# Adherence to Mediterranean diet is associated with methylation changes in inflammation-related genes in peripheral blood cells

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## ABSTRACT

Epigenetic processes, including DNA methylation, might be modulated by environmental factors such as the diet, which in turn have been associated with the onset of several diseases such as obesity or cardiovascular events. Meanwhile, Mediterranean diet (MedDiet) has demonstrated favourable effects on cardiovascular risk, blood pressure, inflammation, and other complications related to excessive adiposity. Some of these effects could be mediated by epigenetic modifications. Therefore, the objective of this study was to investigate whether the adherence to MedDiet is associated with changes in the methylation status from

peripheral blood cells. A subset of 36 individuals were selected within the PREvención con DIeta MEDiterránea (PREDIMED)-Navarra study, a randomised, controlled, parallel trial with three groups of intervention in high cardiovascular risk volunteers, two with a MedDiet and one low-fat control group. Changes in methylation between baseline and five years were studied. DNA methylation arrays were analysed by several robust statistical tests and functional classifications. Eight genes related to inflammation and immunocompetence (*EEF2, COL18A1, IL4I1, LEPR, PLAGL1, IFRD1, MAPKAPK2, PPARGC1B*) were finally selected as changes in their methylation levels correlated with adherence to MedDiet and because they presented sensitivity related to a high variability in methylation changes. Additionally, *EEF2* methylation levels positively correlated with concentrations of TNF-α and CRP. This report is apparently the first showing that adherence to MedDiet is associated the methylation of the reported genes related to inflammation with a potential regulatory impact.

**Key words: Mediterranean, diet, adherence, methylation, DNA, epigenetics**

## INTRODUCTION

Individual phenotypical features results from the interplay among genetics, epigenetics and environmental factors, including the diet [30]. In this context, epigenetic marks involve heritable changes that cannot be explained through variations in DNA sequence and can potentially be transmitted to the offspring [29]. Epigenetics signatures include DNA methylation, histone modifications, nuclear proteins action as epigenetic regulators, genomic imprinting, non-coding RNAs such as microRNAs (miRNAs), and non-covalent mechanisms [30]. These epigenetic modifications may lead to chromatin structure impairments in terms of accessibility and compactness, which may regulate gene expression and provide mechanisms for cellular diversity [38]. Actually, epigenetic marks are influenced by environmental factors, which have been associated to several diseases and complications such as inflammation, obesity, insulin resistance, type 2 diabetes, cardiovascular diseases, immune diseases, apart from being implicated in embryogenic development, aging and cancer [10].

DNA methylation is one of the most studied epigenetic processes and, in mammals, primarily occurs by the addition of a methyl group in the carbon 5' position of a cytosine, which is adjacent to a guanine, forming a methylated CpG dinucleotide [38, 40]. Interestingly, nutrients and components of the diet are able to modify gene expression at the transcriptional level through changes in DNA methylation [3, 10, 18]. Indeed, Milagro *et al*., (2013) reviewed nutritional factors that may cause metabolic effects acting through epigenetic processes, such as methyl donors, vitamins, fatty acids or polyphenols [30]. For instance, some studies have evidenced the influence of n-3 and n-6 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic [8, 14], docosahexaenoic [8, 23] and arachidonic [21], on DNA methylation. Folic acid supplementation in juvenile-pubertal period in rodents induced

epigenetic and phenotypic changes, associated with an increase of PPAR-α gene methylation in liver and a decrease of insulin receptor methylation in adipose tissue, with reciprocal changes in gene expression [5]. Another example is genistein, which reduces methylation of *WNT5a* and *BTG3* promoters in both cancerous and renal cells [24].

Additionally, obesity and accompanying comorbidities have been highly associated to epigenetic changes induced by the dietary intake [19]. For example, subjects with greater response to calorie restriction in a weight loss intervention presented a hypomethylation of the promoter region of tumour necrosis factor α (TNF-α) and leptin (LEP) in peripheral blood mononuclear cells [6] and in subcutaneous adipose tissue [11]. Thus, the progress in the epigenetic field and the study of nutritional biomarkers is contributing to define new roles of nutrients in health and disease, for the prevention of these diseases and implementation of precision treatment strategies [17, 22].

Traditionally, Mediterranean diet (MedDiet) has been associated with a protective effect against cardiovascular diseases [45], but no large primary prevention trials with clinical events as end-point was performed until 2003 when PREvención con DIeta MEDiterránea (PREDIMED) study arose with the purpose of analysing the effect of a MedDiet intervention on prevention of cardiovascular diseases [16]. The results of the trial showed that the MedDiet was associated with a decrease of cardiovascular events and with a favourable effect on blood pressure, insulin sensitivity, lipid profile, lipoprotein particles, inflammation, oxidative stress and metabolic syndrome manifestations [15, 27, 31].

Our hypothesis was that phenotypic changes observed after an intervention with MedDiet could be associated with epigenetic modifications in certain genes. Because of that, the objective of this epigenome-wide study was to investigate whether the adherence to MedDiet along the intervention is related to methylation changes in PREDIMED-Navarra participants.

## MATERIALS AND METHODS

### Study design and participants

The current trial was conducted within the framework of the PREDIMED study, which was a multicenter, randomized, primary prevention feeding trial with blinded assessment of end points carried out in Spain with the aim of evaluating the effects of the MedDiet on primary cardiovascular prevention (www.predimed.es). The protocol and recruitment methods have been described in detail elsewhere [16, 26]. All participants provided written informed consent, and the protocol was approved by the Research Ethics Committees at all recruiting centres in compliance with the Helsinki Declaration guidelines. This trial has been registered with the International Standard Randomised Controlled Trial Number (ISRCTN of London, England: 35739639).

Eligible participants were men aged 55–80 and women aged 60–80 years without any previous history of cardiovascular disease. At baseline, participants should fulfil at least one of the following two criteria: type-2 diabetes or three or more cardiovascular risk factors: smoking, hypertension, elevated low-density lipoprotein, cholesterol levels, low high-density lipoprotein cholesterol levels, overweight or obesity, or a family history of premature coronary heart disease. Participants were randomized to one of three nutrition interventions: a MedDiet supplemented with extra virgin olive oil (EVOO), a MedDiet supplemented with mixed nuts, or a low-fat diet (control group). All groups received dietary instructions and a previously validated fourteen-item dietary screener (p14) was used to assess adherence to MedDiet at baseline and at the last visit [37]. This questionnaire comprises 14 questions about frequency of consumption of several recommended foods in MedDiet (**Supl. Table S1**). Each question can give one or zero points depending on whether the recommendations are accomplished or not, obtaining a maximum of 14 points which represent the highest adherence to MedDiet, and a minimum of zero points. Other questionnaires (medical conditions, food consumption and composition, physical activity) were completed as described elsewhere [16]. Data of anthropometric measures and body composition (body weight, waist circumference, body mass index) and blood pressure were collected in the same consultations [31]. Plasma, serum and buffy-coat were stored and biochemical features (glycaemia, cholesterol levels, and triglycerides) were measured as previously published [31]. Composition of different types of leukocytes (neutrophils, lymphocytes, monocytes, eosinophils and basophils) in whole blood was analysed by ABX Pentra 60 hematology analyser (Horiba, Madrid, Spain).

For the present secondary analysis, 36 participants were selected from the recruitment centre at the University of Navarra. These volunteers were selected following different criteria. Firstly, they were sifted by smoking (non-smokers or former-smokers) and by age (between 60 and 70 years old). Then, six women and six men were randomly chosen from each diet. Data from patients (questionnaires, anthropometry, blood pressure, p14 score, blood samples and biochemical measures) were collected at baseline and at five years of intervention. Methylation analysis was also performed at baseline and at five-year follow-up.

**DNA extraction and DNA methylation analyse**

After overnight fasting, venous blood samples from baseline and five years of intervention were drawn on EDTA tubes. Samples were centrifuged at 2000 x*g*, 4 °C, 15 min and buffy-coats were collected. Aliquots were coded and kept refrigerated until they were stored at -80 °C. DNA was extracted from buffy-coat with MasterPure™ DNA Purification Kit for Blood (Epicentre, Madison, WI, USA) according to manufacturer's instructions and shipped on dry ice to Unidad de Genotipado y Diagnóstico Genético from Fundación Investigación Clínico de Valencia (INCLIVA), where microarray preparation, hybridization and scanning was performed.

As previously described [25], DNA was quantified using PicoGreen double-stranded DNA (dsDNA) Quantification Reagent® (Invitrogen, Carlsbad, CA, USA). EZ DNA methylation kit (Zymo Reaearch, Irvine, CA, USA) was used to bisulphite modification of 500 mg of genomic DNA according with manufacturer's protocol. Bisulphite-treated genomic DNA was amplified and hybridized using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) and scanned using the Illumina hiScanSQ platform. The intensity of the images was extracted with the GenomeStudio Methylation Software Module (v 1.9.0, Illumina, San Diego, CA, USA).

**Treatment of methylation raw data, Ingenuity Pathway Analysis and selection**

Microarray data were normalized in R by a categorical subset quantile normalization method using the pipeline developed by Touleimat & Tost (2012) in a previous paper [41]. Afterwards, Pearson correlations between methylation changes (five years – baseline) and p14 differences (five years – baseline) were calculated in order to choose those CpGs that were differentially methylated and also correlated with the changes in adherence to MedDiet, assessed by p14. Methylation change was chosen instead of methylation at five years as a way of correction, in order to avoid the influence of methylation at baseline. From all the significant CpGs obtained (n=12990), and in order to control the type I error rate, a more restrictive selection was performed using the r-value from correlations >|0.5|, which corresponds to a $p<0.0019$. Then, the selected CpGs were submitted to a two-winged analysis. Initially, they were analysed with Ingenuity Pathway Analysis (IPA) software, (Qiagen Redwood City, CA, USA, www.ingenuity.com), using predefined pathways and functional categories of the Ingenuity Knowledge Base in order to detect associated pathways and relevant gene regulatory networks [43]. Pathway analyses were performed with IPA Core Analysis module. Canonical pathways with a $p<0.05$ after Fisher's test were defined as a statistically significant overrepresentation of input genes in a given process. Secondly, CpGs were selected because: 1) they presented the highest standard deviation of the mean of methylation changes, and subsequently, the highest variability (>5% of methylation changes); this method is considered a robust and consistent process of filtering beta values of methylation with high sensitivity to changes [39]; and 2) there was previous evidence that these genes where the CpGs were located expressed in blood cells. Afterwards, a manual search in the scientific literature for the genes was performed in order to classify them into biological function groups for further analysis. A diagram of the analytic process is reported in the Supplementary Material (**Fig S1**).

**ELISA analysis**

Soluble intercellular cell adhesion molecule-1 (sICAM-1, Ref. DCD540), vascular cell adhesion molecule-1 (VCAM-1, Ref. DVC00), C-reactive protein (CRP, Ref. DCRP00), TNF-α (Ref. HSTA00D) and LEP (Ref. D0BR00) were measured using standard enzyme-linked immunosorbent assay (ELISA) in EDTA plasma samples at baseline and at five years according the manufacturer's specific protocol.

All ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MA, USA). Protein concentrations were measured by Multiskan Spectrum from Thermo Scientific (Waltham, MA, USA). The selection of these molecules was based on the observed decrease of VCAM-1, sICAM-1 and CRP in the MedDiet groups in PREDIMED trial [7, 15, 35] and the described relationship between TNF-α and the action of two selected genes (eukaryotic elongation factor 2 (*EEF2*) and mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (*MAPKAPK2*). LEP was chosen because it is the ligand of LEP receptor encoded by another selected gene (*LEPR*).

**Statistical analysis**

Participants were characterised comparing the differences (five years-baseline) of some anthropometric and biochemical features, blood pressure and p14 score among the three groups using ANOVA test. Tukey's multiple comparison test was applied for p14 score analysis.

Differences in the composition of types of leukocytes between five years and baseline were correlated (Pearson) with p14 changes and with methylation changes of the eight CpGs selected. Benjamini-Hochberg correction was applied to control the false discovery rate.

For ELISAs statistical analysis, participants were categorised into two groups of p14 at five years by the median (Q1 and Q2) and then, a Student T test, Mann-Whitney U test or Median test, as appropriate, was carried out in order to compare the quantity of protein in both groups. One-tailed p-values were selected as the MedDiet has been previously described with inflammatory beneficial effects [27] and therefore, a decrease (or at least, no differences) was expected in the concentrations of inflammatory biomarkers in the Q2 group. Correlations (Pearson or Spearman, when appropriate) between the methylation data of selected CpGs and protein concentration obtained in ELISAs were also performed. For analysing the correlations, data from both baseline and five years were included.

In general, the Shapiro-Wilk analysis was employed to test normality. R Studio [34] was used for the analysis of Pearson correlations during the selection process. Statistics and graphs were performed using STATA version 14.0 (Stata Corp, College Station, TX, USA) and GraphPad Prism 6 (Graph-Pad Software, San Diego, CA, USA). The significance level was set at $p<0.05$.

**RESULTS**

Participants showed no differences among the three intervention groups in age and changes of weight, waist circumference, body mass index, glycaemia, cholesterol levels, triglycerides and arterial pressure (**Table 1**). Variations among groups in composition of

types of leukocytes were also not observed (data not shown). However, when analysing the adherence to MedDiet assessed by p14, at baseline, all groups presented a similar p14 with non-significant differences. However, after five years, the MedDiet groups showed a significantly higher p14, and, consequently, a higher adherence to MedDiet in comparison with the control group (MedDiet groups in conjunction (11.8) vs. low-fat diet (9.8); $p<0.001$). When analysing each group separately, both MedDiet groups presented significant differences in p14 between five years and baseline, whereas in the control group such differences were not found (**Fig 1A**). Regarding inflammation biomarkers (**Supl. Table S2**), results showed a significant decrease of TNF-α ($p=0.024$), sICAM-1 ($p=0.033$) and CRP ($p=0.044$) in the group with higher p14 at five years of intervention, while a trend towards a decrease for VCAM-1 molecule ($p= 0.051$) was found (**Fig 1B**). However, non-significant differences were observed for LEP values (data not shown).

Pearson correlations allowed the identification of CpGs that were differentially methylated and correlated with p14 changes. From all the significant CpGs, 316 were selected (**Supl. Table S3**) with the criteria of $r>|0.5|$, corresponding to a $p<0.002$. These selections are explained in detail in *Materials and methods.* These 316 CpGs were two-winged analysed combining computational and literature approaches.

For the first approach, the 316 CpGs were further studied by IPA in order to feature the associated canonical pathways (**Fig 2**). Some of the pathways were related to inflammation, such as *Role of JAK1, JAK2 and TYK2 in Interferon Signalling, STAT3 Pathway, Mitochondrial L-carnitine Shuttle Pathway, AMPK Signalling* and *Role of JAK family kinases in IL-6-type Cytokine Signalling*), whereas others were involved in metabolism such as *Histamine Biosynthesis, GDP-L-fucose Biosynthesis I, Glycine Biosynthesis I* and *Coenzyme A Biosynthesis.*

For the second approach, another selection strategy was performed as described in materials and methods. From all the 316 CpGs, 50 were selected (**Supl. Table S4**). Afterwards, the 50 CpGs were classified into biological function groups (**Fig 3**). Those functions were immunocompetence/inflammation, adipogenesis, diabetes/insulin secretion, metabolism, methylation, angiogenesis, cell dynamics, cell survival/cell death, gene expression, cell differentiation/cell growth, blood pressure regulation, and ubiquitination. Some of the genes were categorised in more than one group because they presented different functions depending on factors such as the tissue where they were expressed. Further investigation was focused on eight genes (**Supl. Table S2**) from the immunocompetence-function group based on bibliographic search. The selected genes were: *EEF2, COL18A1 (*collagen type XVIII alpha 1)*, IL4I1* (interleukin 4-induced gene-1)*, LEPR, PLAGL1* (pleiomorphic adenoma gene-like 1)*, IFRD1* (interferon-related

developmental regulator 1)*, MAPKAPK2* and *PPARGC1B* (peroxisome proliferator-activated receptor gamma, coactivator 1 beta). Correlation graphs between methylation and p14 changes of these eight genes are reported (**Fig 4**). Interestingly, *EEF2*, *IL4I1* and *PPARGC1B* presented a negative association whereas *COL18A*, *LEPR*, *PLAGL1*, *IFRD1* and *MAPKAPK2* had a positive association with p14. Methylation changes included from 60 to -40 for *EEF2* as the maximum interval and from 10 to -30 for *PPARGC1B* as the minimum interval.

Some inflammation-related molecules such as TNF-α, VCAM-1, sICAM-1 and CRP, and in addition LEP, were measured. The purpose of this assay was to assess whether a higher or a lower methylation induced changes in these protein concentrations. As a result, correlations between measured proteins and methylation data at baseline and at five years were performed. Results showed correlations between (A) *LEPR* methylation and concentration of LEP, (B) *EEF2* methylation and concentration of TNF-α, and (C) *EEF2* methylation and concentration of CRP (**Fig. 5**).

Since methylation measured in peripheral blood cells (PBCs) can depend on variations in the types of leukocytes, correlation studies were performed between each type of cell (five years-baseline) and p14 changes or methylation changes of the selected eight CpGs, showing no association for any comparison.

## DISCUSSION

The current study demonstrates for the first time that following a MedDiet is associated with the differential methylation of the selected genes: *EEF2*, *COL18A1*, *IL4I1*, *LEPR, PPARGC1B*, *MAPKAPK2*, *IFRD1* and *PLAGL1*. Along these lines, other preliminary studies focused on selected genes (*FTO* and *TCF7L2*) in the PREDIMED-Valencia trial (n=195 individuals) reported that intervention with MedDiet was associated with changes in methylation at 1-year [12]. Likewise, when focusing on *FNDC5* (irisin gene) (n=181), higher adherence to MedDiet at baseline was associated with statistically significant differences in methylation at baseline [13]. Thus, this study adds further insights to previous reports where environmental factors, including diet, were able to modify the epigenome [3].

Phenotypical changes in anthropometric and biochemical measurements among the participants in the three dietary groups were not reported. Regarding adherence to the MedDiet, our results showed that, although baseline p14 scores were similar among the participants, intervention with MedDiet led to a significant increase in this score. In contrast, participants in the low-fat diet presented non-significant differences. Thus, the p14 score was demonstrated to be a good method for assessing adherence to the MedDiet [37]

The MedDiet has shown prevention capacity against cardiovascular diseases and associated risk factors such as inflammation, hypertension, and hyperlipidaemia, among others [15, 27]. On the other hand, variations in DNA methylation have been related to several diseases such as obesity, type 2 diabetes, cancer, and cardiovascular diseases [10]. Hence, the MedDiet could be associated with variations in DNA methylation, which in turn may cause changes in the expression of some genes associated with these diseases. In fact, the genes with higher variability in methylation changes selected from our study were linked to functions that are intimately related to those diseases. Such functions are immunocompetence/inflammation, adipogenesis, diabetes/insulin secretion, metabolism, methylation, angiogenesis, cell dynamics, cell survival/cell death, gene expression, cell differentiation/cell growth, blood pressure regulation, and ubiquitination. Canonical pathway screening from IPA showed that genes with methylation changes that correlated with p14 changes were mainly associated with pathways of inflammation and metabolism. From all these groups, eight genes from immunocompetence/inflammation were selected for further bibliographic research as methylation levels were measured in DNA extracted from PBCs. The studied eight genes were *EEF2*, *COL18A1*, *IL4I1*, *LEPR, PPARGC1B*, *MAPKAPK2*, *IFRD1* and *PLAGL1*. All of them have been described performing functions associated with inflammation, which can be related to the described favourable effects of the MedDiet on the immune system [27]. For example, *EEF2* controls TNF-α production in macrophages, regulating the inflammatory response and triggering several intracellular signalling cascades that influence cell survival, death, differentiation, proliferation, and migration [20]; *IL4I1* can inhibit the proliferation of CD3-stimulated T lymphocytes [2, 48]; and *MAPKAPK2* is involved in the production of TNF-α and other cytokines, and in the granulocyte infiltration [36]. In this context, several studies derived from PREDIMED trial have demonstrated that inflammatory biomarkers, such as Interleukin 6, VCAM-1, sICAM-1, and CRP, decrease in MedDiet groups [7, 15, 35]. In fact, the quantitative analysis of some of these molecules (CRP, sICAM-1, VCAM-1) and TNF-α in this study confirms that individuals with higher adherence to MedDiet presented lower inflammatory biomarkers. Therefore, the beneficial inflammatory effects of the MedDiet could be mediated by changes in the methylation levels of genes related to inflammation. Indeed, an association between *EEF2* methylation and both inflammatory biomarkers TNF-α, and CRP, was observed in the study, suggesting that changes in *EEF2* methylation drive to a variation in *EEF2* expression, which might result in the regulation of the production of inflammatory molecules. In fact, EEF2 has been described controlling TNF-α elongation [20]. Therefore, an increase in *EEF2* methylation would produce changes in its expression and thus, in the production of TNF-α. Moreover, methylation of *LEPR* and concentration of LEP were also associated, suggesting a likely regulation of the interaction between the receptor and its ligand through epigenetic mechanisms. Zhang et al. (2001) described that there is a LEPR-mediated feedback suppression on *LEP* expression [49]. Hence, in order to be in accordance with the obtained results, an increase in *LEPR* methylation would lead to an increase in *LEPR* expression and thus, to a decrease in LEP production.

The mentioned gene functions also include actions related to adipogenesis and metabolism. For example, *LEPR* absence leads to severe obesity and metabolic disorders [33]; *PPARGC1B* is associated with type 2 diabetes [42] and related to brown adipocytes [32]; and *IFRD1* is a unique mediator of nutrient absorptive and metabolic adaptation following gut resection and its overexpression in the intestine alters growth, metabolic rate, adiposity, and intestinal triglyceride absorption [44]. Nevertheless, the methylation levels were measured in PBCs. It may be possible that these cells could be acting as proxies for other tissues that are less accessible but clinically important [4] such as adipocytes. The analysis of these tissues in order to study whether the methylation levels match those observed in PBCs would be a step forward for identifying epigenetic biomarkers. In this context, further investigation of nutritional biomarkers would be useful to identify susceptibility to suffering from diseases, or even to guide personalized nutritional interventions [11].

Methylation changes in this study have shown both a positive and a negative association with p14 changes. Traditionally, increases in methylation have been associated with lower gene expression and viceversa. However, it has recently been described that this relationship is not always valid as it depends on the location of the CpG inside the gene and in the gene itself. Thus, some genes present higher or lower expression when there is an increase or decrease in their methylation, respectively; however, this pattern is not universal [28, 46].

Nevertheless, there are some limitations in this study. Firstly, it could be useful to increase the number of participants to raise the statistical power and more reliable results. Secondly, type I error cannot be discarded due to the high number of test performed in this study and that corrections for multiple comparisons were implemented, but an specific effort has been made to analyse data with different robust biological criteria and statistical strategies. Thirdly, although correlation between the different types of leukocytes and the variables investigated in this study was assessed in order to avoid influences in methylation changes, correction for cell type was not applied. Since there were no associations between the types of leukocytes and the variables studied, correction for cell type was not carried out. Finally, gene expression could be evaluated and related to methylation changes in order to determine how the diet influences the general health status. Although this analysis was not performed, it has been described in the literature that some foods and components of the diet are able to modify the expression of some of the genes selected in the present study in other contexts. For instance, two isothiocyanates obtained from broccoli (sulforaphane and iberin) increased expression of *PLAGL1* in stromal cells, possibly being related to a reduced risk of prostate cancer [9]. Diet supplementation with flaxseed increased endostatin (fragment of *COL18A1*) levels in breast tissue cultures [1]. As for fucoxanthin, a carotenoid found in brown seaweed, macroalgae, and diatoms, Yoshiko & Hoyoku (2007) described an increase in *IFRD1*

expression in HepG2 cells [47]. Therefore, it is likely that the methylation changes observed in this study correlated with changes in the expression of the selected genes, although a further analysis is needed to corroborate this hypothesis.

In conclusion, this study shows that the MedDiet is associated with changes in the epigenome through methylation mechanisms in PBCs in a notable way in at least 50 genes. Some of these changes in methylation levels occurred in genes related to inflammation, but with other possible functions related to adipogenesis, metabolism, angiogenesis, and diabetes, among others. Therefore, MedDiet may be exerting a beneficial effect on health through anti-inflammatory actions that might be potentially mediated by epigenetic mechanisms. Further studies with a larger sample size are needed to confirm these findings.

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**Table 1**

| | Low-fat diet (n=12) | MedDiet + EVOO (n=12) | MedDiet + nuts (n=12) | p |
|---|---|---|---|---|
| **Female, n (%)** [1] | 6 (50) | 6 (50) | 6 (50) | |
| **Age at baseline, years** | 64.6 (3.9) | 63.5 (1.7) | 63.2 (2.1) | 0.425 |
| **Δ Weight, kg** | 0.1 (2.7) | 1.3 (4.9) | 1.6 (4.4) | 0.637 |
| **Δ Waist circumference, cm** | 0.0 (3.0) | 3.7 (6.8) | 0.8 (5.8) | 0.213 |
| **Δ BMI, kg/m2** | 0.1 (1.1) | 0.4 (1.9) | 0.7 (1.7) | 0.312 |
| **Δ Glycemia, mg/dl** | -1.7 (15.3) | -8.8 (53.9) | 0.18 (37.6) | 0.825 |
| **Δ HDL-cholesterol, mg/dl** | 2.7 (6.7) | -0.1 (8.0) | 1.9 (13.9) | 0.750 |
| **Δ LDL-cholesterol, mg/dl** | -16.6 (24.7) | -9.0 (29.5) | 5.8 (28.1) | 0.185 |
| **Δ Total cholesterol, mg/dl** | -16.1 (33.1) | -11.8 (33.4) | 11.5 (36.9) | 0.217 |
| **Δ Triglycerides, mg/dl** | -10.9 (45.4) | -13.6 (63.7) | 19.4 (22.4) | 0.243 |
| **Δ Systolic arterial pressure, mmHg** | 1.2 (18.0) | 0.9 (22.7) | 4.8 (11.8) | 0.740 |
| **Δ Diastolic arterial pressure, mmHg** | 0.1 (9.6) | -4.3 (10.2) | -0.7 (9.3) | 0.509 |

Values are Mean (SD), except [1] for which is represented as n (%). P-values are achieved by an ANOVA test among the three groups. A significant p-value is considered $p<0.05$.

BMI: Body Mass Index; HDL: high-density cholesterol; LDL: low-density cholesterol; MedDiet: Mediterranean diet; EVOO: extra-virgin olive oil

**Figure 1**

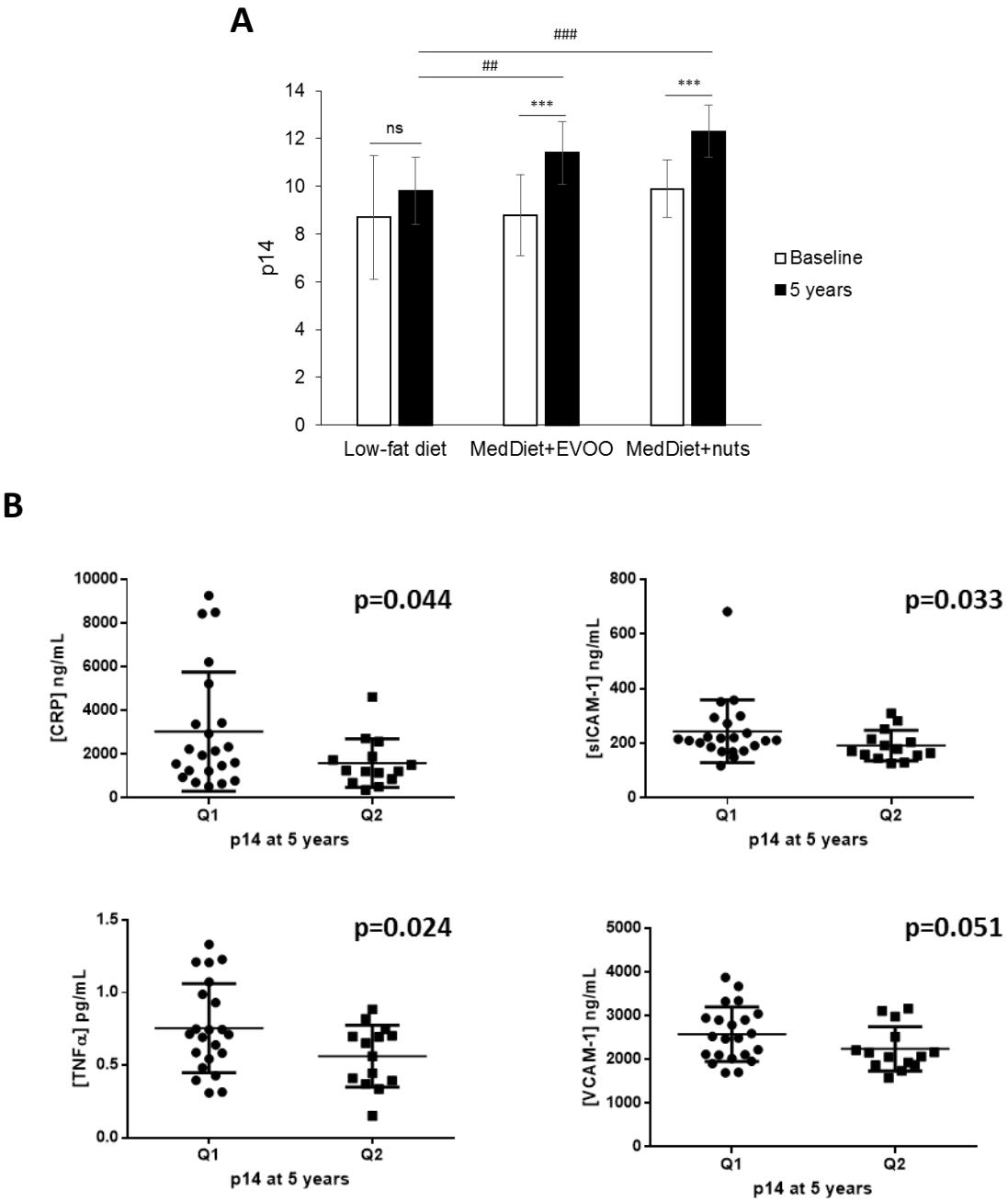
A
###
##
***
***
ns
p14
14
12
10
8
6
4
2
0
Low-fat diet
MedDiet+EVOO
MedDiet+nuts
Baseline
5 years
B
[CRP] ng/mL
10000
8000
6000
4000
2000
0
p=0.044
Q1
Q2
p14 at 5 years
[sICAM-1] ng/mL
800
600
400
200
0
p=0.033
Q1
Q2
p14 at 5 years
[TNFα] pg/mL
1.5
1.0
0.5
0.0
p=0.024
Q1
Q2
p14 at 5 years
[VCAM-1] ng/mL
5000
4000
3000
2000
1000
0
p=0.051
Q1
Q2
p14 at 5 years

**Figure 2**

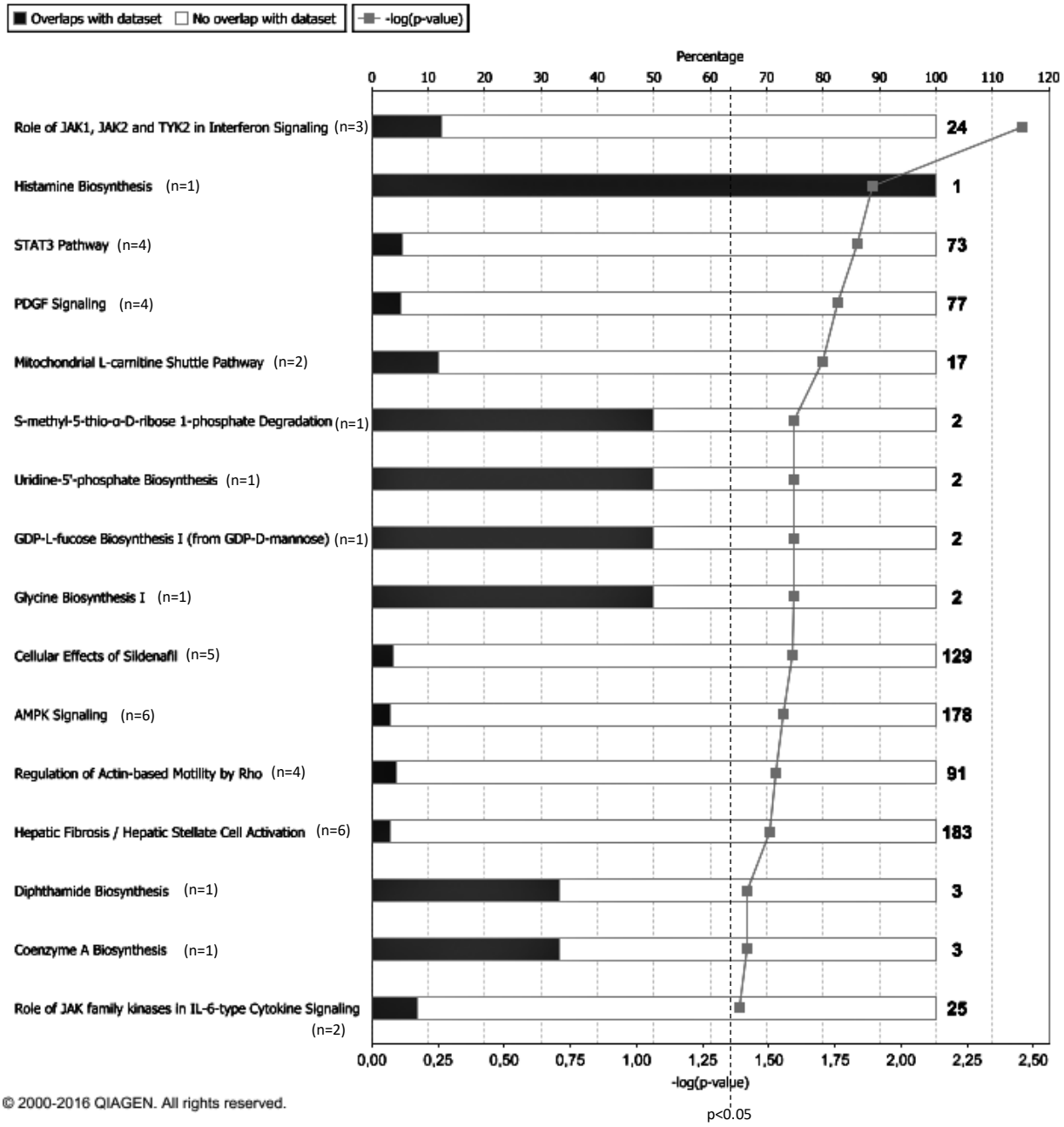

**Figure 3**

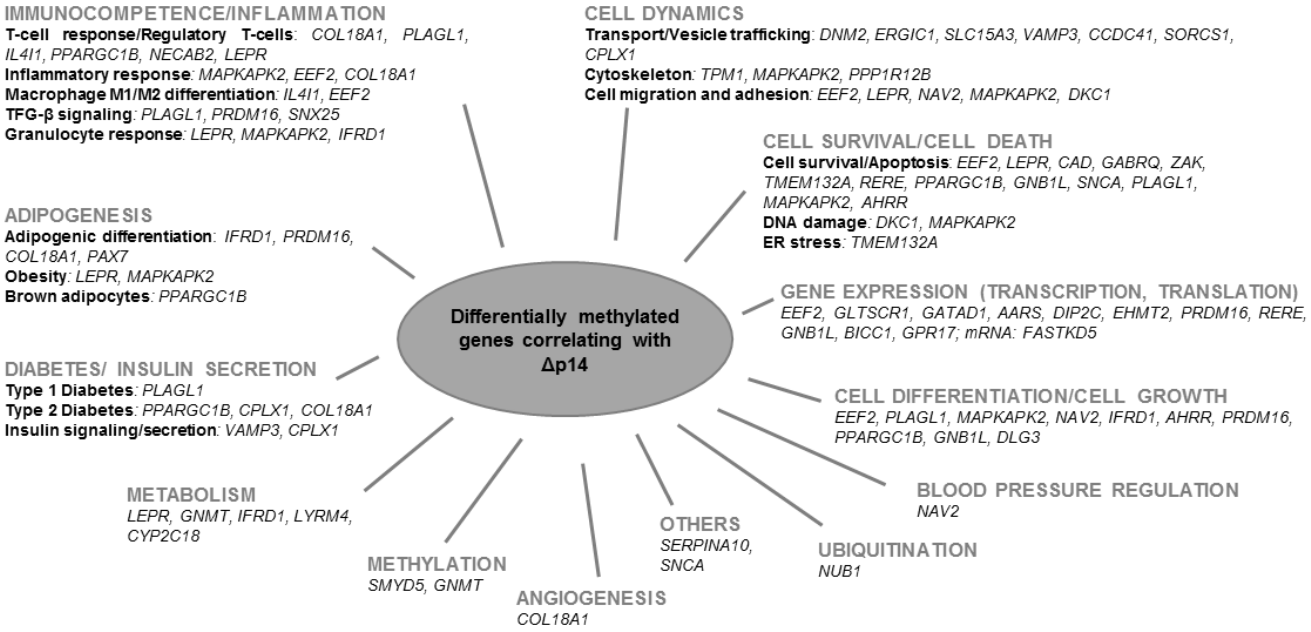


**Figure 4**

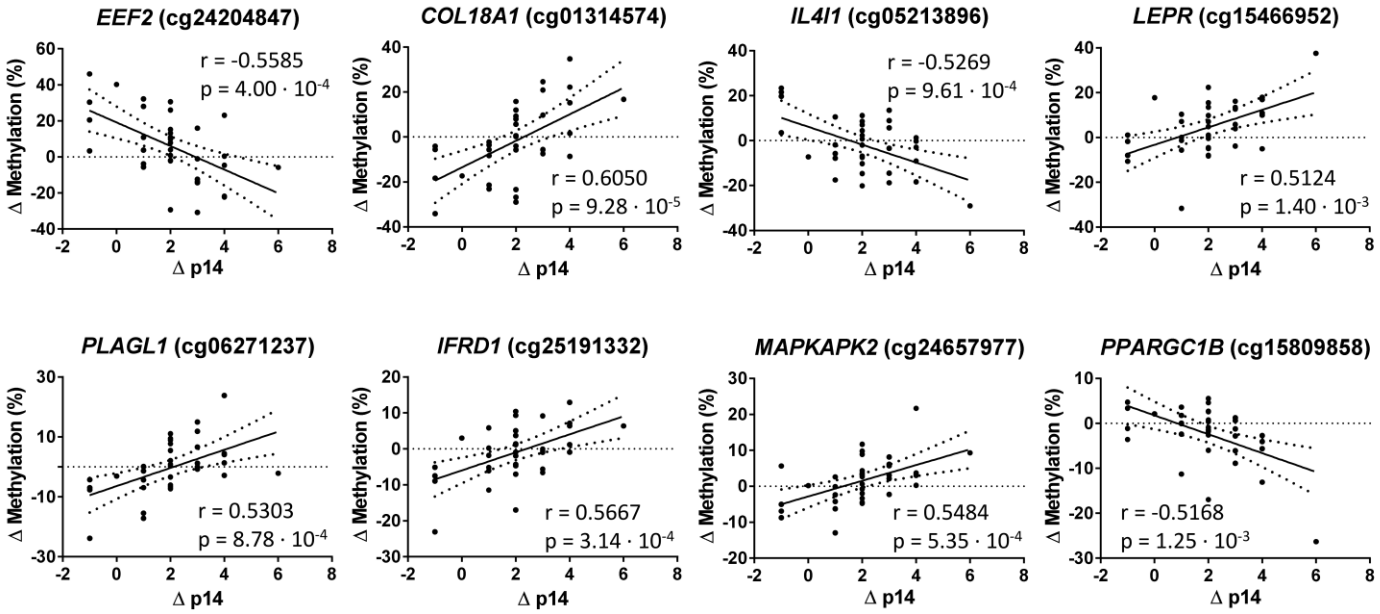


**Figure 5**

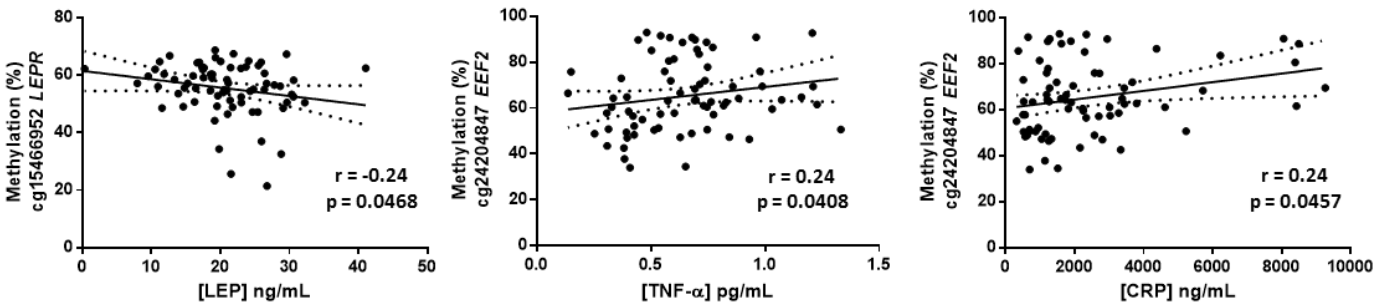

**Table 1: Characteristics of the study population and differences between the three dietary groups after five years of follow-up.**

**Figure 1: p14 values and inflammatory biomarkers after five years follow-up. A) p14 values at baseline and five years of each dietary group**. Values represent Mean ± SD. Symbols represent *** p<0.001 (Student T test, 5 years vs. Baseline); ## p<0.01, ### p<0.001 (ANOVA + Tukey's test, respect to control). **B) Protein concentration variation depending on p14 at five years.** p14 at five years is divided by the median in two groups, Q1 and Q2. Lines in the distribution represent mean and SD. One-tailed p-values were calculated by Student T-test or Mann-Whitney U test, when appropriate. p<0.05 was considered significant. CRP: C-reactive protein; MedDiet: Mediterranean diet; EVOO: extra-virgin olive oil; sICAM-1: soluble intercellular cell adhesion molecule 1; TNF-α: Tumor necrosis factor α; VCAM-1: vascular cell adhesion molecule 1.

**Figure 2. Canonical pathways associated with differentially methylated CpGs that correlate with Δp14 (Ingenuity Pathway Analysis).** The graph presents the canonical pathways ordered by –log(p-value) and the percentage of genes from our list (316 CpGs, in black) that are in one specific pathway (total number of genes in the right part of the graphs). In grey is represented the –log(p-value), indicating that all of them are significant (p<0.05).

**Figure 3. Manual curation of top 50 genes.** The first 50 genes corresponding to the CpGs with higher standard deviation of the total CpGs that were differentially methylated and correlated with p14 changes (Δp14) are classified by biological functions after a bibliographic research.

**Figure 4: Correlation graphs (Pearson) of selected genes representing the association between differences in methylation (Δ Methylation) and differences in adherence to MedDiet (Δ p14).** Dot lines on both sides of the solid line (linear regression for correlation) represent 95% confidence band. *COL18A1*: Collagen Type XVIII Alpha 1; *EEF2*: Eukaryotic Elongation Factor 2; *IFRD1*: Interferon-related developmental regulator 1; *IL4I1*: Interleukin 4-induced gene-1; *LEPR:* Leptin receptor*, MAPKAPK2:* Mitogen-activated protein kinase (MAPK)–activated protein kinase 2; *PLAGL1*: Pleiomorphic adenoma gene-like 1; *PPARGC1B*: Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Beta.

**Figure 5: Statistically significant correlation graphs (Spearman) representing the association between methylation of a selected CpG with the protein concentration obtained by ELISA.** Dot lines on both sides of the solid line (linear regression for correlation) represent 95% confidence band. CRP: C-reactive protein; *EEF2*: Eukaryotic Elongation Factor 2; LEP: Leptin; *LEPR*: Leptin receptor; TNF-α: Tumor necrosis factor α.