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Gene expression signature induced by grape intake in healthy subjects reveals wide-spread beneficial effects on peripheral blood mononuclear cells

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ABSTRACT

Using a transcriptomic approach, we performed a pilot study in healthy subjects to evaluate the changes in gene expression induced by grape consumption. Blood from twenty subjects was collected at baseline (T0), after 21 days of grape-rich diet (T1) and after one-month washout (T2). Gene expression profiling of peripheral blood mononuclear cells from six subjects identified 930 differentially expressed transcripts. Gene functional analysis revealed changes (at T1 and/or T2) suggestive of antithrombotic and anti-inflammatory effects, confirming and extending previous finding on the same subjects. Moreover, we observed several other favourable changes in the transcription of genes involved in crucial processes such as immune response, DNA and protein repair, autophagy and mitochondrial biogenesis. Finally, we detected significant changes in many long non-coding RNAs genes, whose regulatory functions are being increasingly appreciated. Altogether, our data suggest that a grape diet may exert its beneficial effects by targeting different strategic pathways.

1. Introduction

It is well known that fruit and vegetable consumption improve human health. However, the molecular mechanisms behind the protective effects of these foods are still not entirely elucidated. For this reason, *in vivo* studies on gene expression changes in response to supplementation with dietary compounds are increasing considerably (Corella, Coltell, Macian, & Ordovás, 2018; Pokimica & García-Conesa, 2018).

Grape (*Vitis vinifera* L.) is one of the most typical fruit of the Mediterranean diet with a high content of polyphenols. Compelling evidence suggests that grape and grape-derived products exert beneficial effects thanks to their antioxidant, anti-inflammatory, anti-cancer, antimicrobial, antiviral, cardioprotective, neuroprotective and hepatoprotective activities (Ali, Maltese, Choi, & Verpoorte, 2010; Dell'Agli et al., 2013; Giovinazzo & Grieco, 2015; Leifert & Abeywardena, 2008; Nassiri-Asl & Hosseinzadeh, 2009; Rasines-Perea & Teissedre, 2017; Xia, Deng, Guo, & Li, 2010). Concerning polyphenols, whose healthy properties are generally attributed to their well-recognised antioxidant activity, evidence is accumulating that they can influence the expression of many genes, including those involved in inflammation, cell signalling, metabolism, cell survival and proliferation (Goszcz, Duthie, Stewart, Leslie, & Megson, 2017; Nosrati, Bakovic, & Paliyath, 2017; van Breda & de Kok, 2018).

Several recent publications reported the influence of single nutrients or dietary patterns on gene expression profiles in peripheral blood mononuclear cells (PBMCs) [reviewed in Afman, Milenkovic, & Roche, 2014]. Interestingly, the biological pathways influenced by these food compounds are largely overlapping and include the chemokine signalling, cell adhesion, NF-κB signalling pathway and apoptosis. Altered gene expression has been demonstrated with quercetin (Boomgaarden et al., 2010), hesperidin (Milenkovic, Deval, Dubray, Mazur, & Morand, 2011), grape seed extract (Tomé-Carneiro et al., 2013), resveratrol (De Groote et al., 2012), isoflavones (Niculescu, Pop, Fischer, & Zeisel,

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2007; van der Velpen et al., 2013), olive oil (Camargo et al., 2010; D'Amore et al., 2016) and mixtures of antioxidant molecules composed by resveratrol, green tea extract, alpha tocopherol, vitamin C, n-3 PUFA and lycopene (Bakker et al., 2010). In these studies, the daily intake of single or multiple nutrients was in the range of hundreds mg/day.

Recent findings suggest that polyphenols also affect the expression of non-coding RNAs (ncRNAs), a large family of molecules able to fine-tuning gene expression at the epigenetic, transcriptional or post-transcriptional level (Fu, 2014; Milenkovic, Jude, & Morand, 2013; Salviano-Silva, Lobo-Alves, Almeida, Malheiros, & Petzl-Erler, 2018).

In previous studies, we showed that skin extracts of several table grapes reduce the prothrombotic properties of endotoxin-stimulated PBMCs (Carrieri et al., 2013; Milella et al., 2012). Based on these findings, we designed a pilot study in twenty healthy subjects to investigate the effect of grape consumption on (i) prothrombotic and inflammatory changes in blood cells and plasma, using functional and immunologic assays (biochemical study) and (ii) gene expression profiles of PBMC using a transcriptomic approach. The biochemical study showed significant anti-thrombotic and anti-inflammatory effects of grape diet (Ammollo et al., 2017). Here we report the results of the transcriptomic study, which was performed on six out of the 20 subjects enrolled. The data obtained show extensive changes in the gene expression profile of PBMC, which may provide clues to decipher the molecular mechanisms behind the beneficial effects of the grape diet.

2. Materials and methods

2.1. Table grape

The table grape used in our experiments was the black grape *Autumn royal*, a grape cultivar grown in the Apulian region at Council for Agricultural Research and Economics, Research Centre for Viticulture and Enology (CREA VE), Turi (BA), Italy. The *Autumn royal* was chosen for its ripening period and well-balanced antioxidant, micronutrient and phytochemical composition (Carrieri et al., 2013). During harvest time (September 2014), fresh grape was distributed weekly to the participants.

2.2. Study design

We performed a pilot study in twenty healthy volunteers (10 females and 10 males; aged 24–48 years). The study protocol was approved by the ethical committee of the University of Bari, and written informed consent was obtained from participants before starting the study. The details on sample size, exclusion criteria and general and laboratory characteristics of subjects were previously reported (Ammollo et al., 2017). Briefly, subjects were asked to intake, on top of their usual diet, 5 g of grape per kg body weight (approximatively 350 g) per day for 21 days, preferably at midmorning and mid-afternoon between meals, after which they had to follow a grape-free diet for 4 weeks (washout). Blood samples were taken before starting the grape diet (T0), at the end of the 21-day diet (T1) and after the 4-week wash-out period (T2).

2.3. Blood samples, RNA isolation and microarray analysis

Blood samples were collected from overnight fasting subjects before 9:00 am. PBMCs were isolated by using the Fycoll–Hypaque method, as described (Semeraro et al., 1983), and stored at −80 °C, until tested.

Total RNA was isolated from PBMCs using the PureLink® RNA Mini Kit, according to the manufacturer's instructions (Ambion by Life Technologies, Carlsbad, CA, USA); RNA concentration and purity were assessed spectrophotometrically.

Samples from six subjects (three males and three females), were used for microarray experiments. For hybridisation, washing and scanning we applied the Two-Color Microarray-Based Gene Expression Analysis (Version 6.7) protocol (Agilent Technologies, Santa Clara, CA, USA).

2.4. Statistical analysis

The array images were analysed using Agilent Feature Extraction software version 12.0 (Agilent Technologies, Santa Clara, CA, USA). Two-Color data were processed and analysed using the GeneSpring version 13.1.1 (Agilent Technologies, Santa Clara, CA, USA) as a Single-Color Experiment Type to have also the T2*vs*T0 comparison. Background subtraction (thresholding), summarisation (geometric mean), log base 2 transformation, normalisation (Percentile Shift) and baseline transformation of signal values were carried out.

Differences in gene expression were calculated by repeated measure ANOVA between T1*vs*T0, T2*vs*T0 e T2*vs*T1 and genes showing significant ($P < 0.05$) fold change ≥ 1.5 were considered differentially expressed (DE).

2.5. Bioinformatic functional analysis

To group genes with a high degree of similarity, we made a clustering analysis using the GeneSpring software. Both hierarchical and K-means clustering analyses were performed.

Up- and down-regulated genes at different timeframes (T1vsT0, T2vsT0 e T2vsT1) were also analysed using the Venn diagram tool available at BEG/Van de Peer Lab web site (bioinformatics.psb.ugent.be/webtools/Venn/).

For the functional interpretation of results, we used the Gene Group Functional Profiling (g:GOSt) and the Compact Compare of Annotations (g:Cocoa) tools available in the g:Profiler web server (update 2018-10-02) (Reimand et al., 2016). Protein-protein interaction networks were generated using the STRING database (Szklarczyk et al., 2017).

2.6. Quantitative real-time PCR

Reverse transcription of 0.5 μg of total RNA was performed using random primers and the SuperScript III Reverse Transcriptase kit (Invitrogen, San Diego, CA, USA), according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed on the stepOne Plus PCR Real Time (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocol using Brilliant III Ultra-fast SYBR® Green qPCR Master Mix (Agilent, Santa Clara, CA, USA) and gene-specific primers (Spandidos, Wang, Wang, & Seed, 2010; Waizenegger et al., 2015). All reactions were performed in triplicate. Relative amounts of all mRNAs were calculated using the ΔΔCt method (Pfaffl, 2001). The ribosomal protein L (RPL) gene was used as endogenous reference for normalisation.

Regression analysis of fold change values was performed to compare qRT-PCR and microarray results.

3. Results

3.1. Effect of table grape intake on PBMC expression profile

The expression profile analysis was carried out on PBMCs from six subjects at baseline (T0), after 21 days of grape-rich diet (T1) and after a 4-week washout period (T2). In total, 930 genes were identified as differentially expressed (DE), including protein-coding and non-coding RNAs. 463 genes were DE in T1*vs*T0 (202 up and 261 down), 849 in T2*vs*T0 (389 up and 460 down) and 12 in T2*vs*T1 (seven up and five down).

The hierarchical and K-means clustering analysis of DE genes was performed to group genes with a high degree of similarity (Fig. 1A and B, respectively). These analyses revealed clear-cut modifications of gene expression profile at the end of the grape-rich diet period (T1) and at the end of the washout period (T2) when the changes in gene

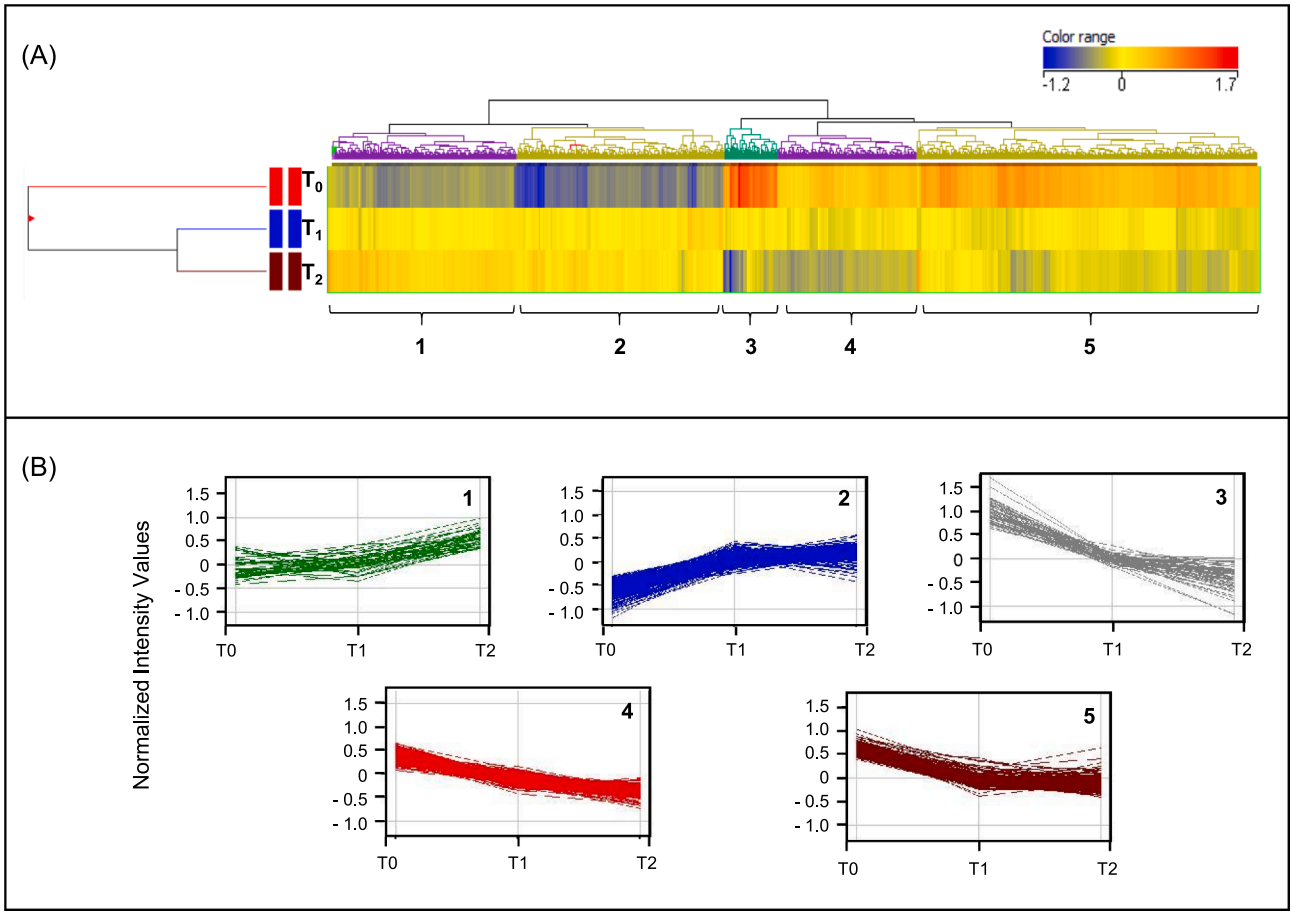

Fig. 1. Clustering analysis of differentially expressed genes. Hierarchical (A) and k-means clustering (B) of differentially expressed genes at the end of the grape-rich diet period (T1) and after 4 weeks of washout period (T2), compared to baseline (T0). In hierarchical clustering analysis, the different colours indicate different expression values as reported in the legend of "colour range". Lowest and highest expression values are in blue and red, respectively.

expression appeared even more pronounced. Based on the different expression patterns derived from the clustering analysis, five main subclusters were identified (Fig. 1). Subclusters 1 and 2 encompassed up-regulated genes and subclusters 3–5 down-regulated genes. The most significant expression changes were in subcluster 3, which included 58 genes. Despite the small number of DE genes, g:GOSt analysis of subcluster 3 revealed a significant enrichment of many down-regulated genes participating in immune-related responses such as lymphocyte activation, cytokine production, cell-cell adhesion and tumour necrosis factor (TNF) and NOD-like receptor (NLR) signalling (Fig. 2A–B). TNF is a primary mediator of inflammation, immunity and apoptosis, phenomena that are implicated in a broad spectrum of human diseases (Wajant, Pfizenmaier, & Scheurich, 2003). The NLR pathway (Saxena & Yeretssian, 2014) activates the nuclear factor NF-κB and the mitogen-activated protein kinases (MAPKs), which drive the transcription of numerous genes involved in both innate and adaptive immune responses.

The Venn diagrams of the down- and up-regulated genes (930 genes in total) at the different timeframes (T1*vs*T0, T2*vs*T0, T2*vs*T1) are shown in Fig. 3. Overall analysis revealed 76 genes DE only at the end of the grape-rich diet (T1, 26 up-regulated and 50 down-regulated), 387 genes DE at both the end of the grape-rich diet and of the washout period (176 up-regulated and 211 down-regulated), whereas 462 genes were DE only at the end of washout period (T2, 213 up-regulated and 249 down-regulated).

3.2. Functional analysis of differentially expressed genes

To provide a framework for the interpretation of our results, we used the Venn diagram tools to classify genes. Class A encompassed genes that were DE at both T1 and T2 (compared to T0), whereas class B and class C included genes DE at either T1 or T2, respectively. This gene classification was used in g:Profiler (g:GOSt) for the interpretation of transcriptomics data, which was made also in the light of functional data we obtained in the very same subjects (Ammollo et al., 2017), as summarized in Fig. 4.

3.2.1. Class A genes analysis

The profile of down-regulated genes in class A harmonizes with the picture stemming from the analysis of subcluster 3, suggesting that the most remarkable and lasting effect of grape intake is on the immune response, through the modulation of TNF and NLR pathways (see Figs. 2 and 4).

Another relevant pathway, which seems to be suppressed by grape intake, is the "AGE-RAGE signalling pathway". AGE (Advanced Glycation End) products are a complex group of compounds produced through the non-enzymatic glycation and oxidation of proteins, lipids and nucleic acids. The interaction of AGEs with their cell receptors induces the generation of free radicals that produce oxidative stress and damage. For this reason, AGEs are considered important mediators of ageing and diseases such as diabetes, atherosclerosis, chronic kidney disease, and Alzheimer's disease (Soman et al., 2013). When activated, the AGE/RAGE signalling triggers multiple intracellular pathways involving NADPH oxidase, protein kinase C, and MAPKs, eventually

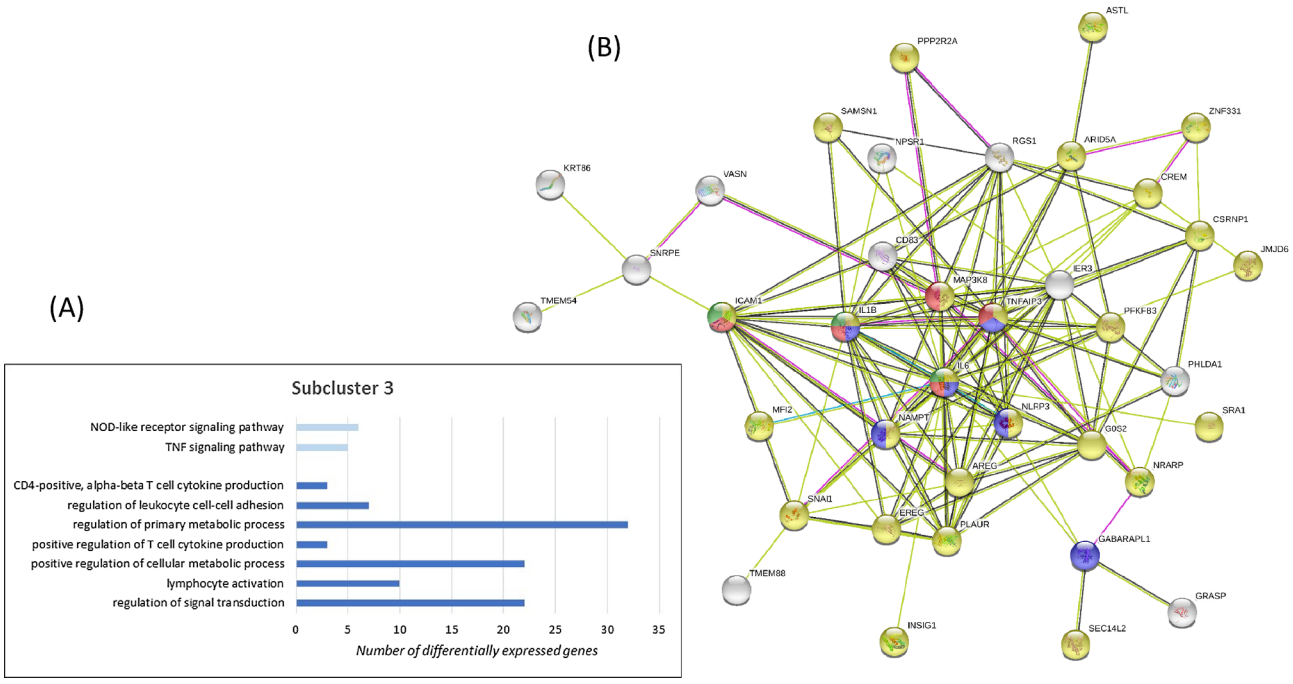

Fig. 2. Subcluster 3 analysis in g:GOSt and STRING. (A) g:GOSt analysis results. Subcluster 3 genes enriched in KEGG pathways (light blue) and in GO:BPs terms (in blue). Genes enriched in regulation of primary metabolic process: PLAUR, JMJD6, ICAM1, CREM, SEC14L2, FAM83D, NAMPT, MAP3K8, AREG, TNFAIP3, AVPI1, G0S2, SNAI1, EREG, IL1B, ZNF331, IL6, CSRNP1, PRDM8, SAMSN1, NLRP3, MFI2, SYAP1, PFKFB3, INSIG1, ASTL, ARID5A, SERTAD1, TAF13, NRARP, SRA1, PPP2R2A. Gene enriched in lymphocyte activation: JMJD6, ICAM1, MAP3K8, CD83, TNFAIP3, IL1B, IL6, SAMSN1, NLRP3, NRARP. Genes enriched in regulation of leukocyte cell-cell adhesion: ICAM1, MAP3K8, CD83, IL1B, IL6, NLRP3, NRARP. Genes enriched in positive regulation of T cell cytokine production: IL1B, IL6, NLRP3. (B) STRING database analysis. Number of recognised nodes (i.e., genes): 46 out of 58. avg. local clustering coefficient:0.565. PPI enrichment p-value: 4.44e-16. Setting for edge visualisation: evidence; line colour indicates the type of interaction. Co-expression: black; experimentally determined: purple: association in curated databases: light blue; co-mentioned in PubMed abstracts: green. Genes enriched in KEGG NOD-like receptor signalling pathway (in blue), genes enriched in KEGG TNF signalling pathway (in red), genes enriched in Reactome Interleukin-10 signalling (in green); genes enriched in regulation of primary metabolic process (in yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resulting in the activation NF-κB and in the overexpression of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, and a variety of atherosclerosis-related genes. Consistent with this view, we found that IL-1 and IL-6, along with the genes of the MAPK signalling pathway, were down-regulated at both T1 and T2.

Up-regulated genes were significantly enriched in diverse GO:BPs and Reactome pathways. Among these, the most interesting genes are those involved in the "TRAIL signalling" (TNF-related apoptosis-inducing ligand), "macroautophagy" and "regulation of signalling by CBL", which are pathways that regulate apoptosis and cellular quality.

3.2.2. Class B gene analysis

The most relevant finding concerning this class of genes is the down-regulation of two major pathways: the "Common Pathway of Fibrin Clot Formation" (gene CD177) and the "Response to elevated platelet cytosolic Ca^{2+} (CD9 and GAS6 genes) (Fig. 4). GAS6 is responsible for platelet activation and regulation of thrombotic response (Law, Graham, Di Paola, & Branchford, 2018), and CD9 for platelet activation and aggregation, cell adhesion, cell motility and tumour metastasis. These data are in line with our previous findings in the same subjects (Ammollo et al., 2017) and provide a plausible molecular basis for the

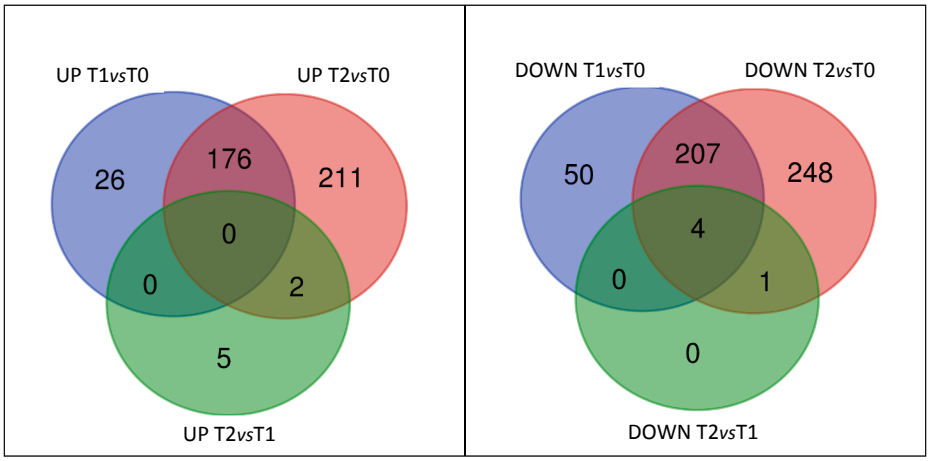

Fig. 3. Venn diagrams of up- and down-regulated gene lists at T1*vs*T0, T2*vs*T0 and T2*vs*T1 timeframes. The down- an up-regulated genes at different timeframes have been analysed using the Venn diagram tool, available at the BEG/Van de Peer Lab web site (bioinformatics.psb.ugent.be/webtools/Venn/).

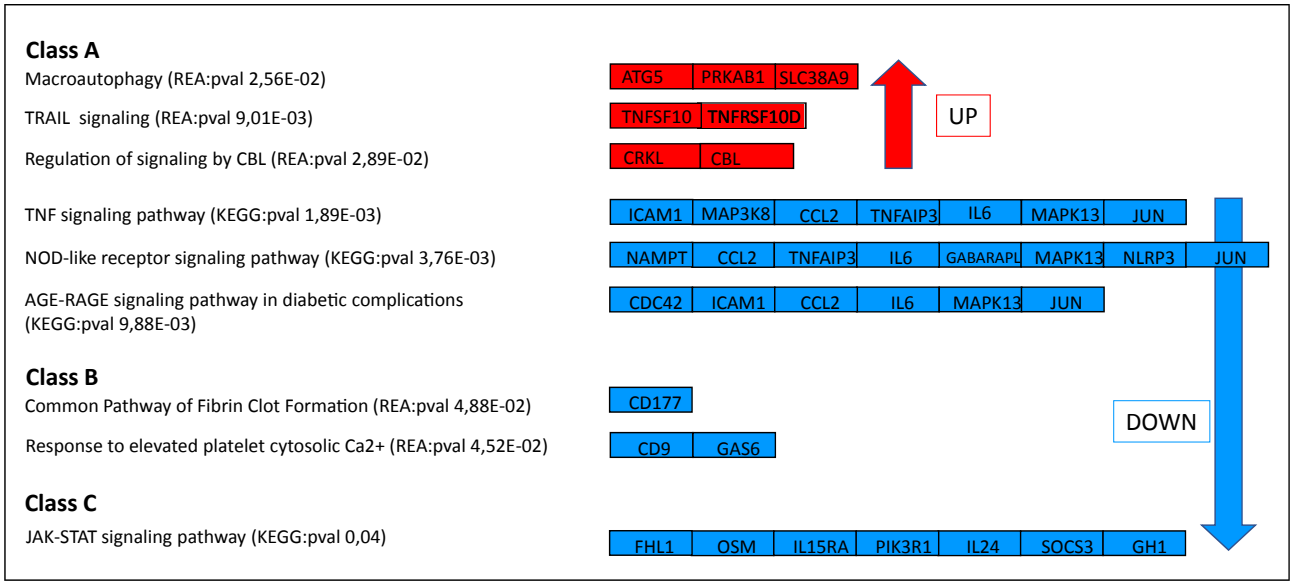

Fig. 4. Summary of g:GOSt analysis of Class A, B and C genes. Most relevant pathways in which genes of Class A, B and C are enriched. Up-regulated genes are depicted in red, down-regulated genes in blue. REA: Reactome; KEGG: Kyoto Encyclopedia of Genes and Genomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antithrombotic activity of grape intake.

3.2.3. Class C gene analysis

We found an enrichment of genes involved in the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway, which plays a major role in cell development and homeostasis (Seif et al., 2017). When cytokines bind their receptors, they activate the JAK tyrosine kinases, which in turn activate STATs that modulate the expression of a series of genes at the nuclear level. Of relevance, among the genes belonging to this pathway, is the downregulation of SOCS3, a member of the STAT-induced STAT inhibitor (SSI) family, also known as suppressor of cytokine signalling (SOCS), which suggests the activation of the JAK-STAT signalling. This hypothesis is supported by the behavior of other members of this family, such as SOCS1, a class A gene that is also down-regulated, and JAK2, STAT2, BCL2L2 (all in Class C) and MYC (Class A), which are all all up-regulated, consistent with the view that the JAK-STAT signalling remains active.

To obtain additional information on the biological meaning of our data, we performed a comparative analysis of the three gene Classes using g:Cocoa. We, therefore, evaluated the total statistical significance across all functional categories and ordered the results accordingly. Contrary to g:GOSt analysis, g:Cocoa identified up-regulated genes of Class C as the most meaningful (data not shown). The comparative analysis of Class A, B and C genes confirmed that the effects of grape intake are related to the immune response and cellular metabolism and homeostasis as general processes. Furthermore, we found that modulation of some specific pathways takes place only after the washout period, suggesting a delayed and long-lasting effect of grape intake on DNA and protein damage repair as well as on the organelle biogenesis and maintenance. The most significant pathways were “signal transduction”, “protein metabolism”, “gene expression (transcription)”, “immune system” and “cell cycle”. Class C also included genes involved in “interleukin receptor SHC signalling”, “SUMOylation of DNA damage response and repair proteins”, “Transcriptional activation of mitochondrial biogenesis”, “Metabolism”, “Organelle biogenesis and maintenance” (Fig. 5).

3.3. Effects of grape intake on the expression of lncRNAs

Long non-coding RNAs (lncRNAs), a large and heterogeneous class of RNA molecules longer than 200 nucleotides, have a broad spectrum of regulatory functions, including the regulation of transcription and RNA processing, nuclear-cytoplasmic transport, translational control and the modulation of chromatin structure (Salviano-Silva et al., 2018).

The functional analysis of our data revealed the presence of 35 up-regulated and 170 down-regulated lncRNAs (Table 1). The Venn diagrams provide a picture of how their expression levels were changed at different timeframes (Fig. 6). Notably, the largest number of down-regulated lncRNAs were found at the end of the washout period. Because of the paucity of information presently available in public web servers such as g:Profiler or others, we manually searched articles in PubMed which addressed the possible function of the lncRNA genes that were DE in our study. Available references for these genes are reported in the Supporting Table S1 along with gene name, type of expression (down- or up-regulation) at different timeframes, Class type, hyperlink to the NCBI entry and, when available, number and hyperlink to the references in PubMed. Due to their crucial role as gene regulators, most lncRNAs are believed to be involved in different types of cancers. Interestingly, the majority of lncRNAs down-regulated in our study are over-expressed in many types of tumours among which colorectal, gastric, hepatocellular, pancreatic, oesophagal, and oral squamous cell cancers.

3.4. qRT-PCR validation of gene expression changes induced by grape

To validate microarray experiments, we performed a qRT-PCR on eight DE genes, which were selected based on their key role in gene expression, cell cycle progression, differentiation or immune response. The gene tested in qRT-PCR (four up-regulated and four down-regulated) are reported in the legend to Fig. 7. As can be seen, qRT-PCR results were strongly correlated with microarray data (R^2 = 0.993 T1vsT0; R^2 = 0.955 T2vsT0).

4. Discussion

To our knowledge, this is the first *in vivo* study that analyses the expression profiles of PBMCs in response to supplementation with fresh table grape. The daily portion of grape contained approximately 400 mg of total phenolics, which is similar to the amount of polyphenols in a single dose of extra virgin olive oil in D’Amore’s study (Fu,

source	term name Biological pathways (Reactome)	term ID	n. of term genes	corrected p-value	UP_CLASS_C	UP_CLASS_A	DOWN_CLASS_A	DOWN_CLASS_C	UP_CLASS_B
rea	Interleukin receptor SHC signaling	R-HSA-912526	27	3.27e-02	3				
rea	SUMOylation of DNA damage response and repair proteins	R-HSA-3108214	76	4.32e-02	4				
rea	Transcriptional activation of mitochondrial biogenesis	R-HSA-2151201	55	1.22e-02	4				
rea	Reactome	0000000	10578	9.90e-27	108	87	83	74	
rea	Signal Transduction	R-HSA-162582	2650	2.87e-05	24	22	25	23	
rea	MAPK family signaling cascades	R-HSA-5683057	284	5.04e-03			7		
rea	Death Receptor Signalling	R-HSA-73887	140	4.00e-02	5				
rea	Metabolism of proteins	R-HSA-392499	2089	2.44e-02	19		17		
rea	Gene expression (Transcription)	R-HSA-74160	1395	2.75e-03	17		14		
rea	RNA Polymerase II Transcription	R-HSA-73857	1257	1.71e-02			13		
rea	Vesicle-mediated transport	R-HSA-5653656	698	8.94e-03		10			
rea	Membrane Trafficking	R-HSA-199991	605	2.64e-03		10			
rea	Immune System	R-HSA-168256	2019	3.40e-06	26	23		22	
rea	Cytokine Signaling in Immune system	R-HSA-1280215	687	3.97e-04	12		10	12	
rea	Growth hormone receptor signaling	R-HSA-982772	24	1.54e-02				3	
rea	Signaling by Interleukins	R-HSA-449147	464	4.90e-04			9	10	
rea	Interleukin-7 signaling	R-HSA-1266695	36	4.38e-02			3		
rea	Interleukin-4 and 13 signaling	R-HSA-6785807	111	6.81e-03				5	
rea	Interferon Signaling	R-HSA-913531	195	1.78e-03	7				
rea	Innate Immune System	R-HSA-168249	1094	1.01e-02	14				
rea	Neutrophil degranulation	R-HSA-6798695	476	1.46e-02	9				
rea	Adaptive Immune System	R-HSA-1280218	785	2.37e-02		10			
rea	Class I MHC mediated antigen processing & presentation	R-HSA-983169	369	2.63e-02		7			
rea	Metabolism	R-HSA-1430728	2087	1.63e-06	27				
rea	Organelle biogenesis and maintenance	R-HSA-1852241	281	1.85e-02	7				
rea	Cell Cycle	R-HSA-1640170	628	2.25e-02	10		9		
rea	Cell Cycle, Mitotic	R-HSA-69278	522	2.94e-02	9		8		

Fig. 5. Snapshot of Class A, B and C gene analysis by g:Cocoa. Reactome pathways enriched in the comparative analysis of Class A, B and C genes performed by g:Cocoa. UP_CLASS_C, UP_CLASS_A, DOWN_CLASS_A, DOWN_CLASS_C, UP_CLASS_B columns are ordered by g:Cocoa on the basis of the functional similarity among genes and total statistical significance. Down-regulated genes of Class B were not included by g:Cocoa because of the lack of statistical difference when compared with genes belonging to other classes.

Table 1
Number of genes differentially expressed at different timeframes.

Timeframe[a]	UP-regulated		DOWN-regulated		#Total genes
	#pc genes[b]	#lncRNAs[c]	#pc genes[b]	#lncRNAs[c]	
T1*vs*T0	178	24	186	75	463
T2*vs*T0	362	27	317	143	849
T1*vs*T2	6	1	5	0	12

[a] T0, baseline; T1, 21 days after grape diet; T2, 4 weeks after a wash-out period.
[b] pc, protein coding genes.
[c] lncRNAs, long non-coding RNAs.

2014) and in the same order of magnitude as the concentration(s) of single or combined nutrients tested in other *in vivo* studies (Bakker et al., 2010; Boomgaarden et al., 2010; Camargo et al., 2010; D'Amore et al., 2016; De Groote et al., 2012; Milenkovic et al., 2011; Niculescu et al., 2007; Tomé-Carneiro et al., 2013; van der Velpen et al., 2013). Notably, hundreds of genes were found to be up- or down-regulated after a 3-week grape-rich diet and even more after an additional 4-week washout period. The reason why some genes are DE at the end of the washout (T2) but not after the grape diet period (T1) is unknown. Some studies suggest that the bioactive compounds ingested with the diet may accumulate into the cell membrane beyond the nutrient intake and modify the membrane composition and fluidity (Tedesco et al., 2000;

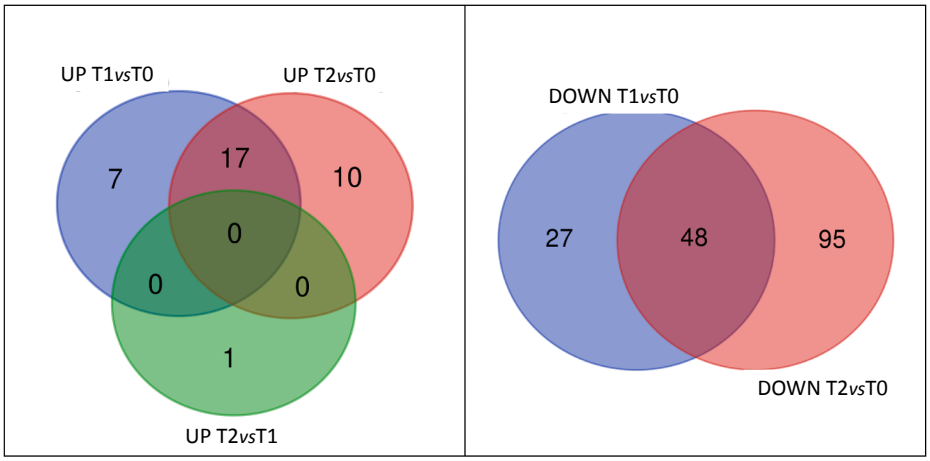

Fig. 6. Venn diagrams of up- and down-regulated long non-coding RNAs at T1*vs*T0, T2*vs*T0 and T2*vs*T1 timeframes. Diagrams were obtained using the Venn tool available at the BEG/Van de Peer Lab web site (bioinformatics.psb.ugent.be/webtools/Venn/).

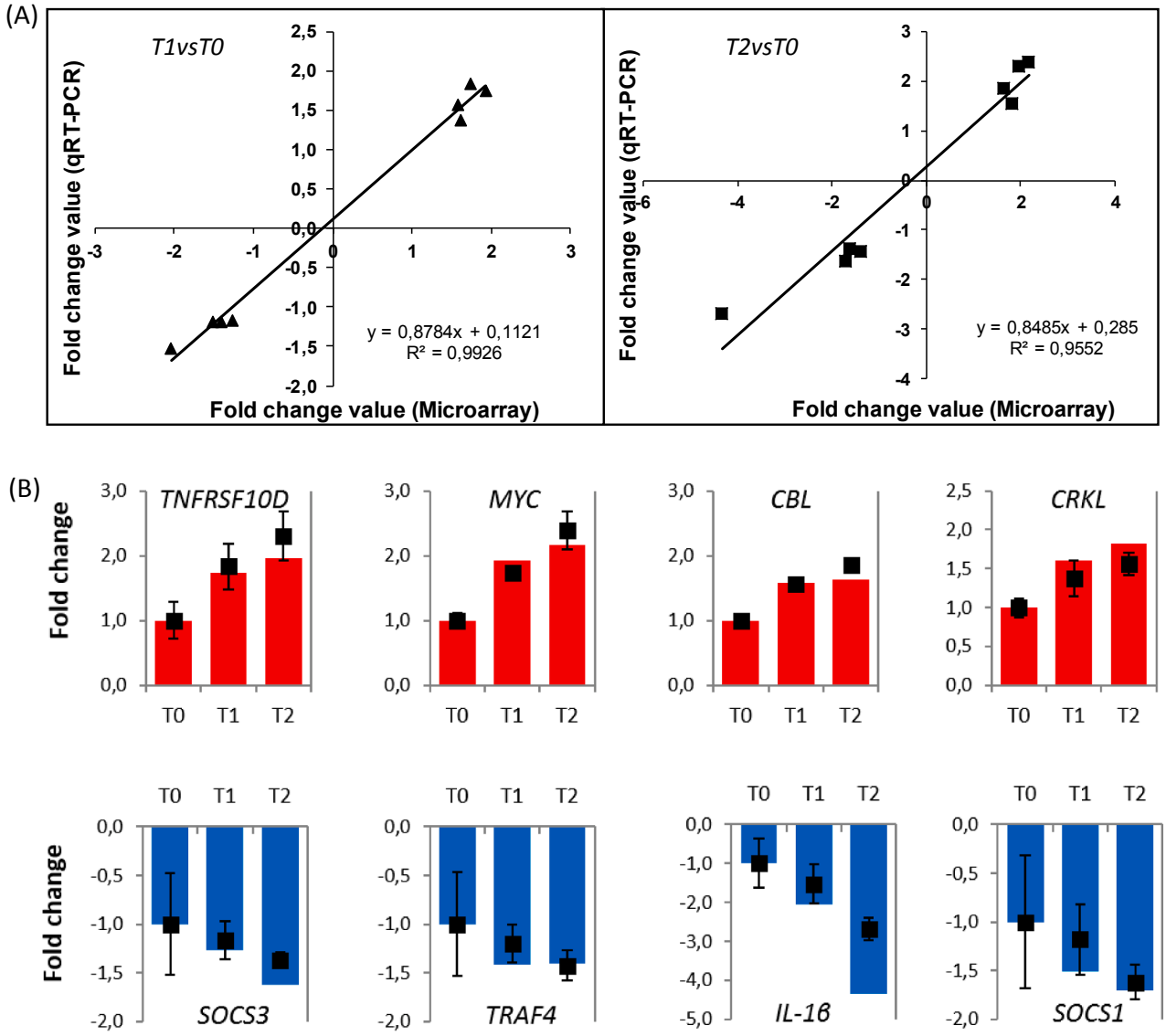

Fig. 7. qRT-PCR validation of selected genes. (A) Regression analysis of values obtained by microarray and qRT-PCR assays at *T1vsT0* and *T2vsT0*. (B) mRNA levels of up-regulated (red) and down-regulated (blue) genes analysed by microarray (coloured bars) and qRT-PCR (black squares). Validated up-regulated genes were: i) TNFRSF10D (TNF Receptor Superfamily Member 10), a receptor for the cytotoxic ligand TNFSF10; ii) MYC, a proto-oncogene that encodes for a transcription factor involved in cell cycle progression, apoptosis and cellular transformation; iii) CBL, a proto-oncogene coding for a RING finger E3 ubiquitin ligase required for targeting substrates for degradation by the proteasome; iv) CRKL, a gene encoding for a protein kinase implicated in signal transduction pathways that act as major convergence point in tyrosine kinase signalling. Validated down-regulated genes were: v) SOCS1 and vi) SOCS3 (Suppressor of Cytokine Signaling 1 and 3); vii) TRAF4 gene (TNF Receptor Associated Factor 4), that encodes for a member of the TNF receptor-associated factor (TRAF) family and that regulates activation of NF-κB in response to signaling through Toll-like receptors; viii) IL-1β (Interleukin 1 Beta) gene, that encodes for a potent proinflammatory cytokine and it is involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis. Results are the mean ± SEM of 4 independent determinations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Youdim, Shukitt-Hale, MacKinnon, Kalt, & Joseph, 2000), which in turn may impact on cell behaviour, including gene response to external signals. Alternatively, the delayed response might be the consequence of an indirect effect of grape, e.g. through the modulation of regulatory genes which act on other genes. Whatever the mechanism, the delayed and long-lasting effect of dietary supplementation is supported by previous finding by our own group, on the same subjects studied here (Ammollo et al., 2017), and by others (Alvarez-Suarez et al., 2014).

In our previous study (Ammollo et al., 2017), we showed that the grape-rich diet reduced the thrombin generation capacity of the blood. This effect appeared to be due to a decrease in circulating procoagulant microparticles and not to a decrease of PBMC tissue factor (TF), which is believed to be the primary trigger of coagulation in thrombotic conditions (Semeraro & Colucci, 1997). Interestingly, the lack of TF increase, documented originally by an immunologic assay (Ammollo et al., 2017), was confirmed here by the unchanged levels of TF gene expression in the very same PBMC. The findings of this study suggest that the antithrombotic activity induced by grape intake might at least partly be due to the down-regulation of CD177, CD9 and GAS6 genes, which belong to the broad category of genes involved on clot formation and platelet activation. Moreover, we found that the grape-rich diet induced the up-regulation of two genes that inhibit platelet activation, namely ENTPD1 and MRVI, along with the down-regulation of THBS, an adhesive glycoprotein gene that plays a role in platelet aggregation, angiogenesis and tumorigenesis.

Concerning the anti-inflammatory activity of the grape-rich diet, we previously reported a decreased release of IL-1β from PBMCs in response to lipopolysaccharide stimulation (Ammollo et al., 2017).

Consistent with this finding, both array analysis and qRT-PCR (Fig. 7) showed a downregulation of the IL-1β gene (see Class A genes). Furthermore, the functional analysis of down-regulated genes in subcluster 3 and Class A showed a significant enrichment in GO:BPs related to leukocyte cell adhesion and cytokine production. In addition, we found a decreased expression of IL-6 (Class A), IL-33 (Class C) and of some chemokines such as CCL2 (Class A), a strong chemoattractant of monocyte/macrophage, CXCL16 and CXCR4 (Class C), responsible for the recruitment of monocytes into the arterial wall during atherosclerosis, and ICAM1 (Class A), an endothelial adhesion molecule essential for leukocyte transendothelial migration during inflammation. In agreement with these results, we also observed a downregulation of proinflammatory genes such as TNF, NLR and genes of JAK/STAT pathway. Altogether these results suggest that table grape intake improves cell defences, mitigating the low-grade generation of proinflammatory signals, that can be induced in healthy subjects by many subtle stimuli coming from daily life and which contribute to processes like atherosclerosis and ageing. Importantly, these beneficial effects of the grape-rich diet are not undermined by an increase in glucose levels as previously shown (Ammollo et al., 2017).

The present microarray data add to our previous study (Ammollo et al., 2017) as it suggests that there may be additional protective mechanisms induced by table grape intake, beyond mitigation of inflammation and thrombosis. Among them, of particular relevance are autophagy, DNA repair and mitochondrial biogenesis. Our findings hint that table grape intake activates autophagy, a central pathway for cell homeostasis and cell remodelling because of its role in the process of cellular quality control through the removal of waste and damaged proteins and organelles. Several natural compounds with anti-inflammatory or antioxidant activity can intensify basal autophagy (e.g., curcumin, vitamin D and resveratrol (Han et al., 2012; Chen et al., 2013; Uberti et al., 2014; Rigacci, 2015). We found an up-regulation of genes involved in CBL and “macroautophagy” pathways (Fig. 4). In particular, the ubiquitin-protein ligase CBL and the protein kinase CRKL were up-regulated (Class A), as also confirmed by qRT-PCR (Fig. 7). We also found other DE genes involved in the three fundamental mechanisms of autophagy: ubiquitination, the formation of the autophagosome and fusion of autophagosomes with lysosomes.

Regarding ubiquitination, the ubiquitin-conjugating enzyme UBE2E was up-regulated and the deubiquitinase TNFAIP3 was down-regulated.

Moreover, we observed an up-regulation of genes coding for ATG proteins, such as ATG5, which contributes to the autophagy vesicle formation, and ATG4C, which is required for cytoplasm-to-vacuole transport. The fusion process of autophagosomes with lysosomes is mediated by syntaxin (STX) family membrane receptors. In our samples, STX7 (Syntaxin 7, Class A) and STXBP4 (Syntaxin Binding Protein 4, Class C) were both up-regulated.

DNA repair and organelle biogenesis also appeared to be stimulated by grape consumption (Fig. 5). The genes of “SUMOylation of DNA damage response and repair proteins” and of “transcriptional activation of mitochondrial biogenesis” pathways were found to be overexpressed at the end of the washout period. Sumoylation plays an essential functional role in DNA damage response. Several recent reports describe various contributions of sumoylation to genome integrity, both alone and in combination with other posttranslational modifications (Hendriks, 2015). Specific functions of sumoylation in DNA repair are the stimulation of BRCA1 ubiquitin ligase activity, which helps to recruit repair proteins to double-strand breaks and to release the homologous recombination protein RAD52 from DNA (Cremona, Sarangi, & Zhao, 2012). In our study, both BRCA1 and RAD52 were overexpressed at the end of the washout period, highlighting a long-lasting protective effect of grape.

Mitochondrial biogenesis is essential for both normal cell physiology and response to major inflammatory states (Piantadosi & Suliman, 2012). Two genes involved in this process were up-regulated in PBMCs: TFB1M, encoding a dimethyltransferase that is part of the basal mitochondrial transcription complex, and GABPA, encoding one of three GA-binding protein transcription factor subunits involved in the activation of cytochrome oxidase expression and nuclear control of mitochondrial function.

Another finding of our study is a large number of lncRNAs whose expression is modulated by grape intake. This observation is potentially relevant as it might help to elucidate the role of lncRNAs in the complex scenario of gene and genome regulation. Moreover, because of the alleged role of lncRNAs in cancer and metabolic disorders, our data might offer clues to uncover hitherto unknown mechanisms through which fruits and vegetables may protect from these severe diseases.

In our study, RNA was derived from blood mononuclear cells, which are considered a valid tool for nutrigenomic studies and believed to mirror the dietary effects on *in vivo* gene expression, providing useful information about systemic health (De Mello, Kolehmanien, Schwab, Pulkkinen, & Uusitupa, 2012; Olsen, Skeie, & Lund, 2015). On the other side, it should be kept in mind that PBMCs consist of different cell types (B and T lymphocytes, natural killer cells and monocytes), which may display different gene expression patterns. Therefore, we cannot exclude that a change in PBMC composition, possibly induced by the grape diet, might have contributed, at least in part, to the gene expression changes observed in this study.

5. Conclusions

Our pilot study in volunteers lends further support to the concept that table grape intake induces health benefits. More importantly, the transcriptomic findings suggest that the mechanisms underlying these beneficial effects may involve numerous strategic processes such as immune response, autophagy, DNA repair and mitochondrial functionality. More extensive studies are warranted to validate the hypotheses raised by our work.

Ethics statement

The following authors: Rosa Anna Milella, Marica Gasparro, Fiammetta Alagna, Maria Francesca Cardone, Silvia Rotunno, Concetta Tiziana Ammollo, Fabrizio Semeraro, Apollonia Tullo, Flaviana Marzano, Domenico Catalano, Donato Antonacci, Mario Colucci, Domenica D’Elia, of the original manuscript “Gene expression signature induced by grape intake in healthy subjects reveals wide-spread beneficial effects on PBMCs” declare that this work involves the use of human subjects, and ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained for experimentation with the subjects. The study protocol was approved by the ethical committee of the University of Bari, and written informed consent was obtained from participants before starting the study as reported in Ammollo et al. (Ammollo et al., 2017). The privacy rights of human subjects was observed.

Declaration of Competing Interest

The authors have declared no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103705.

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