Genes and miRNA expression signatures in peripheral blood mononuclear cells in healthy subjects and patients with metabolic syndrome after acute intake of extra virgin olive oil

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Extra virgin olive oil (EVOO) consumption has been associated with reduced cardiovascular risk but molecular mechanisms underlying its beneficial effects are not fully understood. Here we aim to identify genes and miRNAs expression changes mediated by acute high- and low-polyphenols EVOO intake. Pre- and post-challenge gene and miRNAs expression analysis was performed on the peripheral blood mononuclear cells (PBMCs) of 12 healthy subjects and 12 patients with metabolic syndrome (MS) by using microarray and RT-qPCR. In healthy subjects, acute intake of EVOO rich in polyphenols was able to ameliorate glycaemia and insulin sensitivity, and to modulate the transcription of genes and miRNAs involved in metabolism, inflammation and cancer, switching PBMCs to a less deleterious inflammatory phenotype; weaker effects were observed in patients with MS as well as in healthy subjects following low-polyphenol EVOO challenge. Concluding, our study shows that acute high-polyphenols EVOO intake is able to modify the transcriptome of PBMCs through the modulation of different pathways associated with the pathophysiology of cardio-metabolic disease and cancer. These beneficial effects are maximized in healthy subjects, and by the use of EVOO cultivars rich in polyphenols. Nutrigenomic changes induced by EVOO thus legitimize the well-known beneficial effects of EVOO in promoting human health and, potentially, preventing the onset of cardiovascular disease and cancer.

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1. Introduction

Adherence to Mediterranean diet (MeD) has been shown to significantly protect against cardiovascular and cancer risks [1]. One of the main components of the Mediterranean diet is extra virgin olive oil (EVOO), which is also considered a functional food. EVOO contains high levels of monounsaturated fatty acids (MUFA; oleic acid: 70–80%), and a number of biologically active minor components (e.g. phenolic compounds) playing a role in health promotion [2–4]. Several studies have shown that olive oil intake is effective in lowering blood pressure in hypertensive patients, reducing lipid and DNA oxidation, ameliorating lipid profile and insulin-resistance, inflammation, and endothelial dysfunction, thus leading to protection against cardiovascular disease (CVD) [3–5]. EVOO has also shown a favorable effect on tumorigenesis and cancer progression due to its ability to modify cell membrane composition, signal transduction pathways, transcription factors, and oncogenes [6]. Few studies have been conducted in humans to elucidate the nutrigenomics responses of inflammatory cells to the...
acute intake of super-physiological doses of EVOO; these studies were focused mainly on mRNA changes thereby the adaptive modulation of the overall transcriptome (genome and miRNome) in response to EVOO is lacking so far [7]. Moreover, it is still unresolved whether beneficial changes in modulation of genes involved in proliferative, antioxidant, and inflammatory pathways are mediated by oleic acid or executed by minor components.

MicroRNAs (miRNAs) are small 19–23 nucleotide RNA molecules that act as regulators of RNA degradation and/or blocking protein translation of specific sets of target proteins [8], thus acting as master transcriptional regulators of genetic networks, cellular, and physiological processes. As it occurs for genes, miRNA regulation is tightly modulated, and miRNA dysregulation has been documented in models of disease (e.g. metabolic syndrome, MS; atherosclerosis; cancer) [9]. Additionally, miRNA have been proposed to be secreted by certain cells (e.g. macrophages and platelets) exerting regulatory effects on other cell types in an “endocrine” or “paracrine” fashion [10]. However, this is just the sunrise of a proper understanding of all the functions regulated by these small RNA sequences as well as for their use as effective targets for treatment.

Here we performed a study aimed to characterize the effects of EVOO on the miRNome and transcriptome of circulating inflammatory cells (peripheral blood mononuclear cells, PBMCs) that could underlie the molecular mechanisms exerted by EVOO in the prevention of the cardio-metabolic risk. PBMCs have been recognized as carriers of systemic signals, and the adaptive changes of their transcriptome have been proposed either as biomarkers of a wide range of pathological conditions (e.g. inflammatory disease, MS, and cancer) or tools to investigate response to medication, and nutritional changes [11,12].

We aimed to identify genes and miRNAs expression changes mediat-ed by acute EVOO intake expression patterns in the PBMCs of healthy subjects, and patients with MS. Our results show that acute intake of EVOO rich in polyphenols is able to ameliorate glycemia and insulin sensitivity, and to promote the transcription of genes and miRNAs involved in the anti-inflammatory, and anti-cancer responses as well as in the energy homeostasis. Interestingly, these effects are partially lost in patients with MS as well as by the administration of low-polyphenols EVOO thus pointing to the importance of the phenolic fraction and to the health status of the organism that receives EVOO in order to achieve the best transcriptional response of PBMCs.

2. Methods

2.1. Chemical profile of extra virgin olive oil

The two EVOOs selected for the present study were both produced in Apulia, and similar in fatty acid composition (Supplementary Table 1); differing mainly for the content in polyphenols (the “Coratina” cultivar had higher content in polyphenols when compared to the “Peranzana” cultivar). We used the same stocks of EVOO to treat all the participants included in each treatment arm.

2.2. Study population

Patient recruitment was performed in the Clinic of Nutrition (Head: Prof. Antonio Moschetta) of the Clinica Medica “Augusto Murni”, Aldo Moro University of Bari, Italy (Director: Prof. Giuseppe Palasciano). Twelve healthy subjects (6M:6F; mean age 29 ± 2 yrs.), and 12 patients at the first diagnosis of MS (6M:6F; mean age 35 ± 3 yrs.) were recruited for this study (baseline characteristics are shown in Supplementary Table 2). The diagnosis of MS was assessed in accordance to the Adult Treatment Panel III (presence of three or more criteria) [13]. The presence of MS complications (CVD, cerebrovascular diseases) as well as of other diseases were the exclusion criteria. None of the subjects was a smoker and/or underwent pharmacological therapy (except for anti-hypertensives drugs in MS patients). Subjects with a daily consumption of alcohol over 25 g/day, and intake of anti-oxidant supplements during the last 2 months prior to study enrollment were excluded. In all subjects, background information was collected, and physical examination was performed. Cardiovascular risk was assessed using the scoring system of the Progetto Cuore [14]. The study protocol was approved by the Ethical Committee of the Azienda Ospedaliero-Universitaria Policlinico di Bari, Italy.

2.3. Study design, dietary control and virgin olive oil administration

Prior to both dietary interventions (ingestion of high-polyphenols and low-polyphenols EVOO), patients followed a 1-week washout period in which no olive oil consumption was allowed. During the first 4 days subjects were asked to control anti-oxidant intake, while during the 3 days prior to the intervention they followed a strict low-phenolic compound diet. Female volunteers were scheduled to start the intervention at the beginning of their follicular phases.

After 12 h fasting, subjects underwent EVOO intake [50 mL (44 g)] at 8 am in a single dose. All the subjects consumed the total amount of EVOO administered. During the following 4 h, subjects abstained from food and drinks (with the exception of water that was allowed in small quantities), and were instructed not to perform physical exercise (Supplementary Fig. 1).

2.4. Sample collection, biochemical measurements, and PBMC isolation

After overnight fasting and 4 h after EVOO administration, samples for serum biochemistry and whole blood (18 mL) for PBMC isolation were collected. PBMCs were isolated immediately after collection of blood using a standard, previously validated protocol [11]. Cells were then stored at −80 °C until RNA extraction.

2.5. RNA sample preparation

RNA was extracted from the PBMCs or cell subpopulations pellet using QIAzol® Lysis Reagent (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. To avoid possible DNA contamination, the RNA was treated with DNAase-1 (Ambion, Foster City, CA). RNA purity (A260/A280 > 1.75), and the concentration was checked by spectrophotometer, while the RNA integrity was assessed by Bio-Rad ExperionTM (Bio-Rad, Hercules, CA). Only samples with Relative Quality Index (RQI) > 8 were used for microarray analysis while samples with RQI > 6.5 were used for quantitative RTqPCR. Samples were stored in aliquot at −80 °C prior to use.

2.6. Microarray analysis of gene and miRNA expression profiles

Microarray gene and miRNAs expression analysis was conducted on RNA extracted from PBMCs. For GE expression: whole RNA (400 ng) was used for cRNA synthesis using the Illumina Total Prep RNA Amplification kit (Ambion, Austin, TX, US) following the manufacturer’s instructions, and randomly hybridized to the array. Genome-wide expression profile analysis of PBMCs was performed using the Illumina whole genome direct hybridization assay (HumanHT-12 v4 Expression BeadChip Kit). All the bead chips (GE) were read on the same microarray platform at one time (Illumina iScan System, San Diego, CA, USA). For miRNA expression: whole RNA (400 ng) was studied using the Illumina Universal MicroRNA Expression Profiling (Human V2 MicroRNA Expression profiling kit & Illumina Universal Bead Chips). All the bead chips (GE/miR) were read on the same microarray platform (Illumina iScan System, San Diego, CA, USA). Upon the manufacturer instructions, data were processed using the Illumina Genome Studio Software through specific algorithms of filtration and cleaning of the signal. All the items with detection p value >0.001 were excluded. Data from PBMCs were normalized together with the quantile method [15]. Background was subtracted. Final output consisted of normalized
fluorescence intensity of each probe (AVG signal), representing the expression levels of each gene. This initial screening allowed us to identify 8708 genes to be exported from Genome Studio Software on which, after transformation log2-transformed data, we thus performed the statistical analysis. The identification of up- or down-regulated genes by high-polyphenols EVOO ingestion was performed by comparing gene expression in PBMCs at baseline and after 4 h of EVOO intake, in both healthy subjects and MS patients.

2.7. Reverse-transcription and quantitative RTqPCR

For genes, according to the manufacturer’s instructions, 190 ng of RNA were reverse-transcribed in a volume of 20 μL using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Real-time quantification was done using previously-validated TaqMan® Assays (Applied Biosystems, Foster City, CA), according to manufacturer instructions. Quantitative normalization of cDNA in each sample was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cyclophilin as internal controls. For miRNAs: quantification of miRNA was done using TaqMan miRNA Assays (Applied Biosystems). Briefly, 10 ng of RNA were reverse-transcribed and real-time quantification was done using previously-validated TaqMan® Assays (Applied Biosystems, Foster City, CA), according to the manufacturer instructions. Quantitative normalization was performed using U6snRNA and RNU48 as internal controls.

For all experiments the PCR assays were performed in 96 well optical reaction plates using the ABI 7500HT system (Applied Biosystems). Briefer, 10 ng of RNA were reverse-transcribed and real-time quantification was done using previously-validated TaqMan® Assays (Applied Biosystems, Foster City, CA), according to the manufacturer instructions. Quantitative normalization was performed using U6snRNA and RNU48 as internal controls.

For all experiments the PCR assays were performed in 96 well optical reaction plates using the ABI 7500HT system (Applied Biosystems, Foster City, CA). All reactions were run in triplicate. Relative quantification was performed using the ΔΔCT method.

2.8. Statistical analysis

The datasets underwent a restrictive screening for excluding the presence of outliers, before starting the analyses. In order to minimize the effect of variation caused by non-biological factors, all the genetic (GE) values were normalized directly in illumina Genome Studio Software with the quantile method, and then were analyzed with classical statistical approaches to evaluate differences among groups and correlations between clinical and prognostic variables and levels of expression of genes. In particular, for continuous variables the difference among paired samples was assessed with Wilcoxon signed-rank test on the Log2 normalized data. Comparison of the effects of EVOO among the two study populations (CTRL vs. MS) was performed by using Mann-Whitney test on fold changes. The raw p-values were then adjusted by the Benjamini-Hochberg procedure to control the false discovery rate (FDR), the expected proportion of incorrectly rejected null hypotheses (“false discoveries”) [16,17]. Data are shown as means ± SD (Supplementary Table 3A). All the analyses were performed using SAS (Release 9.2, 2008) and R (version 2.12.2). These datasets are publically available on GEO (Ref: GSE75027; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75027).

2.9. Bio-informatics functional analyses

After statistical analysis, differentially expressed genes within groups were studied using the Core Analysis Function of Ingenuity Pathway Analysis (Ingenuity System Inc., USA). IPA (Ingenuity System, USA, www.ingenuity.com) was used to determine the functional annotation and the significant Biological Functions related to the differentially expressed genes, and to compare the pathway enrichment among groups [18]. Networks showing relationships and interactions between differentially expressed genes and others that functionally interact with them, were generated and ranked in terms of significance of genes participating to the pathway (p < 0.01). Only genes significant at Mann-Whitney (or FDR), and gene-class testing (pathway analysis) were considered biologically relevant [17], and selected for further qPCR confirmations. To integrate mRNA and miRNA expression data, we analyzed putative miRNA:mRNA pairs using another feature of IPA (that interrogates Target Scan Human database) regarding their fold change, where the expression of the miRNA is the opposite of its mRNA target (i.e. miRNA down-regulated vs. mRNA up-regulated or genes up-regulated vs. miRNA down-regulated; see Supplementary Table 4).

3. Results

3.1. Metabolic effects of EVOO in healthy subjects and patients with MS

We recruited 12 healthy controls who underwent oral administration of a single dose of 50 mL of high-polyphenols and low-polyphenols EVOO preceded by a 7-days washout period. The laboratory findings before and after high- and low-polyphenols EVOOs intake are summarized in Table 1. Four hours after high-polyphenols EVOO administration, we found a decrease in serum glucose, insulin and
HOMA-IR (Table 1). No significant differences were observed in the other assessed metabolic variables (serum total cholesterol, LDL-c, HDL-c, and TGs). On the other hand, low-phenols EVOO did not induce significant changes in serum biochemistry in the same subjects (Table 1). In order to check if the metabolic effects of high-polyphenols EVOO are maintained also in metabolic disease, we recruited 12 patients with MS undergoing the same dietary intervention. The effects on glucose metabolism induced by high-polyphenols EVOO were not confirmed in patients with MS (Table 2).

3.2. Gene expression profiling in PBMCs after high-polyphenols EVOO intake and validation by RTqPCR

In order to study the effects induced by high-polyphenols EVOO on PBMCs at transcriptional level, we performed gene expression microarrays in both healthy controls and patients with MS. At the paired analysis (T0, baseline values, vs. T1, 4 h after EVOO administration), high-polyphenols EVOO induced the modulation of 2438 annotated genes (1376 up-regulated and 1062 down-regulated - out of total 2447 differentially modulated: p < 0.05) in the PBMCs of healthy subjects, and of 954 annotated genes (403 up-regulated and 551 down-regulated - out of total 963 differentially modulated: p < 0.05) in the PBMCs of patients with MS. Of these hits, 389 annotated genes (195 over-expressed and 194 under-expressed) were consistently changed in both groups (CTRL and MS). The complete list of the genes differentially modulated either in the CTRL group or in the MS group is shown in Supplementary Table 3A; these results are summarized in Figs. 1A and 2A.

In order to provide a framework for interpretation of our results, we functionally clustered significant (p < 0.01) biological pathways using the Core Function of the IPA Software [19] (Figs. 1B and 2B, and details in Supplementary Table 3B and 3C for pathways up-regulated and down-regulated, respectively). High-polyphenols EVOO intake induced dramatic changes in the mRNA abundance of a number of genes involved in the modulation of PBMCs functions. The pathway enrichment (Figs. 1B and 2B) highlights the modulation of the immune responses (e.g. up-regulation of CD28 Signaling in T Helper Cells, and Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes; suppression of the NFAT in the regulation of the immune response and of PI3K Signaling in B Lymphocytes), and of different inflammatory signals (suppression of both B Cell and T Cell Receptors, NF-κB, IL1/3/8, RANK and thrombin signaling cascades as well as of the LPS-stimulated MAPK activation, and of the NRF2-mediated Oxidative Stress Responses), a negative modulation of cell proliferation and cancer (up-regulation of the DNA Damage Response systems, suppression of different pathways involved in cell and cancer proliferation such as ERK/MAPK, CXCR4, HGF/EGF, HIF1α signaling cascades). Also pathways involved in metabolism and cardio-metabolic risk appear to be modulated by high-polyphenols EVOO intake, as shown by predicted suppression of Protein Kinase A, PI3K/Akt signaling, CREB, mTOR, cholesterol biosynthesis, as well as of mechanisms involved in Cardiac Hypertrophy, Adipogenesis, and Circadian Rhythms; on the other hand glucose metabolism (both gluconeogenesis and glycolysis as well as the biosynthesis of Acetyl-CoA by the Pyruvate Dehydrogenase Complex), and pathways involved in the metabolism of different amino acids (Aspartate, Cysteine, Isoleucine, Leucine, Methionine, Valine, Tryptophan) appeared to be up-regulated. Also a set of transcripts coding for proteins participating to the transcriptional machinery of different nuclear receptors appeared to be modulated by high-polyphenols EVOO (e.g. both androgen and estrogen receptors, glucocorticoid receptor, and retinoid acid receptors). Intriguingly, the fatty acid sensor peroxisome proliferator-activated receptor alpha (PPARα), and its coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α or PPARGC1A), although not modulated at mRNA level, are predicted as activated both by the pathway analysis and by IPA “upstream regulator” prediction tool (that predicts the status of activation of a specific modulator on the basis of the differential modulation of its known targets; Supplementary Fig. 2); this is also paralleled by an up-regulation of the transcripts encoding the retinoid X receptors (RXRα and RXRβ, see Supplementary Table 3A) forming heterodimers with PPARs. Interestingly, we previously described that PPARs and RXRα are suppressed in the PBMCs of patients with MS [11]; this could explain, at least in part, the lack of reactivity to EVOO challenge in the PBMCs of MS patients. In fact, PBMCs of patients with MS showed a reduced number of genes significantly modulated by high-polyphenols EVOO (Figs. 1A and 2A) and reduced (vs. CTRL) changes in the genes being significantly regulated by EVOO (Supplementary Table 3A); as a consequence of these weaker changes, also most of the pathways differentially modulated in the CTRL group were much less enriched in the MS patients.

Overall, we depicted here the gene expression adaptive changes induced by an acute oral administration of phenol-rich EVOO in PBMC. This modulation of the transcriptome is more evident in healthy subjects and parallels the beneficial effects in glucose metabolism observed in their biochemistry following acute EVOO intake.

3.3. RTqPCR confirmation of the gene expression changes induced by high polyphenols EVOO in PBMCs of healthy subjects and patients with MS

In order to confirm by RTqPCR the most important hits modulated by high-polyphenols EVOO, we considered “biologically relevant” only those genes “statistically significant” (p < 0.05, fold change > 1.3) and enriched in “significantly modulated” (p < 0.01) pathways; we then selected hits for confirmation by RTqPCR on the basis of their biological relevance (Fig. 3). We confirmed the up-regulation of: A) acyl-CoA dehydrogenase, C-4 to C-12 straight chain (ACADM) transcript (acyl-CoA dehydrogenase which catalyzes the first reaction of fatty acid β-oxidation [20]); B) and of acetyl-CoA acetyltransferase 1 (ACAT1); involved in the glaryl-CoA degradation pathway, and in the maintenance of lipid homeostasis promoting cholesterol esterification [21]; C) retinol X receptor beta (RXRβ); RXRs mediate the function of a large number of other nuclear hormone receptors and are involved in different metabolic pathways, and exert anti-inflammatory, and anti-cancer actions [22]; D) heat shock 70 kDa protein 1A (HSP1A) plays an essential role in the energy production, folding/unfolding of proteins, cell-cycle control and signaling, and the protection of cells against stress/apoptosis [23]); E) peroxiredoxin 3 (PRDX3) plays an important role in the antioxidant defense system, and homeostasis within mitochondria [24]. On the other hand, we confirmed the suppression of: F) sterol-C5-desaturase (SCDS) participates to cholesterol biosynthesis pathway, and cell proliferation networks [25]; G) interleukin-1 receptor–associated kinase 3 (IRAK3) is involved in the regulation of humoral immune response and in the NF-κB, and IL-8 signaling [26]; H) chemokine CXC motif chemokine receptor (CXCR4) is a chemokine receptor implicated in the regulation of lymphocyte migration, autoimmunity, and cancer progression [27]); I) cyclin K (CCNK) cell cycle, and division [28]); J) hypoxia inducible factor 1, alpha subunit (HIF1A is the master modulator of the cellular responses to hypoxia, but is also involved in inflammation, and cancer cell survival [29]); K) cryptochrome 2 photolyase-like (CRY2 acts as a negative feedback loop component of the circadian clock [30]); L) argonaute RISC catalytic component 2 (AGO2 is involved in mRNA biogenesis thus being a
GENES DOWN-REGULATED BY HIGH-POLYPHENOLS EVOO

A

CTRL

MS

868

194

357

PATHWAYS DOWN-REGULATED BY HIGH-POLYPHENOLS EVOO

B

Protein Kinase A Signaling
Molecular Mechanisms of Cancer
Glucocorticoid Receptor Signaling
Huntington’s Disease Signaling
NRF2-mediated Oxidative Stress Response
Protein Ubiquitination Pathway
RAR Activation
Cardiac Hypertrophy Signaling
ERK/MAPK Signaling
NF-kB Signaling
CREB Signaling in Neurons
PPAR/GXRX Activation (Predicted activation)
Androgen Signaling
p53 Signaling
Breast Cancer Regulation by Statins
IL-8 Signaling
Superpathway of Inositol Phosphate Compounds
Thrombin Signaling
B Cell Receptor Signaling
Estrogen Receptor Signaling
Role of NFAT in Regulation of the Immune Response
CXCR4 Signaling
GNRH Signaling
HGF Signaling
Relaxin Signaling
P2Y Purinergic Receptor Signaling Pathway
Synaptic Long Term Potentiation
Renal Cell Carcinoma Signaling
Adipogenesis pathway
Gx12/13 Signaling
Corticotropin Releasing Hormone Signaling
T Cell Receptor Signaling
SAPK/JNK Signaling
Actin Cytoskeleton Signaling
HIF-1α Signaling
IL-1β Signaling
RANK Signaling in Osteoclasts
mTOR Signaling
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes
Prolactin Signaling
Neurotrophins/Trk Signaling
LPS-stimulated MAPK Signaling
IL-3 Signaling
PI3K/AKT Signaling
Pyridoxal 5′-phosphate Salvage Pathway
EGF Signaling
Thrombospondin Signaling
Unfolded protein response
Circadian Rhythm Signaling
Superpathway of Cholesterol Biosynthesis
Autophagy
Polyamine Regulation in Colon Cancer
Cholesterol Biosynthesis III (via Desmosterol)
Cholesterol Biosynthesis II (via 24,25-dihydroxycholesterol)
Cholesterol Biosynthesis I
Chondroitin and Dermatan Biosynthesis
Dihydrorochoic acid Biosynthesis
D-myo-inositol (1,3,4,5)-trisphosphate Biosynthesis
1D-myo-inositol Hexakisphosphate Biosynthesis II (Mammalian)
D-myo-inositol (1,4,5)-trisphosphate Degradation
GDP-mannose Biosynthesis
Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism

Number of genes

MS
CTRL
central modulator of RNA post-transcriptional modification, and protein synthesis [31]). As shown by microarray analysis, most of the changes induced by high-polyphenols EVOO in healthy subjects were less pronounced or absent in the PBMCs of patients with MS.

In order to investigate whether the modulation on genes involved in proliferative, anti-oxidant, and inflammatory pathways observed in the PBMCs of healthy subjects were mediated by the minor components (polyphenols) of EVOO or by its major components (mono- and poly-unsaturated fatty acids), we compared changes in the expression of the selected candidate genes after acute intake of phenol-rich EVOO to phenol-poor EVOO that did not show any evident metabolic benefit. Most of the genes previously validated by RTqPCR (and discussed above) were not changed after low-polyphenol EVOO challenge (data not shown); on the other hand, in line with the results obtained following high-polyphenol EVOO, a significant increase of HSPA1A and RXRβ, and a decrease of CCR4 were observed (Supplementary Fig. 3), thus pointing to this genes as potentially modulated by fatty acids rather than other components of EVOO.

Overall, we confirmed here that EVOO is able to induce changes in the PBMC transcriptome in key genes for lipid metabolism, inflammation, proliferation, and cancer. The effects are maximized in healthy subjects (when compared to patients with MS), and by the use of EVOO cultivars rich in polyphenols.

3.4. miRNAs expression profiling in PBMCs after EVOO intake and validation by RTqPCR

Since one of the genes modulated by EVOO is AGO2, which is involved in miRNA processing, we also studied miRNA expression profiles using microarrays (containing 1,146 oligonucleotide probes complementatory to mature forms of miRNAs and annotated on miRBase). Of the 14 miRNA (6 up-regulated and 8 down-regulated) potentially modulated according to the miRNA arrays (Fig. 4A), only 8 miRNAs were expressed above the sensitivity limit of the TaqMan miRNA assays (RTqPCR, Fig. 4B–I). In agreement with AGO2 suppression, most of the miRNA confirmed were suppressed in our database. In details, we confirmed the suppression of: 1) miR-146b-5p (over-expressed in human atherosclerotic plaques [32], and up-regulated in human monocytes/macrophage stimulated with oxidized-LDL [33]); 2) the oncogenic miR-19a-3p; 3) miR-181b-5p (involved in the modulation of inflammation and cancer [35]); 4) miR-107 (known to be suppressed by fatty acids, and associated to obesity, insulin resistance and fatty liver [36]); 5) miR-769-5p; and miR-192-5p (that has been associated to the impairment in glucose metabolism, fatty liver, and acute myocardial infarction [37,38]). Among up-regulated miRNAs, we were able to confirm the over-expression of only two miRNAs, namely the anti-inflammatory miR-23b-3p and the tumor-suppressor miR-519b-3p (Fig. 4H and 4I). The other miRNAs had expression levels below the sensitivity limit of the TaqMan probes. Intriguingly, as we observed for gene expression, and in line with the lack of modulation of AGO2, most of these miRNAs (with the exception of miR-19a-3p) were not significantly modulated in the PBMCs of patients with MS.

In conclusion, our results point to EVOO-induced changes in the miRNome which actually support the modulation of EVOO-associated modulation of AGO2, and further support the idea that acute high-polyphenols EVOO intake may exert transcriptional effects potentially beneficial on insulin resistance (i.e. miR-107), inflammation (i.e. miR-181b-5p, miR-23b-3p), and cancer (miR-19a-3p, miR-519b-3p). Intriguingly, most of these beneficial effects of EVOO were not confirmed in MS patients undergoing the same treatment. Different possible explanations could justify these findings, including the lack of a metabolic adaptation in insulin signaling following EVOO in this cohort or of responsiveness of these cells at gene level (e.g. the absence of modulation of AGO2 that is involved in miRNA processing); more research is needed to further address these hypotheses.

4. Discussion

Nutritional interventions are considered efficient approaches to prevent the onset of a wide range of chronic diseases including type 2 diabetes, obesity, and MS, due to their potential to ameliorate plasma glucose and lipids, as well as blood pressure, thus reducing the risk of CVD [1]. Considering the epidemic of obesity and MS, and the fact that MeD has been proven to be beneficial for cardio-metabolic risk, efforts should thus be made to better understand the relative role of each component of MeD to these benefits. Several cross-sectional, epidemiological studies, randomized intervention trials on human or research in animals have pointed to a candidate beneficial role of EVOO in metabolism, and inflammation [2–5]. Moving from these evidences, we used an integrative approach bridging metabolic characterization of healthy subjects and MS patients with the study of adaptive changes in the genome and miRNome following acute EVOO intake to dissect the molecular mechanisms underlying the beneficial effects of EVOO suggested by previous epidemiological studies. The application of gene/miRNA expression analysis in studying human physiology and disease is relatively new and rapidly evolving. Different gene expression studies in PBMCs clearly show that these cells are suitable not only for providing the molecular signatures of disease but also to identify tools for predicting responsiveness of patients to treatments [12]. Our results confirm this hypothesis. We were able to prove that a single dose of EVOO is able to transiently ameliorate insulin sensitivity in healthy subjects. This was paralleled by transcriptomic changes both at mRNA and miRNA expression level in the PBMCs; on the other hand, EVOO did not show benefits in the biochemistry of patients with MS, and this was parallelled also by weaker transcriptomic changes. Part of these effects may be attributed to the “healthy” fatty acids (MUFAs/PUFAs) abundant in EVOO, and part can be due to the minor contents of EVOO.

It is not surprising that different pathways involved in lipid and glucose metabolism and cardio-metabolic risk are modulated by EVOO. We found of particular interest the modulation of the transcriptional machinery of different nuclear receptors (i.e. glucocorticoid, androgen and estrogen receptors; retinoid acid receptors; PPARs); these transcription factors act as master transcriptional regulators of different cell functions (e.g. metabolism, inflammation, cell proliferation, differentiation and apoptosis, circadian rhythm, etc.). We found particularly intriguing the predicted activation of the fatty acid sensor PPARα, and of its coactivator PGC-1α; this is paralleled by the up-regulation of RXXRs transcripts (both α and β) that form obligate heterodimers with PPARs. The fatty acids contained in EVOO are known to activate PPARs [42], and different polyphenols have been shown to be able to activate the AMPK/SIRT1/PGC1 cascade [43] thus the activation of PPARα/PGC1α is consistent with EVOO challenge. PPARα/RXR heterodimers exert different effects in the cells, promoting the transcription of genes involved in fatty acid oxidation & mitochondrial function (in our dataset we observe the up-regulation of ACO1, and ACADM), gluconeogenesis and glycolysis (both promoted in our dataset), and suppressing inflammation [44,45]. Interestingly, we previously described a suppression of both PPARα and RXRα in patients with MS [11] and this could explain, at least in part, the lack of transcriptional responsiveness to EVOO of the PBMCs isolated from MS patients.

Fig. 2. Pathway enrichment analysis of genes differentially modulated by high-polyphenols EVOO in the PBMCs of healthy subjects and MS patients. (A) Of the 1225 genes down-regulated by high-polyphenols intake (TD, baseline values vs. T1, 4 h after EVOO intake), 868 were down-regulated in healthy subjects, 357 were down-regulated in MS patients while 194 genes showed a common pattern of expression in both groups. (B) Pathways significantly enriched (p < 0.01) in the PBMCs of healthy subjects and MS patients after high-polyphenols EVOO intake. Each box represents the number of genes of the associated pathway after the nutritional intervention that are either down-regulated in healthy subjects (white), or in MS patients (black). Detailed results are provided in Supplementary Table 3C.
The most important transcriptional changes induced by high-polyphenols EVOO were documented in the activation status of pathways involved in PBMCs functions, including the modulation of immune responses and the suppression of pro-inflammatory signals. The anti-inflammatory effects of EVOO are well documented [4,46], and our results support these evidences due to the modulation of a number of pathways and genes involved in the inflammatory response. Indeed, we observed the up-regulation of Fcγ receptor-mediated phagocytosis in macrophages and monocytes pathway (which is essential for the immune response), and the suppression of several genes involved in the regulation of inflammatory response such as IRAK3, which is involved in the regulation of humoral immune response and in NF-κB and IL-8 signaling pathways (it also plays an important role in tumorigenesis through the modulation of the pro-inflammatory microenvironment promoting cancer [26]), and of CXCR4, a chemokine receptor implicated in the regulation of lymphocyte migration, autoimmunity, and cancer [27].

EVOO consumption has been also proposed to be protective for cancer [4]; in line with this, we observed an EVOO-induced modulation of genes involved in cell proliferation and survival, as well as in different key processes of tumorigenesis: up-regulation of the DNA Damage Response systems (protective for cancer), suppression of different pathways involved in cell proliferation (e.g. ERK/MAPK, CXCR4, HGF/EGF, HIF1α signaling cascades), the induction of HSPA1A, which plays an essential role in the energy production, folding/unfolding of proteins, cell-cycle control and signaling, and the protection of cells against stress/apoptosis [23]. Also p53 pathway (essential for cell growth regulation and apoptosis) appears to be modulated by EVOO; this modulation featured the suppression of CCNK mRNA (that controls cell cycle and division) [28], and of hypoxia inducible factor 1, alpha subunit (HIF1A) which is involved in various cellular functions, including hypoxia, inflammation, and cancer cell survival [29]. Although still present, the previously-mentioned effects of EVOO were partially lost in patients with MS suggesting an impairment of the adaptive response in the PBMCs of these subjects. This goes in parallel with the lack of metabolic (glucose metabolism) benefits of EVOO in the same cohort of MS patients, and needs to be further studied to understand the underlying molecular mechanisms.

Of note, EVOO also down-regulated AGO2, a key player in miRNA processing (pre-miRNA cleavage and mature miRNA release) [31] thus pointing to miRNA as possible indirect targets modulated by EVOO. The second part of our work was thus studying the adaptive changes at the level of the miRNome of PBMCs following acute EVOO intake. miRNAs have emerged as key regulators of physiological processes, including inflammation, proliferation, and glucose/lipid metabolism [9], and their dysregulation has been shown to contribute to multiple disease, thus making them attractive therapeutic targets. We have proven that acute EVOO intake induces different effects also at the level of the miRNome, including the promotion of miRNAs exerting anti-inflammatory (i.e. the
miR-23b-3p [39]), and tumor-suppressor (i.e. miR-519b-3p [40,41]) properties, and the suppression of miRNA promoting inflammation (i.e. miR-181b-5p), cancer (miR-19a-3p) and insulin resistance (i.e. miR-107). Interestingly, miR-107 has been already shown to be modulated by lipids in vitro (human enterocytes) by linoleic acid [36], and its over-expression has been associated with insulin resistance (miR-107 controls the post-transcriptional regulation of calveolin-1 [47], a key mediator of insulin pathway [47]) in obesity, metabolic disorders, and fatty liver; the modulation of miR-107 is thus of potential biologic interest in this cohort, since insulin sensitivity appears to be transiently ameliorated in healthy subjects following EVOO intake and, on the contrary, no changes in miR-107 are observed in MS subjects that have no changes in markers of insulin sensitivity. Nevertheless, the paralogues miR-107/miR-103 have also been involved in the regulation of innate immunity, inflammatory responses, and circadian clock, but the biological meaning of their function is yet to be properly understood [36,48–51]. Interestingly, in parallel of the lack of responsiveness of glucose metabolism and transcriptome (including AGO2), also miRNA showed a reduced responsiveness to EVOO in PBMCs extracted from patients with MS.

Overall, our message at a glance is that the intake of EVOO containing high phenols improves insulin sensitivity and modulates different pathways in inflammatory cells of healthy subjects; most of these changes point to a beneficial role of EVOO in promoting health toward its anti-inflammatory, anti-cancer, and anti-oxidant properties. This could be of particular relevance since circulating PBMCs are involved in immune functions and also participate in the pathophysiological events promoting atherosclerosis, and cancer. Our study thus provides new insights to decode the molecular mechanisms legitimating the beneficial effects of high phenols EVOO in promoting human health, and preventing onset of different disease.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Transparency document**

The Transparency document associated with this article can be found, in online version.

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