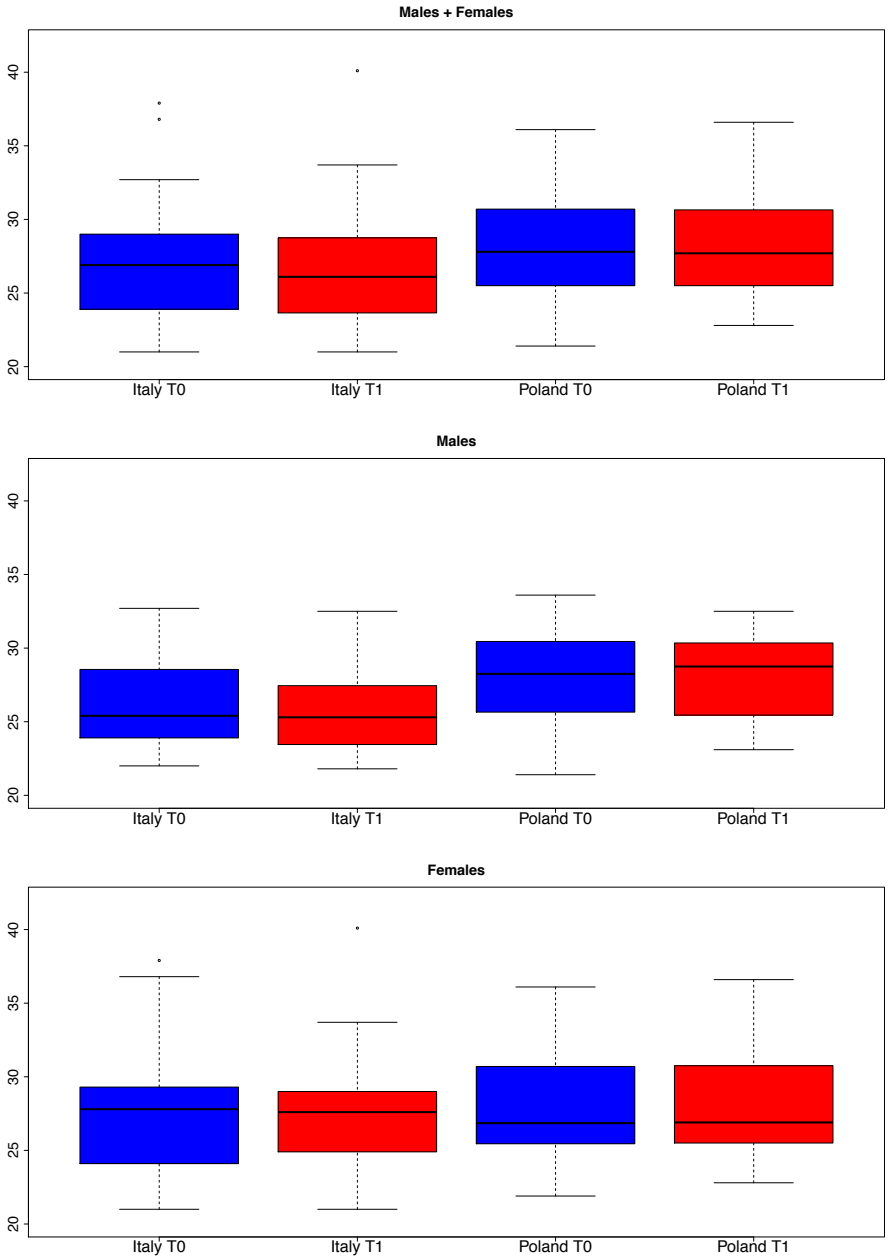


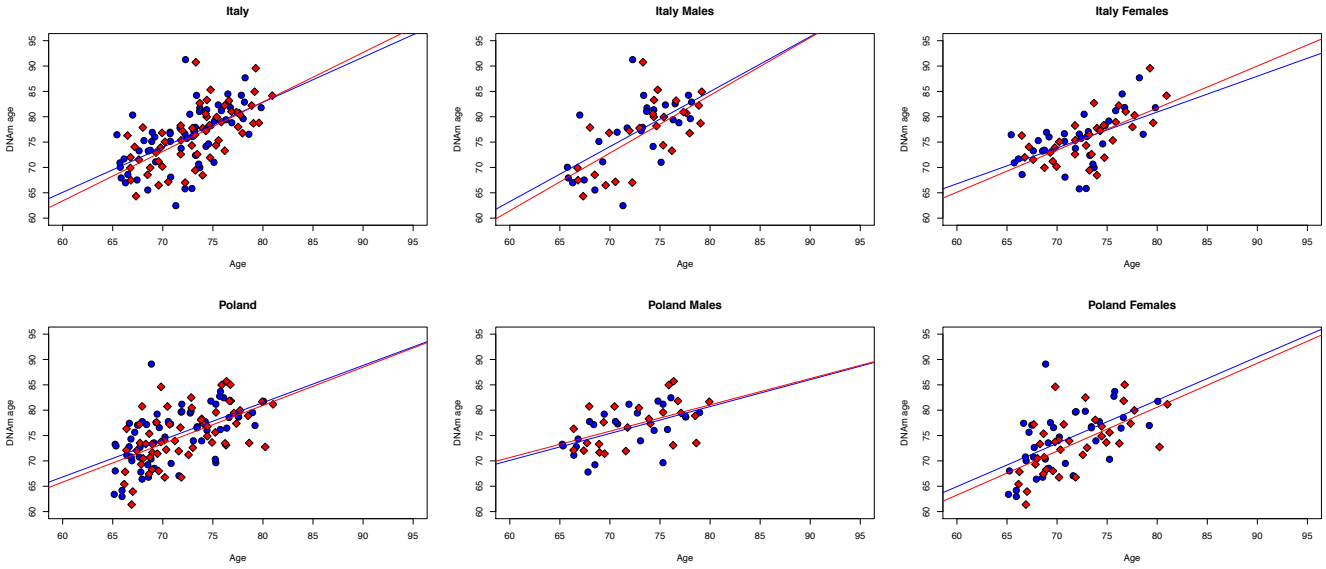
Figure 1.2: Body Mass Index at T0 and T1.

Boxplots of BMI values at T0 and at T1, in Italian and Polish subjects, considering all the subjects (upper panel), only males (middle panel) and only females (lower panel).

At baseline, BMI was significantly higher in Polish males as compared to Italian males. After one year of nutritional intervention, a significant decrease in BMI was observed in Italian males.

1.3.2 *Effect of the nutritional intervention on the epigenetic age acceleration measures*

Epigenetic age (also referred to as DNA methylation age (DNAm age)) was calculated using the online age calculator. As expected, DNAm age was significantly associated with chronological age ($p<0.0001$), both at T0 (before nutritional intervention) and at T1 (after a 12-months Mediterranean-like nutritional intervention), in both Italian and Polish groups (**Figure 1.3)**.



<u>Figure 1.3</u>: Significant association between DNAm age and chronological age at T0 and T1.

Scatter plots of chronological age (x-axis) versus DNAm age (y-axis) in the different groups (T0 = blue; T1 = red). Lines represent fitted linear regressions (<u>Italy T0</u>: $R^2 = 0.57$, p-value = 1.67^{-06}; <u>Italy T1</u>: $R^2 = 0.65$, p-value = 1.41^{-08}; <u>Poland T0</u>: $R^2 = 0.55$, p-value = 5.41^{-06}; <u>Poland T1</u>: $R^2 = 0.56$, p-value = 2.98^{-06}).

For each subject we evaluated the epigenetic age acceleration (AA), that is the deviation between DNAm age and effective chronological age. Positive values of epigenetic AA indicate an epigenetic age older than expected, while negative values indicate an epigenetic age younger than expected on the basis of chronological age. In particular, as described in the Methods section, we considered three measures of epigenetic AA, implemented in the online age calculator, which reflect different aspects of epigenetic aging: 1) AgeAccel; 2) Intrinsic Epigenetic Age Acceleration (IEAA); 3) Extrinsic Epigenetic Age Acceleration (EEAA) (**Table 1.2)**. At T0, in Italian subjects, AgeAccel ranged from -12.38 to 15.62 years, IEAA ranged from -11.56 to 12.27 years and EEAA ranged from -10.90 to 7.41 years. In Polish subjects, AgeAccel ranged from -8.49 to 16.27 years, IEAA ranged from -9.01 to 15.55 years and EEAA ranged from -12.56 to 13.56 years. At T1, in Italian subjects AgeAccel ranged from -8.60 to 14.25 years, IEAA ranged from -8.62 to 9.92 years and EEAA ranged from -8.97 to 7.90 years. In Polish subjects, at T1, AgeAccel ranged from -9.81 to 10.99 years, IEAA ranged from -10.44 to 9.47 years and EEAA ranged from -11.31 to 10.02 years. Baseline measures of epigenetic AA were similar between Italian and Polish subjects (Student's t-test p-value >0.05), but EEAA was significantly higher in Polish males compared to Polish females (Student's t-test p=0.00009) and compared to Italian males (Student's t-test p=0.02).

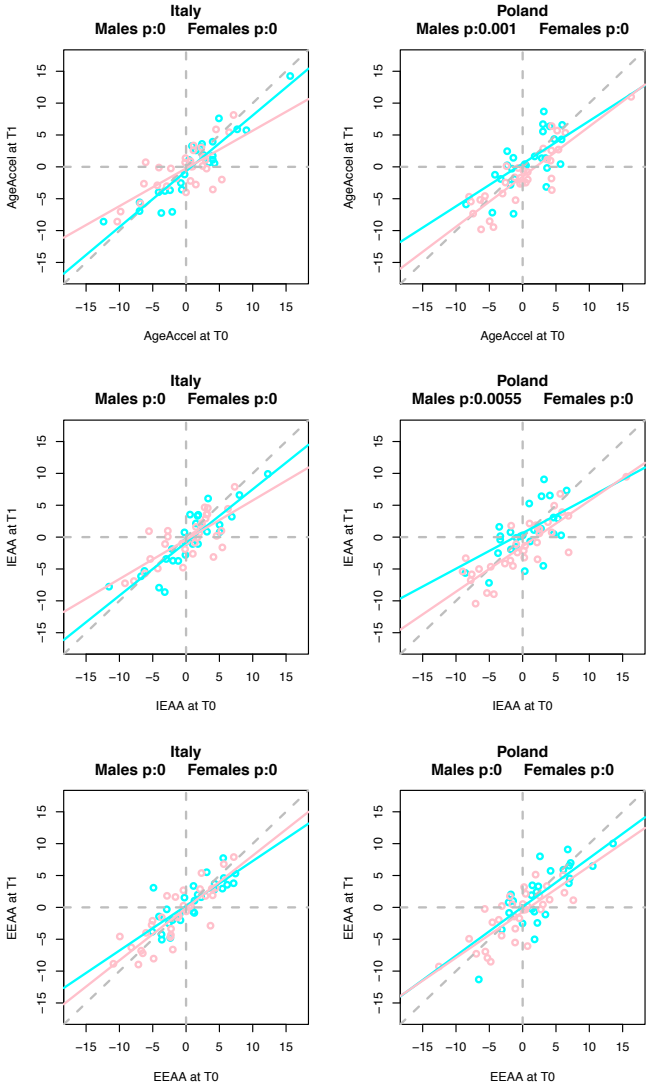
Country			**Italy**		**Poland**	
Subjects (n)			60		60	
Males / Females (n)			27 / 33		24 / 36	
Time			**T0**	**T1**	**T0**	**T1**
DNAm age	(years), mean ± SD	**Males + Females**	75.95 ± 5.9	76.36 ± 5.7	74.95 ± 5.5	74.96 ± 5.5
AgeAccel	(years), mean ± SD		0.35 ± 4.9	-0.12 ± 4.3	**0.30 ± 4.6**	**-0.54 ± 4.6**
IEAA	(years), mean ± SD		**0.49 ± 4.4**	**-0.24 ± 4.0**	0.24 ± 4.7	-0.49 ± 4.4
EEAA	(years), mean ± SD		-0.48 ± 4.4	-0.39 ± 4.2	0.70 ± 4.7	0.16 ± 4.6
DNAm age	(years), mean ± SD	**Males**	76.72 ± 6.9	76.80 ± 6.8	76.23 ± 4.2	77.20 ± 4.3
AgeAccel	(years), mean ± SD		0.97 ± 5.5	0.17 ± 5.2	1.18 ± 3.7	1.30 ± 3.9
IEAA	(years), mean ± SD		0.55 ± 4.8	-0.38 ± 4.6	0.99 ± 3.7	1.19 ± 3.8
EEAA	(years), mean ± SD		0.90 ± 3.9	0.88 ± 3.4	3.47 ± 4.0	2.73 ± 4.1
DNAm age	(years), mean ± SD	**Females**	75.32 ± 5.0	76.00 ± 4.7	74.10 ± 6.1	73.47 ± 5.7
AgeAccel	(years), mean ± SD		-0.16 ± 4.3	-0.34 ± 3.5	**-0.29 ± 5.1**	**-1.76 ± 4.6**
IEAA	(years), mean ± SD		0.45 ± 4.1	-0.13 ± 3.5	**-0.26 ± 5.3**	**-1.61 ± 4.5**
EEAA	(years), mean ± SD		-1.6 ± 4.5	-1.4 ± 4.5	-1.14 ± 4.3	-1.54 ± 4.1

Table 1.2: **DNAm age and measures of epigenetic age acceleration at baseline (T0) and after one year of Mediterranean-like diet (T1).**

SD: standard deviation. DNAm age: DNA methylation age. IEAA: Intrinsic Epigenetic Age Acceleration. EEAA: Extrinsic Epigenetic Age Acceleration.

Statistically significant differences in epigenetic AA measures between T0 and T1 are in bold.

In both Italian and Polish cohorts, epigenetic AA measures at baseline were significantly associated ($p<0.05$) with those obtained after the 12-months tailored nutritional intervention (**Figure 1.4**).



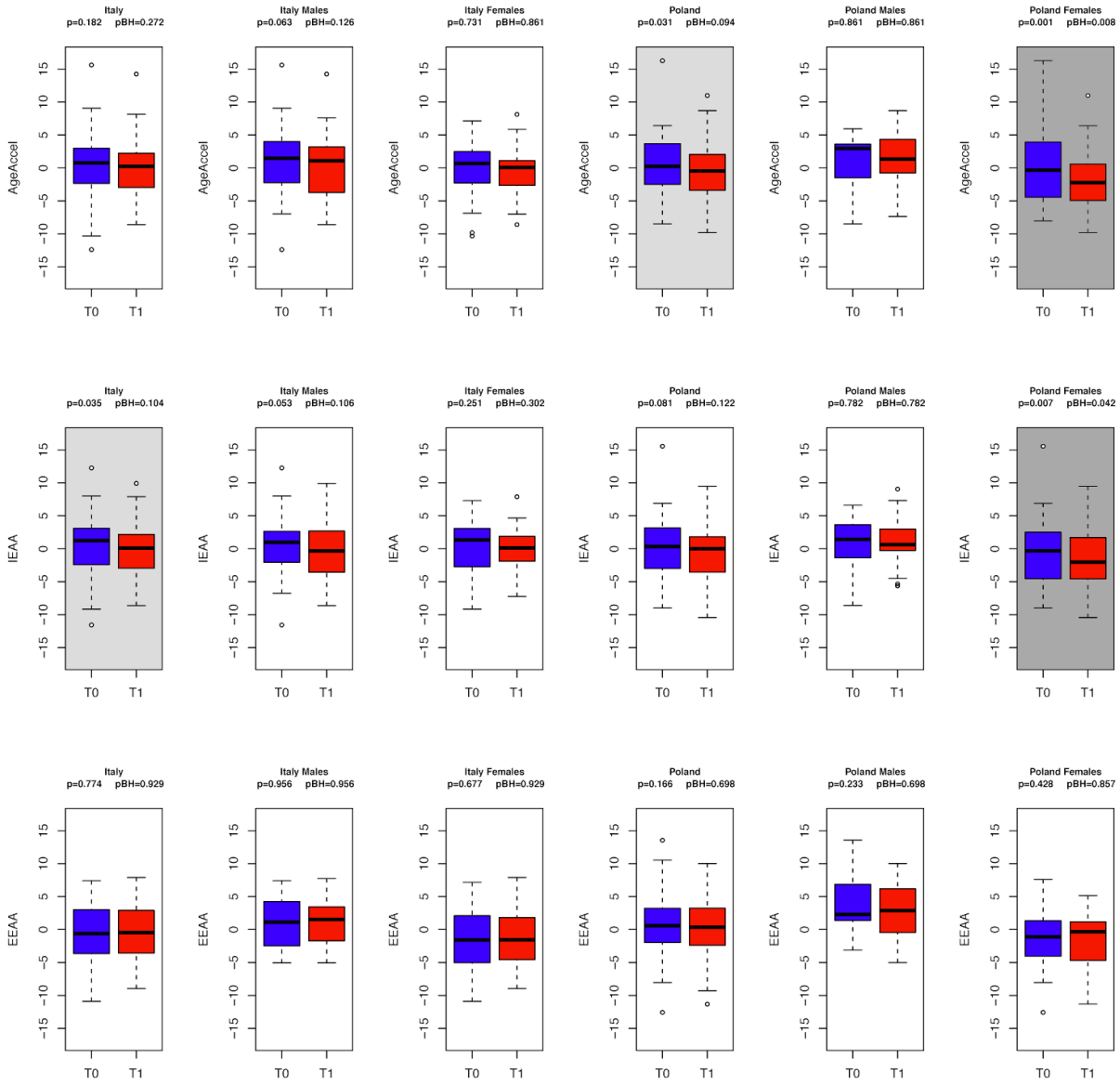
<u>Figure 1.4</u>: Significant association between epigenetic AA measures at baseline and after one year of nutritional intervention.

Scatter plots of epigenetic AA at T0 (x-axis) versus T1 (y-axis). The three epigenetic AA measures (AgeAccel, IEAA and EEEA) are reported respectively in the upper, middle and lower panels. Males and females subjects are indicated with cyan and pink circles respectively. Lines represent fitted linear regression lines models in males (cyan) and females (pink). Grey dotted lines represent the bisector.

<u>AgeAccel Italy</u>: $R^2 = 0.84$, p-value $< 2.2^{-16}$; <u>AgeAccel Poland</u>: $R^2 = 0.79$, p-value $= 3.54^{-14}$; <u>IEAA Italy</u>: $R^2 = 0.81$, p-value $= 6.5^{-15}$; <u>IEAA Poland</u>: $R^2 = 0.75$, p-value $= 3.86^{-12}$; <u>EEAA Italy</u>: $R^2 = 0.84$, p-value $< 2.2^{-16}$; <u>EEAA Poland</u>: $R^2 = 0.80$, p-value $= 1.86^{-14}$.

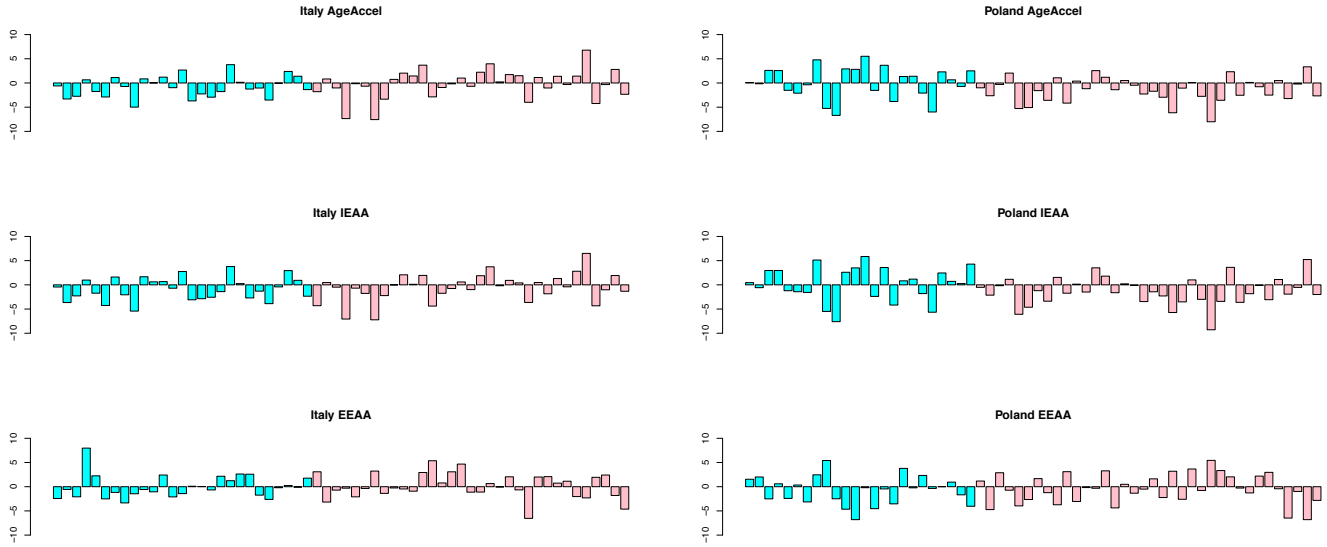
We then used Student's paired-sample t-test to compare the epigenetic AA measures at T0 and at T1. In Italian subjects, no statistically significant differences between T0 and T1 were observed considering AgeAccel, also when subjects were divided according to sex (**Figure 1.5, upper panel**). On the contrary, in Polish subjects, AgeAccel measures were significantly lower at T1 versus baseline (T0) (p=0.0312) (**Figure 1.5, upper panel**). In other words, under the Mediterranean-like diet intervention, there was a statistically significant rejuvenation of the Polish subjects, according to the AgeAccel measure. When we divided samples on the basis of sex, we observed that the effect was predominantly related to a decrease in AgeAccel measures in Polish females at T1 compared to T0 (p=0.0013). Rejuvenation of the Polish females after one year of nutritional intervention was confirmed with the IEAA measure (**Figure 1.5, middle panel**), as analysis returned a significant decrease in IEAA values at T1 versus T0 (p=0.007). Lower IEAA measures were also observed at T1 in Italian subjects as compared to T0 (p=0.0347). The EEAA predictor did not give significant results (**Figure 1.5, lower panel**) in both groups. After correction for multiple testing, the effect remained statistically significant for Polish females, according to AgeAccel (adjusted p-value = 0.008) and IEAA (adjusted p-value = 0.04) measures (**Figure 1.5**).

Figure 1.6 reports, for each subject, the intra-pair difference between AgeAccel at T1 and AgeAccel at T0 (AgeAccel Diff), the intra-pair difference between IEAA at T1 and IEAA at T0 (IEAA Diff), and the intra-pair difference between EEAA at T1 and EEAA at T0 (IEAA Diff). In all three cases, a negative value indicates an epigenetic rejuvenation.



<u>Figure 1.5</u>: A one-year Mediterranean-like diet intervention promotes epigenetic rejuvenation in a country- and sex-specific manner.

Boxplots of epigenetic AA measures at T0 and T1 (upper panel: AgeAccel; middle panel: IEAA; lower panel: EEAA) considering all the subjects, only males and only females. Statistically significant results are greyed.

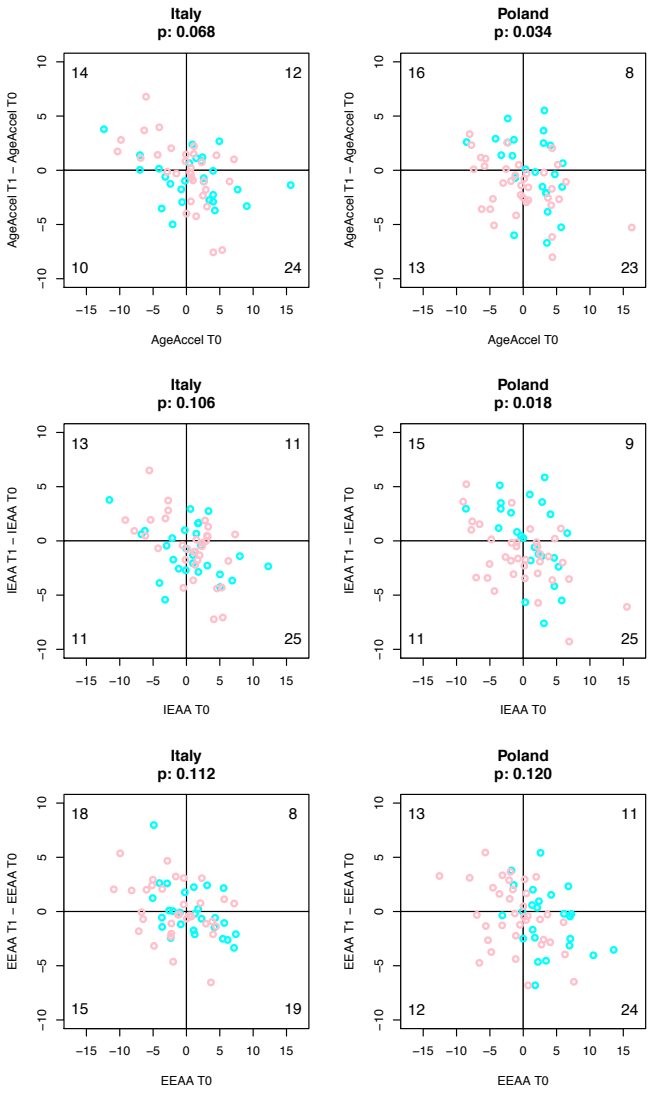


<u>Figure 1.6</u>: Individual-specific response to the nutritional intervention according to epigenetic AA measures.

Barplots of intrapair differences for the three measures of epigenetic age acceleration (Blue: males; Pink: females).

The discrepancy between results observed with AgeAccel and IEAA and those observed with EEAA is of particular interest: the rejuvenation related to the nutritional intervention can be considered as a pure effect on the epigenetic clock, unconfounded by a potential effect of the intervention on the immune system, as IEAA is independent of changes in the composition of blood cells.

Finally, we assessed if AgeAccel Diff, IEAA Diff and EEAA Diff values were related to, respectively, Age Accel, IEAA and EEAA values at baseline (**Figure 1.7**). In both the countries and for all the different AA measures, we found that the majority of subjects showing an epigenetic age rejuvenation (AgeAccel Diff, IEAA Diff or EEAA Diff less than 0) had also baseline AA levels greater than 0 (**Figure 1.7**). Fisher's exact test confirmed that this enrichment was significant for AgeAccel and IEAA measures in Poles, indicating that the effect of the diet tended to be more marked in those subjects that displayed higher epigenetic AA values at T0.



<u>Figure 1.7</u>: Stronger impact of diet on epigenetic AA measures in subjects with higher epigenetic AA values at baseline.

Scatter plots of epigenetic AA measures at T0 (x-axis) versus epigenetic AA measures difference between T1-T0 (y-axis). The three epigenetic AA measures (AgeAccel, IEAA and EEEA) are reported respectively in the upper, middle and lower panels. Males and females subjects are indicated with cyan and pink circles respectively. The number of subjects in each quadrant is reported. Fisher's test exact test was applied to test if there was a difference of proportion of subjects in the 4 quadrants.

1.3.3 *Association between epigenetic age acceleration measures, BMI and adherence to the Mediterranean-like diet*

In order to identify factors associated with the slowdown of the epigenetic AA measures, we first investigated the relationship between BMI and the epigenetic markers. We did not find any significant association between BMI and AgeAccel, IEEA or EEAA.

We also analyzed the association between the epigenetic AA measures and the NU-AGE score measuring the adherence to the Mediterranean-like diet, calculated at T0 and T1. We observed a significant negative association of AgeAccel (p=0.037) and IEAA (p=0.027) with the NU-AGE score, with higher levels of adherence to the Mediterranean-like diet associated with negative epigenetic AA values, that is with epigenetic rejuvenation (**Figure 1.8**).

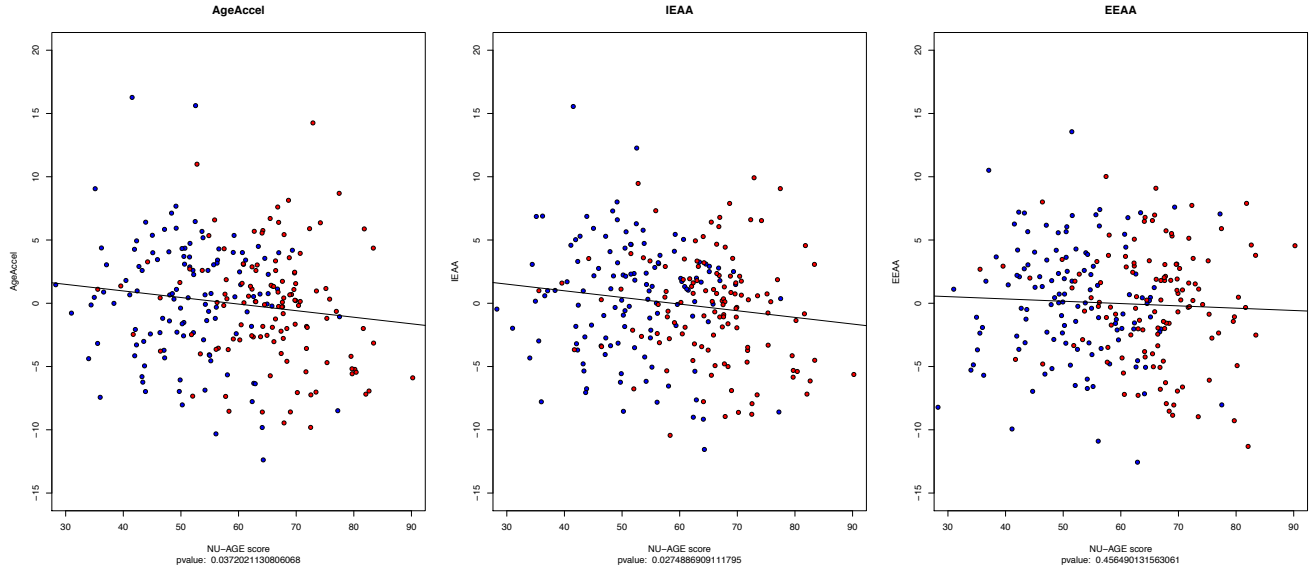


Figure 1.8: Association between epigenetic AA measures and adherence to Mediterranean-like diet.

Scatter plots of epigenetic age acceleration measures (y-axis) and NU-AGE score evaluating adherence to the Mediterranean-like diet (x-axis) (T0 = blue; T1 = red). The line represents a fitted linear regression (AgeAccel: R^2 = - 0.13, p-value = 0.037; IEAA: R^2 = - 0.14, p-value = 0.027; EEAA: R^2 = - 0.05, p-value = 0.457).

1.3.4 *Association between epigenetic age acceleration measures and genotype*

Finally, we evaluated if response to Mediterranean-like dietary intervention, in terms of epigenetic AA, was related to the genetic background of the participants of the study. To this aim, we carried out a genome-wide association study (GWAS) of epigenetic AA measures in our cohort, expressed as AgeAccel Diff, IEAA Diff or EEAA Diff as described above (**Figure**

1.6). The quantile-quantile (QQ) plot of association results demonstrated no genomic inflation. After correction for multiple testing, no significant association was observed at the genome-wide level (Benjamini-Hochberg corrected p-value<0.05). However, small-effect loci with nominal significance (p-value<$1x10^{-4}$) were identified for all of the three measures of epigenetic AA. A total of 68, 49 and 46 single nucleotide polymorphisms (SNPs) were found significantly associated with AgeAccel Diff, IEAA Diff and EEAA Diff respectively (**Table 1.3**). Thirty-one SNPs were common between AgeAccel Diff and IEAA Diff, while there were no SNPs in common between EEAA Diff and AgeAccel Diff or IEAA Diff (**Figure 1.9**). Interestingly, 5 SNPs out of 68 (for AgeAccel Diff) and 6 SNPs out of 49 (for IEAA Diff) showed minor allele frequency differences between Italians and Polish (p-values < 0.05).

SNP	Unadjusted p-value	ID	Common with
Age Accel Diff			
rs2588499	0.000000119	chr2:75708979:C/T:1	Common with IEAA
rs7769262	0.00000162	chr6:63556032:A/C:1	Common with IEAA
rs6930829	0.00000206	chr6:63569602:G/A:1	Common with IEAA
rs9293982	0.00000255	chr6:75558759:A/G:1	Common with IEAA
rs12821987	0.00000612	chr12:97429549:A/C:1	Common with IEAA
rs9597587	0.00000784	chr13:58257265:A/G:1	Common with IEAA
rs6599689	0.00000841	chr10:125106194:A/G:1	Common with IEAA
rs10943071	0.00000896	chr6:63322114:G/A:1	Common with IEAA
rs6942254	0.0000172	chr6:63672200:G/A:1	
rs11568110	0.0000221	chr17:4539673:C/T:1	Common with IEAA
IEAA Diff			
rs9597587	0.000002013	chr13:58257265:A/G:1	Common with AgeAccel
rs2588499	0.000002833	chr2:75708979:C/T:1	Common with AgeAccel
rs9293982	0.000007551	chr6:75558759:A/G:1	Common with AgeAccel
rs9392414	0.00001381	chr6:2686488:C/T:1	Common with AgeAccel
rs12810082	0.00001584	chr12:67192531:C/T:1	Common with AgeAccel
rs12273933	0.0000202	chr11:78950572:G/T:1	Common with AgeAccel
rs12823702	0.00002074	chr12:67203190:C/T:1	Common with AgeAccel
rs7699928	0.0000209	chr4:190088167:C/T:1	Common with AgeAccel
rs9876871	0.00002156	chr3:36812570:C/T:1	Common with AgeAccel
rs7769262	0.00002163	chr6:63556032:A/C:1	Common with AgeAccel
EEAA Diff			
rs6745958	0.00000259	chr2:232870178:G/A:1	
rs1138729	0.000004885	chr2:10271196:A/G:1	
rs10101912	0.000007387	chr8:88218888:C/T:1	
rs7920763	0.000008745	chr10:2740608:A/G:1	
rs557135	0.000009344	chr6:52765760:A/G:1	
rs12800154	0.00001869	chr11:104158392:A/G:1	
rs10132733	0.00001943	chr14:22946231:A/G:1	
rs13379495	0.00001972	chr15:46133052:G/T:1	
rs16949428	0.00002292	chr15:66381722:A/G:1	
rs8028460	0.00002292	chr15:66391818:T/G:1	

<u>Table 1.3:</u> Top 10 of Single Nucleotide Polymorphisms (SNPs) associated with AgeAccel Diff, IEAA Diff and EEAA Diff.

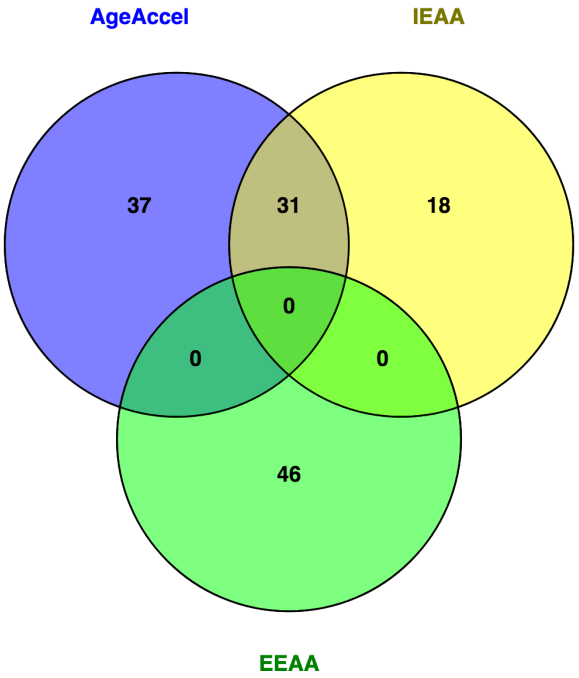


Figure 1.9: Thirty-one SNPs associated with AgeAccel are common with IEAA.

Venn diagram of SNPs associated with AgeAccel (blue), IEAA (yellow) and EEAA (green).

In order to identify pathways that may be relevant to epigenetic AA effects upon Mediterranean-like nutritional intervention, we performed an enrichment analysis using i-GSEA4GWAS. i-GSEA4GWAS is a freely available and widely used open platform which applies an improved gene set enrichment analysis to detect pathways and gene sets associated with traits. i-GSEA4GWAS employs SNP label permutation to correct gene variation to reduce the bias due to different genes with different number of mapped SNPs. This correction ensures to identify gene sets consisting of non-random high-association genes with biological plausibility instead of random high-association genes with large numbers of mapped SNPs (Zhang et al. 2010). In the analysis of AgeAccel Diff associations, we found 60 significant gene sets ($p<0.05$), 13 of which were significant after false discovery rate (FDR) correction (FDR<0.05). IEAA Diff analysis returned 37 significant gene sets ($p<0.05$), 11 of which had a FDR<0.05. We found a large overlap between the enrichment analysis results of the two epigenetic AA measures, in particular for pathways involved in energy metabolism, regulation of cell cycle and of immune functions. On the contrary, enrichment analysis for EEAA Diff did not return any significant result ($p<0.05$).

Gene Set Name	Unadjusted p-value	Gene Set Description
Age Accel Diff		
HSA05130_PATHOGENIC_ESCHERICHIA_COLI_INFECTION_EHEC	< 0.001	Genes involved in pathogenic Escherichia coli infection - EHEC
HSA04115_P53_SIGNALING_PATHWAY	< 0.001	Genes involved in p53 signaling pathway
APOPTOSIS	< 0.001	
SIG_CHEMOTAXIS	< 0.001	Genes related to chemotaxis
MTORPATHWAY	0.0020	Mammalian target of rapamycin (mTOR) senses mitogenic factors and nutrients, including ATP, and induces cell proliferation.
SIG_PIP3_SIGNALING_IN_B_LYMPHOCYTES	< 0.001	Genes related to PIP3 signaling in B lymphocytes
PEPTIDE_GPCRS	0.0020	
HSA04620_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	< 0.001	Genes involved in Toll-like receptor signaling pathway
HSA00100_BIOSYNTHESIS_OF_STEROIDS	0.0020	Genes involved in biosynthesis of steroids
SIG_PIP3_SIGNALING_IN_CARDIAC_MYOCTES	< 0.001	Genes related to PIP3 signaling in cardiac myocytes
HSA04650_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	< 0.001	Genes involved in natural killer cell mediated cytotoxicity
HSA00190_OXIDATIVE_PHOSPHORYLATION	0.0040	Genes involved in oxidative phosphorylation
HSA04512_ECM_RECEPTOR_INTERACTION	< 0.001	Genes involved in ECM-receptor interaction
IEAA Diff		
HSA03010_RIBOSOME	0.0010	Genes involved in ribosome
HSA04620_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	< 0.001	Genes involved in Toll-like receptor signaling pathway
APOPTOSIS	< 0.001	
PEPTIDE_GPCRS	0.0020	
HSA05130_PATHOGENIC_ESCHERICHIA_COLI_INFECTION_EHEC	0.0010	Genes involved in pathogenic Escherichia coli infection - EHEC
HSA00040_PENTOSE_AND_GLUCURONATE_INTERCONVERSIONS	< 0.001	Genes involved in pentose and glucuronate interconversions
APOPTOSIS_GENMAPP	0.0030	
HSA04115_P53_SIGNALING_PATHWAY	< 0.001	Genes involved in p53 signaling pathway
ERKPATHWAY	0.0020	Cell growth is promoted by Ras activation of the anti-apoptotic p44/42 MAP kinase pathway.
HSA05213_ENDOMETRIAL_CANCER	0.0010	Genes involved in endometrial cancer
ST_ADRENERGIC	0.0010	Adrenergic receptors respond to epinephrine and norepinephrine signaling.

Table 1.4: Gene sets associated with AgeAccel Diff and IEEA Diff (with false discovery rate<0.05) according to the enrichment analysis performed with i-GSEA4GWAS *(Zhang et al. 2010)*.

Part two

Development of a new epigenetic biomarker of aging and its application to samples collected within the NU-AGE project

2.1 *Introduction*

The canonical epigenetic clocks (*i.e.* Horvath's and Hannum's models) are based on DNAm values at a large number of CpG sites, measured by Illumina® Infinium microarrays, which are genome-wide platforms for the analysis of DNA methylation. These epigenetic clocks are characterized by their high accuracy and, for Horvath's one, by its wide applicability to different tissues and cells types. Despite these great advantages, these models have some limitations. Their technology is characterized by a high cost, both in terms of equipment and consumables, and by the requirement of complex bioinformatic analysis. This issue could represent a constraint to their use in the context of large human cohorts and to their implementation in clinical settings. There is need for the development of epigenetic biomarkers of aging, based on locus-targeted DNA methylation analysis, assessable with faster, cost effective and easy to apply approaches.

In this direction, alternatives with fewer CpG sites already exist. A model built on DNAm values of whole blood at 3 CpG sites (Weidner's estimator) was published in 2014. This model, based on bisulfite pyrosequencing, was found to significantly correlate with chronological age (Weidner et al. 2014). However, it failed to predict mortality in the Lothian Birth Cohort (Lin et al. 2016). Our group also identified two loci, located in the CpG island of *ELOVL2* and *FHL2* genes, showing very high correlation with chronological age in whole blood (Garagnani et al. 2012). These results have been confirmed in other replicative tissues (Florath et al. 2014; Kananen et al. 2016; Steegenga et al. 2014; Bacalini et al. 2017) and have been applied to teeth samples as well (Giuliani et al. 2016). Methylation levels in these regions were not associated with mortality in the Leiden Longevity Study (Bacalini et al. 2017), but *ELOVL2* and *FHL2* hypermethylation were found associated with the prospective development of breast cancer and colorectal cancer, respectively (Durso et al. 2017). Finally, prediction models based on a small number of CpG sites have been developed recently for forensic age prediction, using pyrosequencing or Agena EpiTYPER® system based approaches (Huang et al. 2015; Zbieć-Piekarska, Spólnicka, Kupiec, Parys-Proszek, et al. 2015; Soares Bispo Santos Silva et al. 2015; Spólnicka et al. 2018, p.2; Freire-Aradas et al. 2016).

The objective of this work was to develop a new epigenetic biomarker of aging for whole blood, based on the combination of a limited number of CpG sites, assessable through Agena EpiTYPER® platform. The performance of this model was then tested in populations characterized by successful or accelerated aging. Finally, this biomarker was applied to samples collected within the framework of the NU-AGE project, in order to evaluate the impact of the one-year nutritional intervention.

2.2 *Methods*

2.2.1 *Samples collection and DNA extraction*

DNA was extracted from whole blood samples of European individuals belonging to previously existing cohorts. The epigenetic model was built and tested on samples collected in apparently healthy subjects of different age (0-98 years old), included in different cohorts. The model was then applied to subjects with Down syndrome, centenarians and their offspring and participants of the NU-AGE project belonging to the nutritional intervention group. Written informed consents were previously obtained from the participants, prior to their inclusion in the different projects. Studies were all approved by local ethics committees. **Table 2.1** summarizes the different cohorts from which the samples were taken from. DNA was extracted from venous blood samples, drawn on EDTA tubes. Genomic DNA extraction was performed with QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany) and 500 ng of DNA were bisulfite converted, using the EZ DNA Methylation Kit (Zymo Research Corporation, Orange, CA, USA) according to manufacturer's instructions.

Subjects	Number	Age range	Brief description of the cohort
Building and testing of the model: healthy subjects			
Controls	315 Females n = 132 Males n = 180 NA n = 3	0–98 years 0-19 years: n = 6 20-39 years: n = 53 40-59 years: n = 94 60-74 years: n = 123 75-98 years: n = 39	Healthy subjects, from which 3 cord blood samples, 26 Down syndrome subjects' siblings, 170 subjects included in the MARKAGE project (Capri et al. 2015), 37 controls belonging to PROPAG-AGEING cohort recruited at the University Medical Center of Göttingen, 30 age-matched controls of centenarians' offspring (PRIN2006 and PRIN2009 cohorts), 17 samples from young collaborators and 32 samples from older adults (79-98 years old) living in the urban area of Bologna.
Application of the model: models of successful or accelerated aging, and nutritional intervention			
Down syndrome subjects	75 Females n = 30 Males n = 45	12–66 years 0-19 years: n = 12 20-39 years: n = 44 ≥ 40 years: n = 19	
Centenarians	106 Females n = 82 Males n = 24	99–112 years 99-104 years: n = 96 105-109 years: n = 7 ≥ 110 years: n = 3	Centenarians recruited in Italy (PRIN2006 and PRIN2009 cohorts)
Centenarians' offspring	143 Females n = 81 Males n = 62	55–89 years 55-69 years: n = 68 70-89 years: n = 75	Centenarians' offspring recruited in Italy (PRIN2006 and PRIN2009 cohorts)
NU-AGE project	233 Females n=128 Males n = 105	65–80 years	Italian (n=124) and Polish (n=109) subjects belonging to the intervention group

Table 2.1: Overview of the cohorts used for the construction and validation of the model.

2.2.2 *Selection of candidate markers*

Identification and selection of relevant CpG sites were firstly based on deep analysis of the literature. We carefully chose potential targets on the basis of the correlation between their methylation levels and chronological age, and on the basis of their association with age-related outcomes. In particular, we considered CpG sites included in previously published epigenetic clocks (Hannum et al. 2013; Horvath 2013; Horvath, Oshima, et al. 2018; Levine et al. 2018); CpG sites showing an increase in inter-individual variability in DNA methylation with age, according to the study by Slieker *et al*. (Slieker et al. 2016); and CpG sites associated with age-related conditions and chronic low-grade inflammation (Ligthart et al. 2016).

Secondarily, selection and validation of relevant CpG sites were based on statistical assessment of their performances in our proprietary Illumina® Infinium 450K datasets on age-related conditions, with particular attention to those in which Horvath's and Hannum's clocks successfully identified age acceleration or deceleration effects (Down Syndrome subjects, centenarians and their offspring).

2.2.3 *EpiTYPER® DNA methylation analysis*

The most relevant CpG sites were subsequently considered for locus-specific DNA methylation analysis with the Agena Bioscience EpiTYPER® system (San Diego, CA, USA; formerly Sequenom). Sequences of the regions of interest, flanking each selected CpG sites, were retrieved from the UCSC genome browser (https://genome.ucsc.edu/), using the genome assembly GRCh37/hg19. Primer design was performed using Agena Bioscience EpiDesigner® software (http://epidesigner.com/), specifically optimized for the EpiTYPER® system.

Locus-targeted DNA methylation analysis was performed according to manufacturer's instructions. Ten nanograms of whole blood genomic bisulfite-converted DNA were amplified using the bisulfite-specific primers, in a 5 µL total volume using a 384-well plate. Unincorporated nucleotides and primers were then removed with the Shrimp Alkaline Phosphatase (SAP) treatment, and reverse transcription/RNaseA cleavage were performed. Finally, 20 µL of RNase-free ddH_2O were added to each sample, as well as 6 mg of Clean Resin in order to eliminate salts of sodium and potassium that could interfere with the analysis. Sample dispensation on a SpectroCHIP was performed by the Nanodispenser, and final detection was done with the mass spectrometer. Methylation data were obtained using the EpiTYPER® analysis software, expressed as continuous values between 0 and 1 (corresponding to 0 and 100% methylation respectively).

2.2.4 *Age prediction*

For each target region, the Agena EpiTYPER® system returns DNA methylation data (expressed as β-values) of a specific number of CpG units (*i.e.* regions containing one or multiple CpG sites) that were included in the predictive model. The first step was the imputation of missing values (retrieved as NA by the Agena EpiTYPER® system), using *mice* (Multivariate Imputation by Chained Equations) R package (Buuren & Groothuis-Oudshoorn 2011). Secondly, methylation β-values were converted to M-values through a logistic transformation included in the Bioconductor package *lumi* (Du et al. 2008). Our model of age-prediction was built using a ridge regression model, included the R package *caret* (Classification and Regression Training) (Kuhn 2008), considering control subjects from 20 to 80 years old (which corresponds to the age range in which chronological age and epigenetic age show a linear relationship according to Horvath's clock). Validation of the prediction model was performed using a 10-fold cross-validation procedure. The outcome of the model was a predicted epigenetic age, expressed in years. Correlation coefficients obtained through linear regression of this predicted epigenetic age and chronological age were calculated in order to evaluate the accuracy of the prediction. We were finally able to define a measure of epigenetic age acceleration (named AgeAccel) as the distance from the regression line between predicted epigenetic age and chronological age in controls. A positive result indicates an epigenetic age acceleration, while negative values indicate an epigenetic age deceleration.

All analyses were performed using R version 3.3.2.

2.3 *Results*

2.3.1 *Selection of candidate CpG sites*

A total of seven CpG probes mapping in six different genes were considered relevant according to the above-mentioned criteria and were then selected for further analysis. Characteristics of the selected CpG sites are shown in **Table 2.2**.

CpG site Illumina®Infinium ID	Location	Associated gene	Correlation status with age
cg16867657	chr6:11,044,877-11,044,877	*ELOVL2*	Positive
cg22736354	chr6:18,122,719-18,122,719	*NHLRC1*	Positive
cg07855221 **cg09253473**	chr17:79,877,314-79,877,31 chr17:79,877,390-79,877,390	*MAFG*	Negative
cg10636246	chr1:159,046,973-159,046,973	*AIM2*	Negative
cg09809672	chr1:236,557,683-236,557,683	*EDARADD*	Negative
cg26372517	chr1:36,039,159-36,039,159	*TFAP2E*	Positive

Table 2.2: List of selected CpG sites and their main characteristics.

ELOVL2 is a gene coding for the Elongation of very long chain fatty acids protein 2, whose methylation status has been previously reported as highly correlated with age, in whole blood and most of human tissues, and considered as a robust epigenetic marker of age (Bacalini et al. 2017; Cho et al. 2017; Florath et al. 2014; Garagnani et al. 2012; Spólnicka et al. 2018; Correia Dias et al. 2019; Zbieć-Piekarska, Spólnicka, Kupiec, Makowska, et al. 2015; Luo et al. 2019). Moreover, our team demonstrated that hypermethylation of *ELOVL2* in blood is associated with the prospective development of breast cancer (Durso et al. 2017) and cg16867657 has been recently included in a new DNA methylation estimator of telomere length (Lu, Seeboth, et al. 2019).

The CpG site cg22736354, associated with the gene *NHLRC1* (NHL Repeat Containing E3 Ubiquitin Protein Ligase 1), is included in several published epigenetic clocks: Horvath's clock (Horvath 2013); Hannum's clock (Hannum et al. 2013); Levine's clock (Levine et al. 2018) and Horvath's new Skin & Blood clock (Horvath, Oshima, et al. 2018). Another CpG site related to this gene has also been reported as an age-related variably methylated position (aVMP) by Slieker *et al*. (Slieker et al. 2016) and *NHLRC1* has been recently included in a DNAm-based forensic age predictor (Vidaki et al. 2017).

Additionally, we evaluated the methylation of *SIRT7* (sirtuin 7) gene, owing to the crucial role of sirtuins in senescence in human cells (Paredes et al. 2018). Two CpG sites (cg07855221 and cg09253473), associated with *SIRT7* and *MAFG* (MAF BZIP Transcription Factor G) genes, were considered relevant and included in our list of targeted loci. SIRT7 age-related hypomethylation was also found in mice livers, with a protective impact of dietary restriction on methylation patterns (Hahn et al. 2017).

We also decided to include in our model the CpG site cg10636246, associated with *AIM2* (Absent in Melanoma 2) gene, coding for an interferon-gamma-induced protein involved in the innate immune response. DNA methylation at this specific CpG site was found to be associated with CRP serum levels in a meta-analysis of epigenome-wide association studies (Ligthart et al. 2016) and is also included in Levine's clock (Levine et al. 2018).

The CpG site cg09809672, associated with *EDARADD* (EDAR Associated Death Domain) gene, is included in several of the diverse epigenetic biomarkers of aging published so far (Bocklandt et al. 2011; Horvath 2013; Hannum et al. 2013; Levine et al. 2018; Horvath, Oshima, et al. 2018). *EDARADD* was also recently included in a panel of genes for age prediction in forensics use (Correia Dias et al. 2019).

Finally, the CpG site cg26372517, associated with *TFAP2E* (Transcription Factor AP-2 Epsilon) gene, which encodes for a protein that acts as a transcription factor, was considered relevant. This CpG site is included in Horvath's clock (Horvath 2013), and was found differentially methylated in our available Illumina® Infinium 450K datasets applied to centenarians subjects and their offspring, who have been previously characterized by a phenomenon of decelerated epigenetic aging (Horvath, Pirazzini, et al. 2015b).

We designed specific EpiTYPER® primers to evaluate the DNA methylation of the above identified CpG sites and of the surrounding ones not assessed by the Infinium platform (**Table 2.3**). Length of the amplicons was between 340 bp (SIRT7/MAFG) and 598 bp (NHLRC1).

Amplicon ID	Forward primer	Reverse primer	Genomic Localization
ELOVL2	AGGAAGAGAGGTAAATTTGTAGGAATAGAGTTATTTTTTT	CAGTAATACGACTCACTATAGGGAGAAGGCTCCCCTCTCCCACAAAAACC	chr6:11,044,680-11,045,053
NHLRC1	TTGAGTTTAGGAGTTTTATGAGGTG	AACAAAAAACAATCCTATTATCCTCA	chr6:18,122,552-18,123,149
SIRT7 / MAFG	GAGGGAGGTAGTAGGGATAATATGG	CTTTAACCAAAACCAAATCTCTCAA	chr17:79,877,158-79,877,497
AIM2	AAAATTTGGTTGATTGTTGATTTTT	CAATACAAATTCTTATCTTCAAAACA	chr1:159,046,805-159,047,299
EDARADD	TTTTTTGGTGATTAGGAGTTTTAGTG	CAAAATTTCAAAAAACAAACCAACT	chr1:236,557,384-236,557,805
TFAP2E	TTATTATAATTGGAGTGTATGGAGTAGG	ACAAAAAAATTAAAAAATCCAACAC	chr1:36,038,876-36,039,325

Table 2.3: EpiTYPER® primers designed for DNA methylation analysis of the six targeted regions.

2.3.2 *Correlation between chronological age and methylation levels*

DNA methylation analysis of the six loci of interest with the Agena EpiTYPER® system retrieved usable DNA methylation values in a total of 73 CpG units, containing 124 unique CpG sites. The distribution of the 73 CpG units was as following: 15 CpG units for ELOVL2, 21 CpG units for NHLRC1, 6 CpG units for MAFG / SIRT7, 7 CpG units for AIM2, 5 CpG units for EDARADD and 19 CpG units for TFAP2E.

For each of the 73 CpG units, we evaluated the association between chronological age and DNA methylation levels in all individuals. The linear regression between age and DNA methylation levels was calculated in control subjects only and was found statistically significant (p-value < 0.05) in 46 CpG units (1 in AIM2, 6 in NHLRC1, 6 in MAFG / SIRT7, 15 in ELOVL2, 4 in EDARADD and 14 in TFAP2E). Three representative examples are presented in **Figure 2.1**.

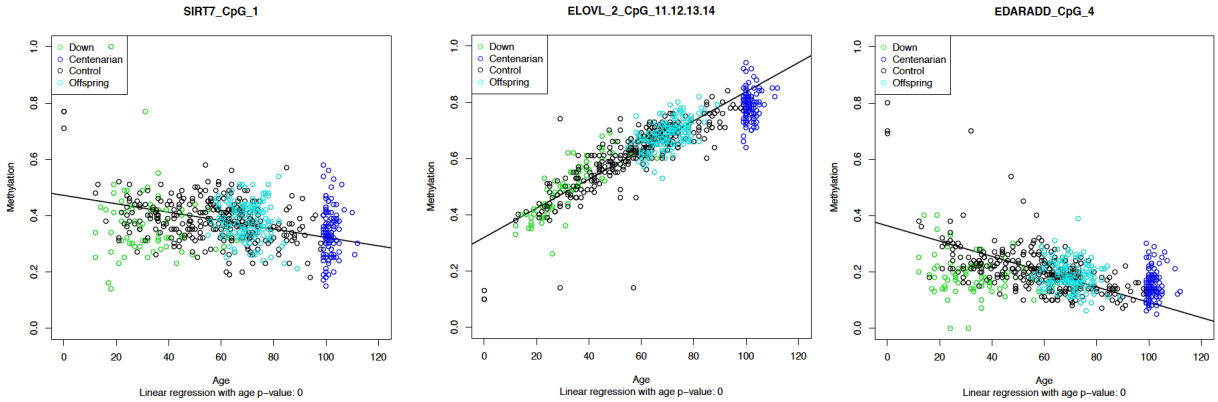


Figure 2.1: Scatter plots of DNA methylation values according to chronological age in 3 CpG sites/units of the amplicons MAFG / SIRT7 (left panel), ELOVL2 (middle panel) and EDARADD (right panel).

For each CpG site/unit, the line represents the linear regression line in controls (SIRT7: R^2 =-0.36, p-value = 5.68^{-11}; ELOVL2: R^2 =0.85, p-value < 2.2^{-16}; EDARADD: R^2 =-0.59, p-value < 2.2^{-16}).

2.3.3 *Statistical prediction model*

The DNA methylation values of the six selected regions obtained in 278 control subjects with age range between 20 and 80 years old were used to build our ridge regression model for epigenetic age prediction. The model provided an accurate estimation of chronological age (R^2 = 0.92, p-value < 2.2e-16) (**Figure 2.2).** The Mean Absolute Deviation (MAD) between predicted epigenetic age and chronological age was equal to 4.70 years.

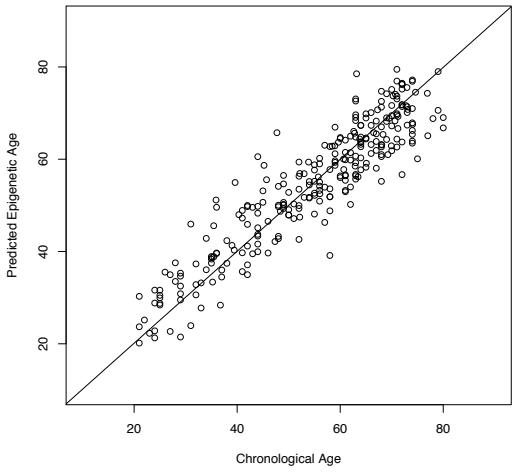


Figure 2.2: Association of chronological age (x-axis) and predicted epigenetic age (y-axis) in control subjects with age range 20-80 years old.

The line represents a fitted linear regression (R^2 = 0.92, p-value < 2.2^{-16}).

2.3.4 *Application of the epigenetic age predictor: age-acceleration and age deceleration models*

Our model was evaluated for its predictivity of biological age by testing its capability to identify age-acceleration and age-deceleration effects in the different cohorts mentioned in the Methods section. Firstly, we applied our model to a cohort of subjects with DS. DS is classified as a progeroid disease and evidence suggests that DS subjects are characterized by an acceleration of the aging process, atypical and segmental, affecting particularly the immune and central nervous systems (Zigman 2013; Franceschi et al. 2019; Gensous et al. 2019). From a biological perspective, DS subjects also exhibit features of accelerated aging, as it has been demonstrated with the Horvath's clock (Horvath, Garagnani, et al. 2015), with the GlycoAge index (Borelli et al. 2015) and with the brain age index (Cole et al. 2017). Here, we applied our model in a cohort of 75 subjects with DS, with chronological age ranging from 12 to 66 years old. We found that DS subjects exhibited a highly significant age acceleration effect, with an average age acceleration (AgeAccel) of 11.2 years (p-value < 0.0001 as compared to age-matched controls (Wilcoxon-Mann-Whitney test)). The magnitude of the age-acceleration effect observed with our model in DS subjects is important and higher than the one observed with the Horvath's model in blood (4.25 years) (Horvath, Garagnani, et al. 2015).

On the contrary, centenarians and their offspring are considered as extraordinary models to study longevity and successful/healthy aging. Centenarians have delayed morbidity, as most of them avoid or largely postpone age-related diseases. Centenarians' offspring, and more generally relatives of long-lived subjects, are healthier than subjects non-related to long-lived parents. They have a significant survival advantage, a higher probability to become themselves long-lived and a lower risk to develop major age-related diseases (Terry, Wilcox, McCormick & Perls 2004; Terry et al. 2003; Terry, Wilcox, McCormick, Pennington, et al. 2004; Bucci et al. 2016). It has previously been demonstrated that long-lived individuals have lower epigenetic age (Horvath, Pirazzini, et al. 2015b; Armstrong et al. 2017). Here, we applied our model to a cohort of 106 centenarians and 123 centenarians' offspring, recruited in Italy. We found that both populations were significantly epigenetically younger than controls: the average AgeAccel measure was equal to – 8.61 years in centenarians (p-value < 0.0001) and equal to – 1.47 years in centenarians' offspring (p-value = 0.047 as compared to age-matched controls) (**Table 2.4**).

Group	Number of subjects	Chronological age (mean±SD)	Epigenetic Age (mean±SD)	AgeAccel (mean±SD)
Down syndrome	75	33.49 ± 12.9	47.87 ± 29.1	+ 11.17 ± 27.0
Centenarians	106	101.52 ± 2.4	85.95 ± 10.7	- 8.61 ± 10.7
Centenarians' offspring	143	70.06 ± 6.7	66.24 ± 9.0	- 1.56 ± 8.6

Table 2.4: **Epigenetic age and measures of epigenetic age acceleration (AgeAccel) in the different groups.**

SD: standard deviation.

Figure 2.3 represents the correlation between predicted epigenetic age and chronological age, while **Figure 2.4** represents the measure of epigenetic age acceleration (AgeAccel) in the different groups.

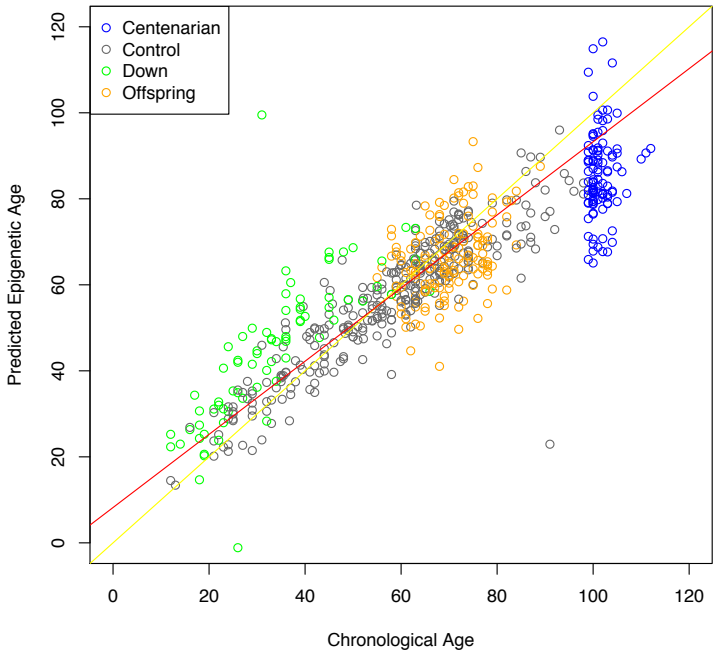


Figure 2.3: **Correlation between chronological age (x-axis) and predicted epigenetic age (y-axis) in the different groups.**

The red represents the linear regression in controls with age range 20-80 years old (R^2 = 0.92, p-value < 2.2^{-16}). The yellow line represents the bisector. Individuals above the red line are characterized by an epigenetic age acceleration phenomenon, whereas individuals below present an epigenetic age deceleration.

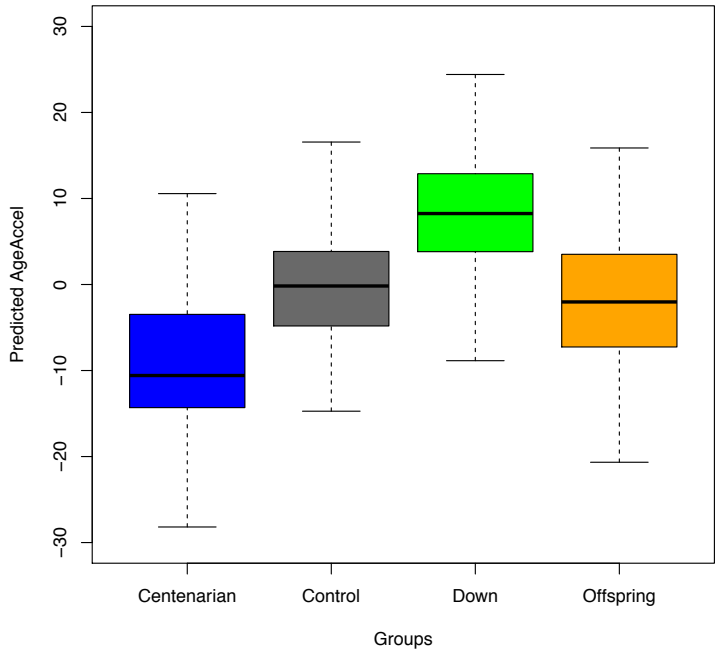


Figure 2.4: Boxplots of predicted epigenetic age acceleration in the different groups.

By definition, controls are characterized by an average epigenetic age acceleration equal to 0.

2.3.5 *Application of the epigenetic age predictor: impact of a nutritional intervention*

We applied our model to the samples collected within the NU-AGE project, in order to evaluate the impact of the one-year nutritional intervention. As compared to the study performed with the Illumina® Infinium 450K platform and presented in Part One, we decided to perform DNA methylation analysis on all available DNA samples from Italian and Polish subjects of the NU-AGE intervention group (Berendsen et al. 2014). Thus, we performed the analysis in 233 individuals, distributed as 124 Italian subjects and 109 Polish ones.

For each individual, we calculated a predicted epigenetic age at baseline (T0) and after one-year of nutritional intervention (T1), as well as a measure of epigenetic age acceleration, AgeAccel (**Table 2.5**). There was no statistically significant difference in terms of chronological age between the groups. However, Polish subjects had significantly higher predicted epigenetic age and AgeAccel values at baseline, as compared to Italian subjects (t-test p-value = 0.047 for predicted epigenetic age and 0.0124 for AgeAccel). Polish females had higher baseline AgeAccel values as compared to Italian females (mean = 1.20 vs -2.28; p-value = 0.036), whereas the difference was not statistically significant between Polish males and Italian males (p-value = 0.174). Predicted epigenetic age were significantly associated with

chronological age (p-value < 0.05) (**Figure 2.5** and **Figure 2.6**), although the correlation was weak (global correlation coefficient for all subjects = 0.273).

Country	**Italy**	**Poland**
Subjects (n)	124	109
Males / Females (n)	60 / 64	45 / 64
Chronological age (years), mean ± SD	72.16 ± 3.8	71.58 ± 4.0
Epigenetic age (years), mean ± SD	67.19 ± 9.7	69.90 ± 10.9
AgeAccel (years), mean ± SD	-2.40 ± 9.2	0.80 ± 10.2

Table 2.5: Baseline characteristics of the Italian and Polish subjects included in the NU-AGE project.

SD: standard deviation.

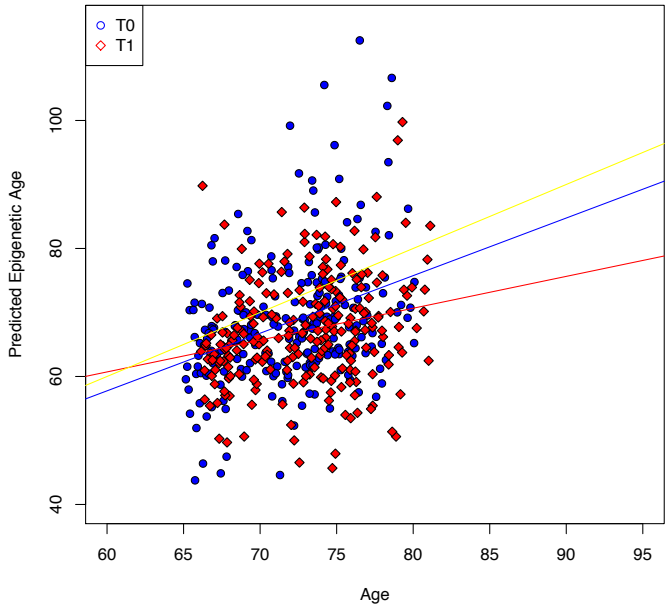
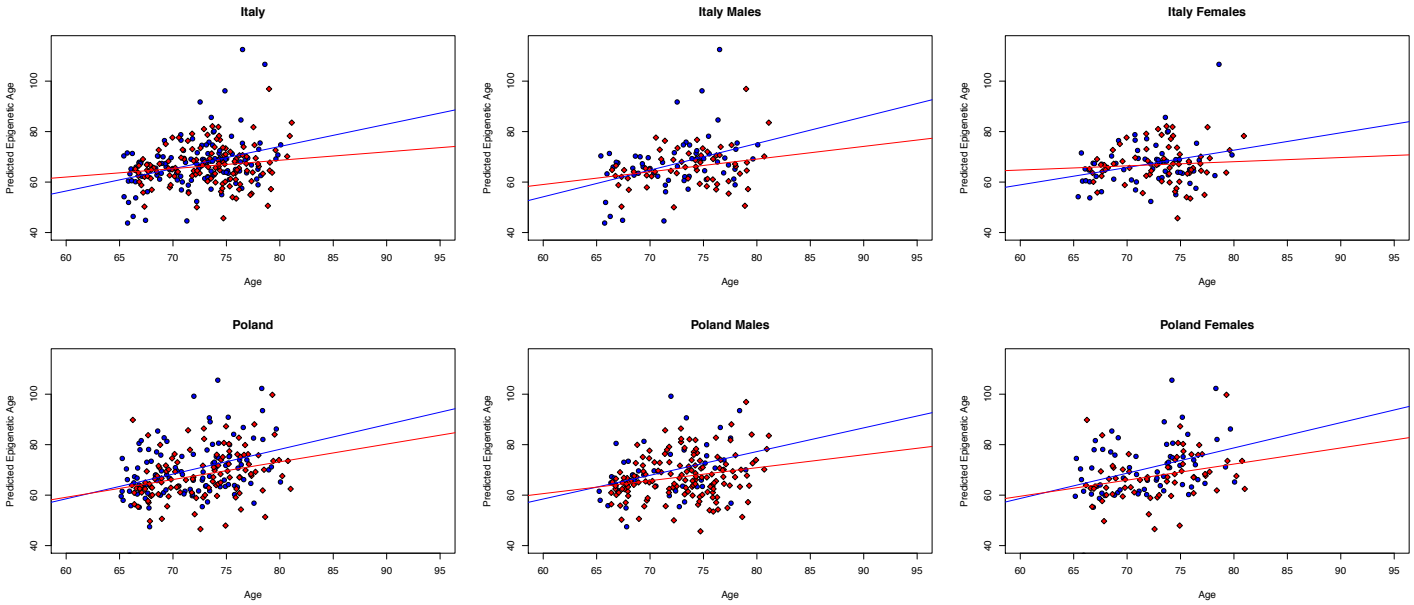


Figure 2.5: Association of chronological age (x-axis) and predicted epigenetic age (y-axis) in all individuals tested, at T0 (blue) and T1 (red).

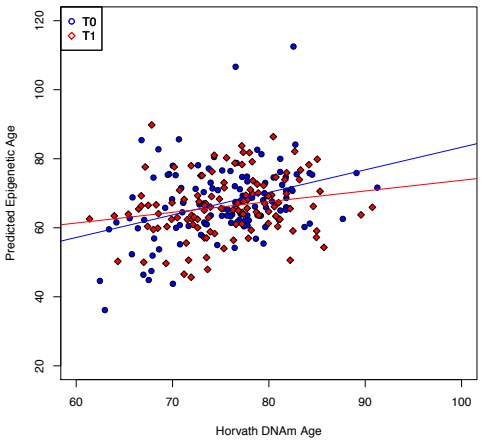
Lines represent fitted linear regressions (T0: $R^2 = 0.34$, p-value = 1.2^{-07}; T1: $R^2 = 0.23$, p-value = 0.0005). Yellow line represents the bisector.



<u>Figure 2.6</u>: Association of chronological age (x-axis) and predicted epigenetic age (y-axis) in NU-AGE subjects at T0 (blue) and T1 (red), according to country and sex.

Lines represent fitted linear regressions (<u>Italy T0</u>: $R^2 = 0.34$, p-value = 0.0001; <u>Italy T1</u>: $R^2 = 0.16$, p-value = 0.07; <u>Poland T0</u>: $R^2 = 0.36$, p-value = 0.0001; <u>Poland T1</u>: $R^2 = 0.30$, p-value = 0.002).

In the 120 subjects for which genome-wide DNA methylation analysis was available, we evaluated the association between epigenetic age predicted with our model and DNAm age predicted with Horvath's model. We observed a statistically significant association between the two sets of values (p-value < 0.001), although the correlation was low (coefficient = 0.29) (**Figure 2.7**).



<u>Figure 2.7</u>: Association of DNAmAge calculated with Horvath's model (x-axis) and predicted epigenetic age (y-axis) in NU-AGE subjects at T0 (blue) and T1 (red).

Lines represent fitted linear regressions (<u>T0</u>: $R^2 = 0.35$, p-value = 7.9^{-05}; <u>T1</u>: $R^2 = 0.20$, p-value = 0.03).

In both Italian and Polish subjects, epigenetic age acceleration measures at baseline were significantly associated ($p<0.05$) with those obtained after the nutritional intervention (**Figure 2.8**).

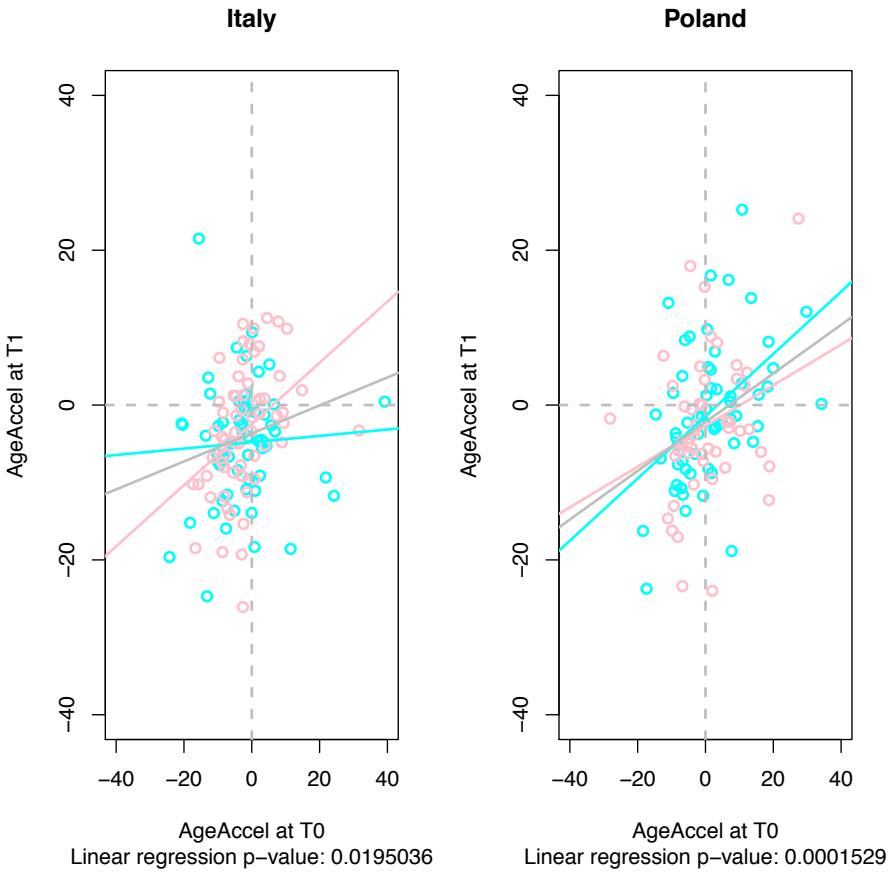
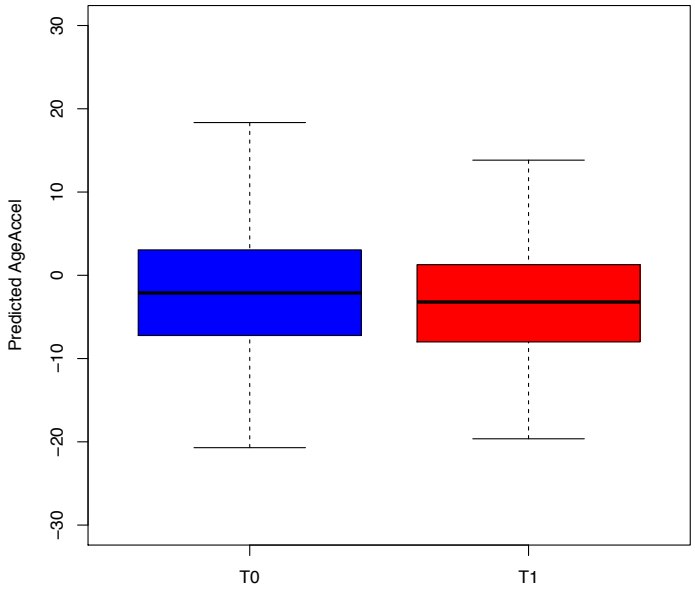


Figure 2.8: Association between epigenetic age acceleration measure at baseline and after one year of nutritional intervention.

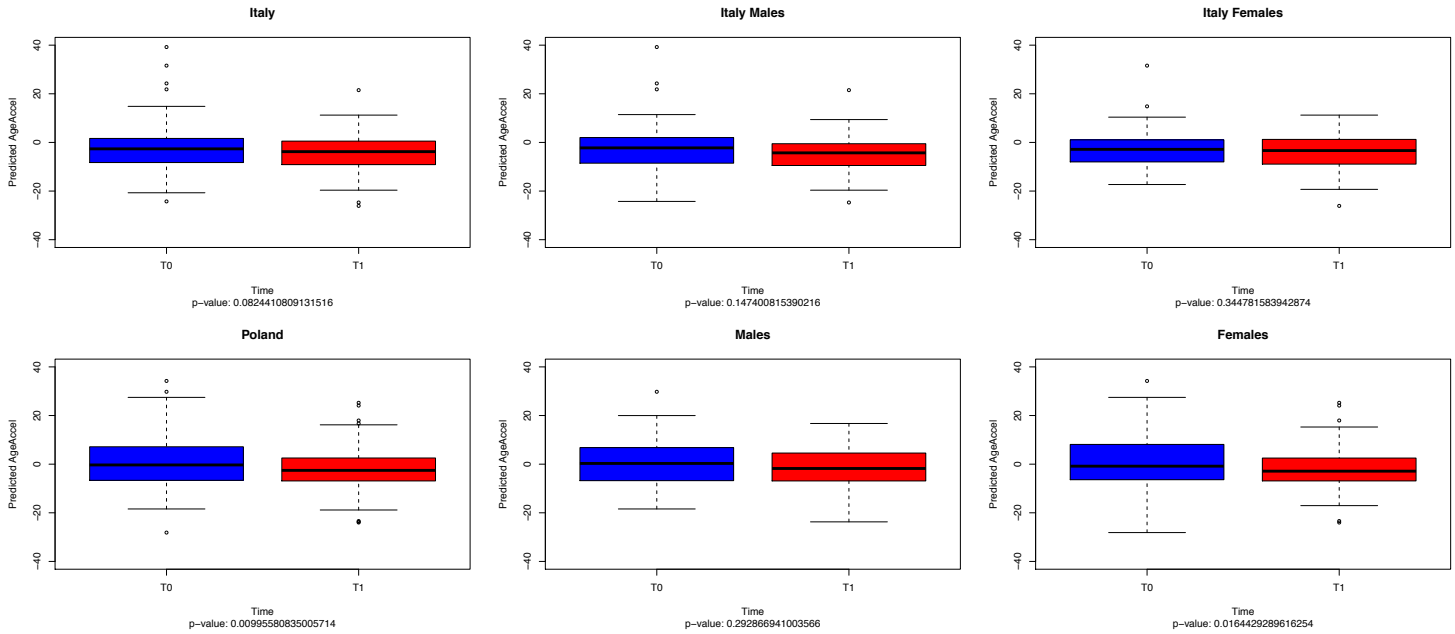
Scatter plots of AgeAccel at T0 (x-axis) versus T1 (y-axis). Males and females subjects are indicated with cyan and pink circles respectively. Lines represent fitted linear regression lines models in all subjects (grey) (Italy: $R^2 = 0.21$, p-value = 0.0195; Poland: $R^2 = 0.35$, p-value = 0.0002), males (cyan) and females (pink). Grey dotted lines represent the bisector (AgeAccel at T0 and T1 equal to 0).

We evaluated the impact of the one-year Mediterranean-like diet on the epigenetic measures of age acceleration, by comparing for each subject AgeAccel values at baseline with the ones obtained at T1. We observed a significant rejuvenation of the individuals after one-year of nutritional intervention (Student's paired t-test p-value = 0.0023) (**Figure 2.9**).



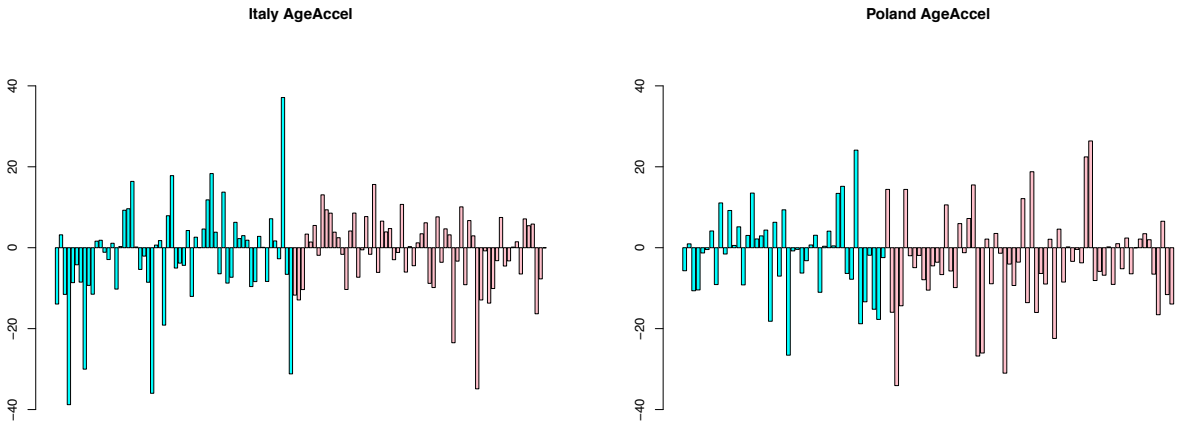
<u>Figure 2.9</u>: Boxplots of predicted epigenetic age acceleration at T0 (blue) and T1 (red).

When we divided samples according to country and gender, we observed that the effect was related to a rejuvenation of Polish subjects (p=0.010), and specially of Polish females (p-value = 0.016) (**Figure 2.10**).



<u>Figure 2.10</u>: Boxplots of epigenetic age acceleration measures at T0 (blue) and T1 (red) considering all the subjects (left panel), only males (middle panel) and only females (right panel).

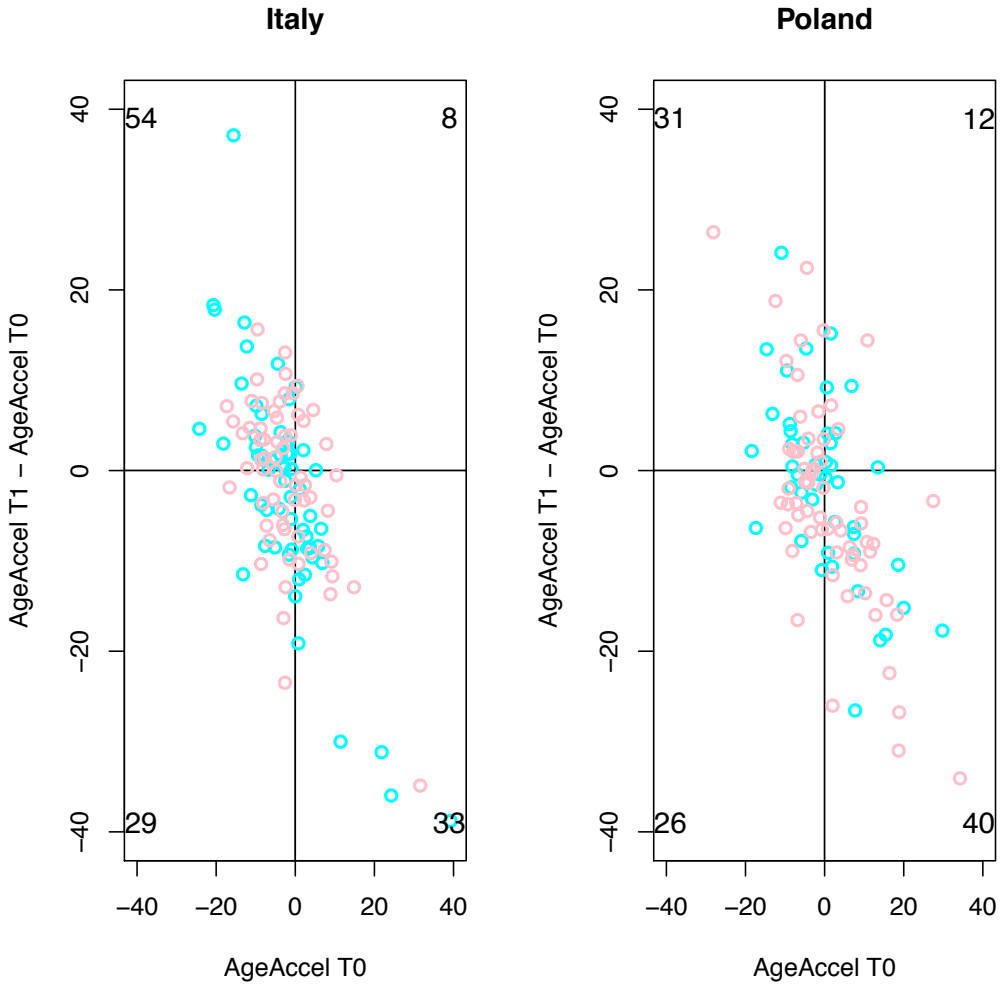
For each subject, we calculated the intra-pair difference between AgeAccel at T1 and AgeAccel at T0 (AgeAccel Diff), defined as AgeAccel at T1 – AgeAccel at T0 (**Figure 2.11**). Here also, a negative value indicates an epigenetic rejuvenation.



<u>Figure 2.11</u>: Individual-specific response to the nutritional intervention according to AgeAccel measure.

Barplots of intrapair differences for AgeAccel measures (Blue: males; Pink: females).

As we did for the epigenetic age acceleration measures obtained with Horvath's model, we assessed if AgeAccel Diff values were related to AgeAccel values at baseline (**Figure 2.12**). In Polish subjects, we observed that the effect of the nutrition intervention was more marked in subjects displaying high values of AgeAccel at baseline. Comprehensively, these results are similar to the ones observed with the measures of epigenetic age acceleration calculated according to the Horvath's clock and presented in the first part of this work.



<u>Figure 2.12</u>: Scatter plots of epigenetic age acceleration measure at T0 (x-axis) versus epigenetic age acceleration measures difference between T1-T0 (y-axis).

Males and females subjects are indicated with cyan and pink circles respectively.

Discussion

It is of critical importance to understand the factors influencing health in old age and to develop strategies which may modulate the aging trajectory, decrease morbidity and increase the number of elderlies in good health. To this regard, lifestyle modifications, and especially nutritional interventions, appear to be promising strategies to impact on aging. In our work, we were particularly interested in the impact of Mediterranean diet, one of the most popular dietary patterns in Southern Europe that has long been associated with health benefits. We evaluated the effects of a one-year Mediterranean-like diet, newly designed according to the nutritional needs of people over 65 years of age and delivered within the framework of the European project NU-AGE (Berendsen et al. 2014), on epigenetic age acceleration measures. We demonstrated that the nutritional intervention can slow down the epigenetic aging rate of blood in specific groups of participants. This rejuvenation effect was observed initially with the measures derived from Horvath's epigenetic clock model and was further confirmed with our gene-targeted model developed with the Agena EpiTYPER® system. Interestingly, the protective effect of the whole diet on the epigenetic age appears to be both country and sex-specific, as Polish, and especially Polish females, appear to benefit the more from the intervention, according to the measures of epigenetic age acceleration. Epigenetic aging rates have been previously described as influenced by race/ethnicity (Horvath, Gurven, et al. 2016) and sex (Horvath, Gurven, et al. 2016; Xiao et al. 2018), and we also demonstrated here that the epigenetic response to an intervention can be influenced by these parameters. It is likely that the observed differences between males and females are not only related to pure biological differences (for example, differences in body composition (Santoro et al. 2018)), but also to anthropologic and cultural components (such as levels of education, cooking or willingness to stick to the nutritional advices for example). While population and sex-specificities appear clearly in this work, inter-individual differences intervene also in the response to the nutritional intervention. Firstly, subjects that were epigenetically older at baseline (*i.e.* subjects with higher epigenetic age acceleration values at T0) had a more marked effect of the nutritional intervention and seemed to benefit the more of the effects of the Mediterranean-like diet. Secondly, according to our GWAS performed in the first part of this work on the epigenetic acceleration measures calculated with Horvath's model, some genetic variants influence the response to the intervention. GWAS results were largely overlapping between AgeAccel and IEAA analysis. Furthermore, enrichment analysis suggested that both epigenetic age acceleration measures were associated to genetic variants in genes involved in pathways related to the regulation of cell metabolism and immune function. Among the gene sets associated to AgeAccel differences between T1 and T0, it is worth to note the presence of the mTOR

pathway, which plays a pivotal role in the regulation of nutrients-sensing and energy metabolism during aging (Lushchak et al. 2019; Cummings & Lamming 2017; Tosti et al. 2018). In animal models, it has been previously demonstrated the influence of a genetic component in the response to a nutritional intervention, such as caloric restriction (Liao et al. 2010). Liao *et al*. observed that lifespan expansion by caloric restriction was not universal in mice and was highly dependent on the strain of the animals, suggesting the important influence of the genotype in the caloric restriction effect (Liao et al. 2010). In humans, this problematic has been poorly investigated. Previous reports have evaluated the association between genetic background and epigenetic age acceleration in different tissues (Lu et al. 2018; Lu et al. 2016; Lu et al. 2017), but little is known about the influence of genetics on the response to a nutritional intervention. In a recent study, the effects of dietary supplementation with folic acid and vitamin B12 on epigenetic age deceleration were found dependent upon gender and *MTHFR* genotype (Sae-Lee et al. 2018). Only the group of women with the *MTHFR* 677CC genotype displayed a deceleration in epigenetic aging upon vitaminic supplementation (Sae-Lee et al. 2018).

Furthermore, our work underlines the importance of DNAm clocks as biomarkers of aging and suggests that they may represent accurate tools to measure the effectiveness of lifestyle-based strategies that prolong healthy living (Mitteldorf 2019). During the last seven years, evidence has accumulated on the robustness of epigenetic clocks, successfully associated with prediction of mortality or morbidity in humans (Horvath & Raj 2018; BLUEPRINT consortium 2016). Epigenetic clock models have been successfully developed in animals, especially in mice and rats models (Wang et al. 2017; Petkovich et al. 2017; Stubbs et al. 2017; Maegawa et al. 2017; Thompson et al. 2018; Meer et al. 2018; Thompson et al. 2017; Polanowski et al. 2014). These animal models are of particular interest because they have the potential to provide new insights on the relationships between DNAm biomarkers and interventions which are intended to increase the lifespan and cannot be easily performed in humans, such as caloric restriction (*i.e.* reduction of caloric intake without causing malnutrition) for example. Thus, it has been demonstrated that animals under caloric restriction are significantly younger regarding their epigenetic age as compared to their untreated counterparts (Maegawa et al. 2017; Wang et al. 2017; Stubbs et al. 2017; Petkovich et al. 2017; Thompson et al. 2018). On the contrary, lifestyle interventions that are known to shorten lifespan in mice (ovariectomy, high fat diet) are associated with significant epigenetic age acceleration (Stubbs et al. 2017).

In this work, we presented a new model, assessable with a different technology than the previously developed ones, that could be of interest in broad clinical settings, in light of the

cost-effectiveness of the technique. After deep analysis of literature and evaluation of potential targets of interest in publicly available and own proprietary Illumina® Infinium datasets, we came to a restricted list of 6 candidate genomic regions. In order to optimize the prediction of chronological age, we decided to include three sites that were present in previously published epigenetic clocks, and we included regions with high levels of correlation between DNA methylation levels and chronological age, such as *ELOVL2*, which has been previously reported as one of the most robust age predictors and incorporated in several forensic age predictors so far (Garagnani et al. 2012; Zbieć-Piekarska, Spólnicka, Kupiec, Makowska, et al. 2015, p.2; Spólnicka et al. 2018, p.2; Jung et al. 2019, p.2). On the other hand, we considered CpG sites that could better reflect differences in the rates of aging, *i.e.* sites that could better reflect the biological age of the individuals. Thus, we considered sites described by Slieker *et al*. as age-related variably methylated positions (aVMPs), *i.e.* sites with a dependency of variance with age (Slieker et al. 2016). Finally, we integrated one site (associated with *AIM2* gene) which showed an important association between DNA methylation and serum CRP levels (Ligthart et al. 2016). Chronic, sterile, low-grade inflammation during aging, designed under the term inflammaging (Franceschi et al. 2000), is known to contribute to age-associated diseases (Franceschi & Campisi 2014) and serum CRP levels can served as sensitive markers of this phenomenon. In a GWAS performed in European and African Americans individuals, Ligthart *et al*. identified 58 CpG sites associated with CRP levels, among which the most significant one was cg10636246, associated with *AIM2* gene and that was thus further considered for our model (Ligthart et al. 2016).

The six selected regions were analyzed with Agena EpiTYPER® technology in a cohort of 315 apparently healthy individuals, previously included in other studies performed in our laboratory. Among these subjects, 278 had a chronological age ranging from 20 to 80 years old and were subsequently used for the construction of our epigenetic age predictor. Our model provides a mean absolute age prediction error of 4.70 years in all healthy subjects and a good correlation between predicted epigenetic age and chronological age. Notably, the application of our model to the NU-AGE cohort overall confirmed the results of Horvath's epigenetic clock, further suggesting that measures of epigenetic age acceleration in humans may respond to changes in lifestyle and thus could be a viable and helpful option to follow the impact of anti-ageing interventions. Further optimizations and refinements of the clocks, which could be tailored for specific indications, specific age range, or specific diseases, are needed. One major limit of the technique used to measure DNA methylation levels with the Agena EpiTYPER® system is the difficulty to normalize and preprocess data before the analysis. While several

normalization procedures and pipelines exist for DNA methylation data obtained with the different Illumina® Infinium platforms (Yousefi et al. 2013; Wang et al. 2015), this is not the case for the gene-targeted approach we used here. Different runs of DNA methylation analysis with the Agena EpiTYPER® system were necessary to obtain data in all the subjects of interest (both controls and cases). Batch variability, variations among technical replicates in the absence of data normalization can introduce potential bias in the model and in its application to other large sample size cohorts, potentially reducing or increasing meaningful findings.

Environmental factors, including diet, are able to modify the epigenome (Bacalini et al. 2014; ElGendy et al. 2018), and cross-sectional associations between epigenetic age acceleration measures and dietary patterns have been previously described (Quach et al. 2017; Levine et al. 2018). However, data from longitudinal studies on a possible rejuvenation of epigenetic age with dedicated therapeutic or lifestyle interventions are few. Two works have been recently published on this topic. Firstly, Pavanello *et al*. examined the hypothesis that an intensive relaxing training of 60 days may influence epigenetic age by turning back the epigenetic clock (Pavanello et al. 2019). They observed a trend to a reduction in DNAm age (estimated with the model proposed by Zbiec-Piekarska *et al*. (Zbieć-Piekarska, Spólnicka, Kupiec, Parys-Proszek, et al. 2015)) after training in 6 healthy subjects ($p = 0.053$), but not in patients after myocardial infarction. Secondly, the effect of a protocol intended to 'rejuvenate the thymus' (TRIIM trial) was examined by Horvath's team (Fahy et al. 2019). The one-year intervention, composed of recombinant human growth hormone, dehydroepiandrosterone and metformin, was delivered to 9 healthy aging men (age range: 51 – 65 years old). A rejuvenating effect on four epigenetic age predictors (Horvath's, Hannum's, Levine's clocks and GrimAge) was observed, with a mean change of about 2.5 years. The intervention was also associated with a protective effect on different immunosenescence biomarkers (reversal of thymic involution, increase in both naïve CD4+ and CD8+ T cells) and the effect persisted six months after discontinuing the treatment. Data regarding the impact of nutritional intervention are lacking and, to our knowledge, our study is the first longitudinal and interventional study to examine effects of such an intervention on epigenetic age acceleration measures in human blood cells. According to our results, a one-year nutritional intervention could be able to rewind the epigenetic AA process in some specific groups. The discrepancy between the slowdown obtained with AgeAccel and IEAA measures on one hand, and the absence of effect observed with EEAA measure on the other hand seems to be of particular interest. Indeed, the three measures of epigenetic AA we studied in the first part of this work do not capture the same features of biological aging. By their very own construction, IEAA is considered as a measure

of epigenetic age acceleration independent of age-related changes in the cellular composition of blood, whereas EEAA is more meant to capture the age-related decline of the immune system. Here, we did not observe any significant impact of the nutritional intervention on this decline according to the EEAA measure. These results therefore suggest that the Mediterranean-like diet has a pure rejuvenating impact on the biological clock, and that this result is unconfounded by a potential effect of the intervention on the immune system.

Collectively, our results indicated that Mediterranean-like diet can promote epigenetic rejuvenation in the elderly, according to two models of biomarkers of epigenetic aging, and that its effect is dependent on different factors including: 1) country / population specific factors, likely influenced by anthropologic and cultural components; 2) sex / gender specific factors and 3) individual-specific factors, for example related to the genetic background and to the baseline epigenetic profile of each individual (**Figure 3**). Further work is required to elucidate how these and other determinants influence the epigenetic aging and how some individuals seem to be more prone to benefit from specific interventions. This will be a key achievement for the development of individualized nutritional interventions aimed at promoting healthy living and, more in general, for the application of a precision medicine approach to anti-aging interventions.

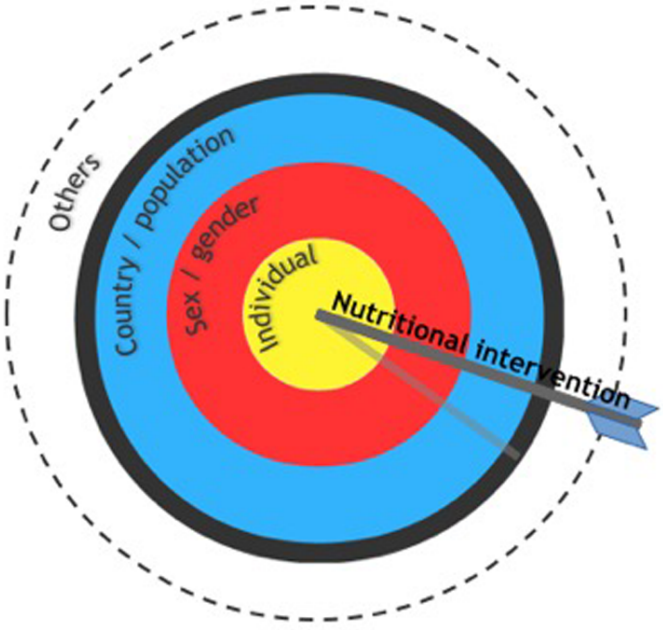


Figure 3: A precision medicine approach for nutritional interventions.

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Abstract

There is a need to identify factors that are able to influence health in old age and to develop interventions that could slow down the process of aging and its associated pathologies. Lifestyle modifications, and especially nutrition, appear to be promising strategies to promote healthy aging. Their impact on aging biomarkers has been poorly investigated. In the first part of this work, we evaluated the impact of a one-year Mediterranean-like diet, delivered within the framework of the NU-AGE project in 120 elderly subjects, on epigenetic age acceleration measures assessed with Horvath's clock. We observed a rejuvenation of participants after nutritional intervention. The effect was more marked in the group of Polish females and in subjects who were epigenetically older at baseline. In the second part of this work, we developed a new model of epigenetic biomarker, based on a gene-targeted approach with the EpiTYPER® system. We selected six regions of interest (associated with *ELOVL2*, *NHLRC1*, *SIRT7/MAFG*, *AIM2*, *EDARADD* and *TFAP2E* genes) and constructed our model through a ridge regression analysis. In controls, estimation of chronological age was accurate, with a correlation coefficient between predicted and chronological age of 0.92 and a mean absolute deviation of 4.70 years. Our model was able to capture phenomena of accelerated or decelerated aging, in Down syndrome subjects and centenarians and offspring respectively. Applying our model to samples of the NU-AGE project, we observed similar results to the ones obtained with the canonical epigenetic clock, with a rejuvenation of the individuals after one-year of nutritional intervention. Together, our findings indicate that nutrition can promote epigenetic rejuvenation and that epigenetic age acceleration measures could be suitable biomarkers to evaluate their impact. We demonstrated that the effect of the dietary intervention is country-, sex- and individual-specific, thus suggesting the need for a personalized approach to nutritional interventions.