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One-Year Supplementation with a Grape Extract Containing Resveratrol Modulates Inflammatory-Related microRNAs and Cytokines Expression in Peripheral Blood Mononuclear Cells of Type 2 Diabetes and Hypertensive Patients with Coronary Artery Disease

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Running title**:** *Gene expression and miRs regulation in PBMCs by resveratrol*

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Abbreviations: RES, resveratrol; GE, grape extract; GE-RES, resveratrol-containing grape extract; T2DM, type 2 diabetes mellitus; PBMCs, peripheral blood mononuclear cells; CCL3, C-C motif chemokine 3; IL, interleukin; TNF-α, Tumor necrosis factor; LRRFIP, Leucine-rich repeat flightless-interacting protein 2; NF-! B, Nuclear factor NF-kappa-B; *miR*, microRNA.

ABSTRACT

Numerous studies have shown that resveratrol (RES) exerts anti-inflammatory effects but human trials evidencing these effects in vivo are limited. Furthermore, the molecular mechanisms triggered in humans following the oral intake of RES are not yet understood. Therefore, the purpose of this study was to investigate the molecular changes in peripheral blood mononuclear cells (PBMCs) associated to the one-year daily intake of a RES enriched (8 mg) grape extract (GE-RES) in hypertensive male patients with type 2 diabetes mellitus (T2DM). We used microarrays and RT-PCR to analyze expression changes in genes and microRNAs (*miRs*) involved in the inflammatory response modulated by the consumption of GE-RES in comparison to a placebo and GE lacking RES. We also examined the changes in several serobiochemical variables, inflammatory and fibrinolytic markers. Our results showed that supplementation with GE or GE-RES did not affect body weight, blood pressure, glucose, HbAlc or lipids, beyond the values regulated by gold standard medication in these patients. We did not find either any significant change on serum inflammatory markers except for a significant reduction of ALP and IL-6 levels. The expression of the pro-inflammatory cytokines *CCL3*, *IL-1β* and *TNF-α* was significantly reduced and that of the transcriptional repressor *LRRFIP-1* increased in PBMCs from patients taking the GE-RES extract. Also, a group of *miRs* involved in the regulation of the inflammatory response: *miR-21*, *miR-181b*, *miR-663*, *miR-30c2*, *miR-155* and *miR-34a* were found to be highly correlated and altered in the group consuming the GE-RES for 12 months. Our results provide preliminary evidence that long-term supplementation with a grape extract containing RES downregulates the expression of key pro-inflammatory cytokines with the involvement of inflammation-related *miRs* in circulating immune cells of T2DM hypertensive medicated patients and support a beneficial immunomodulatory effect that may contribute to treatment.

Keywords: polyphenols, immune cells, transcriptomics, *miRs,* microarrays

1. Introduction

Understanding the specific *in vivo* cell and molecular mechanisms triggered in humans by the regular intake of low doses of dietary polyphenols which may explain the health benefits of these compounds is still an unresolved question. This is no less true for one of the most extensively investigated polyphenols, resveratrol (RES). The knowledge accrued on the bioavailability, bioactivity and cellular mechanisms and pathways allegedly regulated by RES has been thoroughly reviewed [1, 2]. The human trials conducted up to now have evidenced that RES exerts cardioprotective benefits through the amelioration of inflammatory markers as well as of the atherogenic profile, glucose metabolism and endothelial function. These effects have been shown using both high and low doses of RES and in healthy volunteers and medicated patients [3]. However, the specific mechanisms by which these effects may occur are not yet clear. This is a complex issue since the actual molecule(s) or intermediary signals responsible for the effects have not yet been fully identified nor has been well established in which specific tissues, organs or cells the direct action of these molecules or signals occurs. In addition, to obtain tissue samples either from healthy volunteers or patients is not a simple task and does not help to this research. Alternatively, peripheral blood mononuclear cells (PBMCs), constituted mostly by circulating T and B lymphocytes and monocytes, are considered a useful cell source for *in vivo* gene expression and molecular studies in humans [4]. PBMCs are easily collected and are thought to reflect the metabolic regulation occurring in other tissues (i.e. liver or adipose tissue) [5]. These cells are also involved in the immune response and in inflammation-related disorders such as atherosclerosis, playing an important role in the acute manifestation of plaque destabilization [6]. The study of the molecular changes occurring in circulating blood cells in response to dietary modifications can help to understand the mechanisms underlying the benefits of dietary polyphenols. For example, the regular consumption of orange juice or of one of its polyphenol components, hesperidin, for a few weeks has been reported to exert a major impact on leukocytes gene expression in

overweight healthy volunteers. Many of these genes were implicated in cellular processes associated with the inflammatory response and lipid metabolism [7].

In addition to the above, there is the issue of the emerging complexity of the regulatory molecular mechanisms by which cells respond to perturbations of the surrounding environment such as, for example, the exposure to dietary compounds or derived metabolites. One common pattern of cellular response is the early regulation of key transcriptional factors followed by more sustained changes in the transcription of genes and regulatory pathways involved in the specific response (regulatory modules) [8]. Gene expression regulation occurs not only at the level of transcript synthesis but also during mRNA processing and translation. Translation mechanisms are particularly important in the regulation of the metabolic responses to nutrients, where a rapid and effective response is required. Among the translational control mechanisms, non-coding RNAs such as *miRs* have a major impact in gene expression regulation [9]. *MiRs* have been reported to regulate diverse metabolic pathways and to have a role in inflammation and in the pathogenesis of metabolic disorders such as T2DM [10]. Importantly, several polyphenols associated with a decreased risk in CVDs and metabolic diseases have now been reported to modulate the levels of *miRs*. Among those, RES has been shown to modify glucose and lipid metabolism through the regulation of various *miRs* in human monocytes, a process that may be implicated in the anti-inflammatory and anti-carcinogenic properties of this polyphenol [11]. However, these effects were seen in cultured monocytes exposed to very high concentrations of RES (30–50 ! M) which cannot be attained in the human plasma through the dietary intake of RES-containing foods [12]. Recent studies have shown that one-year daily consumption of low doses of a grape extract (GE) containing RES by patients with stable coronary artery disease (CAD) improved serum levels of adiponectin and plasminogen activator inhibitor type 1 (PAI-1) as well as caused a general downregulation of genes involved in inflammation in PBMCs [13]. There are not many studies looking at the molecular mechanisms by which low doses of RES may modulate the inflammatory response *in*

vivo and even less in medicated subjects. Therefore, the purpose of this study was to further investigate the molecular changes associated to the regular intake of low doses of a RES-containing grape extract (GE-RES) in PBMCs isolated from hypertensive patients with T2DM. We analyzed gene expression changes induced by the GE-RES in comparison to a placebo group and to a group consuming the GE lacking RES. In addition, we examined the changes in the levels of several *miRs* which are known to be involved in the regulation of the inflammatory response and explored the relationships between the expression of selected target genes, *miRs* and various serum markers related to inflammation.

2. Materials & Methods

2.1. Patients and study design

This study was carried out with a subset of male type-2 diabetic and hypertensive medicated patients (n=35) which were all participants in a larger intervention study previously described [13]. Briefly, these patients had stable CAD and were treated according to current accepted guidelines for secondary prevention of CVDs. Patients were recruited from the Morales Meseguer University Hospital Cardiology Service (Murcia, Spain). All patients had stable angina or acute coronary syndrome at least 6 months before the inclusion in the study, left ventricular ejection fraction ! 45 %, Class I or II according to the New York Heart Association functional classification and medical therapy according to European Society of Cardiology (ESC) guidelines (statins, β-blockers, antiplatelets, RAS-blockers treatment and oral antidiabetics) for more than 3 months before inclusion. Exclusion criteria were liver enzymes ! 3- fold over normal values or renal failure (creatinine > 1.5 mg/dL), steroid or anticoagulant treatment, habitual intake of food supplements (herbal preparations, 'antioxidant' pills), infectious or neoplastic diseases or other known chronic pathology. The study was a randomized placebo-controlled, triple-blind, dose-response, 1-year follow-up with three parallel arms designated as placebo (maltodextrin), GE (conventional grape extract

lacking resveratrol) and GE-RES (grape extract containing resveratrol). The 35 patients studied here were randomly allocated as follows: placebo (n=9), GE (n=13) and GE-RES (n=13). Table 1 and Table 2 present the baseline clinical characteristics and biochemical variables, respectively, for the 35 participants at the inclusion into the study. The design was approved by the Clinical Ethics Committee from the Morales Meseguer University Hospital (Murcia, Spain; reference of the study 02/07) and by the CSIC's Bioethics Committee (Madrid, Spain). All patients provided written informed consent to participate in the study. This trial was registered at clinicaltrials.gov as NCT01449110.

The extracts used in this study were provided in identical code-labeled capsules, containing 350 mg of either maltodextrin, GE or GE-RES. The composition of the grape extracts has been previously described [14]. Importantly, the phenolic content of the GE and the GE-RES was very similar (151 ± 17 mg and 139 ± 18 mg phenolics per capsule, respectively) but GE-RES also contained 8.1 ± 0.5 mg of RES per capsule. Therefore, the GE and the GE-RES extracts constituted an ideal model to establish the possible specific role of RES against the rest of the polyphenolic constituents of the grape extract. All the extracts used in the study, including GE-RES (Stilvid®), were provided by Laboratorios Actafarma S.L. (Pozuelo de Alarcón, Spain). Patients were instructed to take one capsule per day in the morning, for the first 6 months and two capsules per day for the following 6 months. Patients were also requested to continue with their habitual medication (Table 1), customary lifestyle and diet throughout the 12 months intervention and to avoid the intake of grape-derived products, especially red wine. Potential incidences (dietary habit changes, febrile processes, treatment interruptions, etc.), as well as general adverse effects such as dyspepsia, diarrhea, constipation, nausea or allergic reactions to grape were monitored through questionnaires and phone calls along the study.

2.2. Blood collection and peripheral blood mononuclear cells isolation

Subjects were instructed to fast overnight before each blood collection. Blood samples were collected between 8 and 10 AM to minimize circadian variations. Blood samples were collected in heparinized tubes (BD Vacutainer, Franklin Lakes, NJ, USA) at baseline (day before first pill intake), 6 and 12 months of treatment, processed within two hours after extraction and used to isolate peripheral blood mononuclear cells (PBMCs). Isolation was carried out under sterile conditions to avoid monocytes' activation. Blood was diluted (1:1) with RPMI 1640 cell culture medium and centrifuged by density gradient with Histopaque-1077 (Sigma-Aldrich, Madrid, Spain) according to the manufacturer instructions. After washing the PBMCs (! 2) with phosphate buffer solution (PBS), the cells were lysed, homogenized in RLT buffer (Qiagen, Madrid, Spain) and stored at –80 ºC prior to RNA and protein extraction.

2.3. RNA and protein extraction protocols

Total RNA and protein were isolated from PBMCs using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Madrid, Spain) following the manufacturer recommendations. RNA concentration and purity were checked using the Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies) and the Agilent 2100 Bioanalyzer (Agilent, Madrid, Spain). Only samples with a ratio Abs_{260}/Abs_{280} between 1.8 and 2.1 and RIN (RNA integrity number) values above 8.5 were used in microarray experiments. Purified RNA and protein samples were divided in aliquots and frozen at –80 ºC until further analysis.

2.4. Microarray analysis

A search for potential candidate genes expressed in the PBMCs for which transcription levels may have been altered after the consumption of the GE or the GE-RES was performed using the GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA). This array contains 764,885 distinct probe sets that interrogate approximately 28,869 well-annotated

human genes and a set of 192 microRNAs. Microarrays were performed in RNA samples from six individuals (not pooled) per group (placebo, GE and GE-RES) at 3 different time points (6 chips at baseline, 6 chips at 6 months and 6 chips at 12 months; total number of microarrays = 54) [13]. For each sample, 250 ng of total RNA were processed according to the GeneChip® Whole Transcript (WT) Sense Target Labeling protocol (Affymetrix, Santa Clara, CA, USA). Amplified sense single-strand DNA was obtained using the Ambion® WT Expression Kit (Life Technologies) and 5.5 μg of DNA were fragmented, labeled with the WT terminal Labeling kit, and hybridized for 16 h at 45 ºC onto the chips. GeneChips were washed and stained in the Affymetrix Fluidics Station 450 and scanned using the GeneChip Scanner 3000.

The CEL files were used to extract and normalize the data using Robust Multichip Average (RMA) implemented with the algorithm RMA Sketch for 1.0 ST arrays in the GeneChip Expression Console software version 1.1.2 (Affymetrix). RMA-normalized data were tested for differential gene expression between time points using the Class Comparison tool and an empirical Bayes method (Limma) [15] implemented with Babelomics (http://babelomics.bioinfo.cipf.es/) which performs well for small n microarrays [16]. Using this model, genes differentially expressed after 6 and 12 months of the intervention for each group were defined as those with an adjusted *P*-value < 0.05 and with fold-change ≥ 1.2 (up-regulation) and < -1.2 (down-regulation). Topmost significant differentially expressed genes (fold-change >1.5 and <-1.5) were uploaded into Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, Redwood City, CA). Minimum information about a microarray experiment (MIAME) compliant data have been submitted to the Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/) and can be accessed through GEO Series accession number GSE36930.

2.5. RT-PCR analysis for target genes

Changes in the expression of several selected target genes responding to supplementation with GE-RES were further validated by one-step quantitative RT-PCR (Taqman system, Applied Biosystems, ABI, Madrid, Spain). Primers and probes for the genes were selected from Assays-on-demand (ABI, Madrid, Spain) as follows: *TNF-α* (tumor necrosis factor-alpha) (Hs00174128_m1), *IL-1β* (interleukin 1, beta) (Hs01555410_m1), *LRRFIP-1* (leucine rich repeat (in FLII) interacting protein 1) (Hs01589950_m1), *CCL3* (chemokine (C-C motif) ligand 3) (Hs00234142_m1), *NFKB-1* (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1) (Hs00765730_m1), *NFKBIA* (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) (Hs00153283_m1). Relative expression was measured by one-step quantitative RT-PCR (Taqman system, ABI, Madrid, Spain) run on the ABI 7500 system following the manufacturer's suggested cycling parameters: 48 ºC for 30 min, 95 ºC for 10 min and then 40 cycles of 95 ºC for 15 s, followed by 60 ºC for 1 min. RT-PCR was performed in 25 µL reaction volumes in MicroAmp Optical 96-well plates covered by optical adhesive covers and using Taqman Universal Master Mix (ABI, Madrid, Spain). All data were normalized to the endogenous reference gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (Hs99999905_m1) expression. All assays for a particular gene were undertaken at the same time under identical conditions and in triplicate. Relative expression was calculated by the comparative Ct method and presented as relative expression ratio ($2^{-! ! Ct}$) [17].

2.6. RT-PCR analysis for *miRs*

For *miRs* quantification, total RNA was reverse-transcribed using the miScript Reverse Transcription kit (Qiagen, Madrid, Spain). Relative expression was measured using the miScript SYBR Green PCR kit on a 7900HT Fast Real-Time PCR system following the manufacturer protocol (Qiagen). Specific primers for each *miR* (miScript Primer Assay) were

233 also obtained from Qiagen. Relative expression was calculated as described above using U6
234 for normalization.
235
236 **2.7. Serobiochemical variables, inflammatory and fibrinolytic markers**
237 Biochemical variables were determined in serum using an automated biochemical auto-
238 analyzer (Advia Systems, Siemens Healthcare Diagnostic Inc., Deerfield, IL, USA) as
239 previously described [13]. The tests included the measurement of glucose, creatinine, albumin,
240 bilirubin, total cholesterol (TChol), HDL-cholesterol (HDLc), LDL-cholesterol (LDLc),
241 triglycerides, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate
242 aminotransferase (AST), γ-glutamyl transferase (GGT), creatine phosphokinase (CPK) and
243 urate. Non-HDLc was calculated as Tchol-LDLc. Coagulation parameters were determined in
244 serum using an ACL TOP 700 analyzer (Instrumentation laboratory, Lexinton, MA, USA).
245 Thyroxine (T4) and stimulant hormone of the thyroid gland (TSH) were measured in an Advia
246 Centaur XP system (Siemens Healthcare Diagnostic). Serum levels of inflammation-related
247 markers were measured by enzyme-linked immunosorbent assays (ELISA) using
248 commercially available standard kits following protocols described by the manufacturers. IL-
249 6, IL-10 and TNF-α were measured using ELISA kits from Biolegend (San Diego, CA). In the
250 case of hsCRP (high-sensitivity C-reactive protein) and adiponectin, kits were purchased from
251 AssayPro (Winfield, MO). PAI-1 antigen levels were measured in citrated plasma using
252 PeproTech's (Rocky Hill, NJ) ELISA kits. All samples were tested at least in duplicate.
253
254 **2.8. Levels of TNF-! protein in PBMCs**
255 Protein was extracted from PBMCs at baseline and after 12 months of supplementation and
256 was quantified using the DC protein assay kit (Biorad, Barcelona, Spain) based on the Lowry
257 assay. The levels of TNF-! levels were measured using a human TNF-α ultrasensitive ELISA

kit (Invitrogen, Barcelona, Spain) following the manufacturer's recommended protocol and are presented as pg/mg of protein.

Statistical analyses

Qualitative data are shown as proportions. Quantitative data are expressed as the mean value ± SD or as the median and the 25th to 75th interquartile range (expression data). For RT-PCR results, the statistical analyses were applied to the normalized Ct values: to the endogenous control GAPDH (Ct_{target}–Ct_{GAPDH}) for genes or to the endogenous control U6 (Ct_{target}–Ct_{U6}) for microRNAs. All analyses were performed with SPSS 19.0 (SPSS Inc, Chicago, USA). Assumptions of normality and equal variances were checked to perform the appropriate statistical test. Variables with skewed distribution were logarithm transformed prior to analysis. The accepted level of significance was $P < 0.05$. Baseline data (Table 1 and Table 2) were analyzed to determine possible significant starting inter-group differences. Categorical variables were compared using the Chi–square test and numerical variables were analyzed using one-way analysis of variance (ANOVA) or the non-parametric Krustal-Wallis test when the ANOVA assumptions were not met even after log transformation. For intra-group comparisons and when the data met the required assumptions, we used one-way analysis of covariance (ANCOVA) for repeated measurements. Post-hoc tests were used for additional inter-group comparisons. The covariates used for adjustment were age, body mass index, smoking, diagnosed hypercholesterolemia, ! -blockers, and type of statin. When the assumptions required for ANCOVA were not reached (even after log transformation), intra-group analyses were done using the Friedman repeated measurements test followed by the Wilcoxon test and inter-group comparisons were assessed by the Krustal-Wallis test followed by the Mann-Whitney test. Pearson's correlation coefficients or Spearman's rank correlation coefficients were used to explore the relationship between the expression levels of the genes, microRNAs and selected variables.

3. Results

3.1. Effects of dietary interventions on serobiochemical variables, inflammatory and fibrinolytic markers

All recruited patients completed the study and none experienced adverse effects (intolerance, dyspepsia, allergic reactions, etc) during the one-year consumption of the placebo, GE or GE-RES extract. All participants consumed more than 95% of the expected capsules (compliance levels > 95%). Table 1 and Table 2 show that none of the investigated clinical characteristics and serobiochemical variables and markers presented statistically significant inter-group differences at baseline ($P < 0.05$). Values measured at the end of the 12-month supplementation as well as the significance of the intra-group variation with time are shown in Table 3. Overall these results show that most of the variables and markers examined were not significantly modified after supplementation with placebo, GE or GE-RES. Of specific interest for this group of hypertensive diabetic patients, no reduction was detected in blood pressure, serum lipids, glucose or HbA1c following the consumption of GE or GE-RES. Serum ALP was moderately reduced at the end of the study in the three groups but only significantly in the groups consuming the GE-RES (13.4 %, $P < 0.05$) or the GE (16.1 %, $P < 0.05$). Regarding the inflammatory markers, the most significant results were found for IL-6 which was significantly reduced in the volunteers consuming the GE-RES (13.3 %, $P < 0.05$). In the placebo group we found a significant ($P < 0.05$) decrease in the levels of adiponectin and of IL-10 which was reflected in a significant increase in the Il-6/IL-10 ratio.

3.2. Effects of RES-GE on PBMNCs transcriptomics

Using the analysis model described in the Materials and Methods section, we analysed and compared microarrays results between the experimental groups: placebo, GE, and, GE-RES after 6 and 12 months of supplementation. We specifically focused on those genes and *miRs*

that were significantly altered in PBMCs from volunteers consuming GE-RES at 6 and (or) 12 months and that did not change or were not significant in the placebo and the GE group. A total of 5,241 genes and 31 microRNAs (Supplemental Tables 1 and 2, respectively) were found to be significantly up- or downregulated by the consumption of GE-RES. Most significant changes took place after 12 months of supplementation with GE-RES and were in general moderate changes (fold-change values ranged typically between -2.0 and 2.0). We next used Ingenuity Pathways Analysis (IPA) to identify a number of top functions, biological pathways, upstream regulators and molecules that were most significantly modulated by the consumption of GE-RES. To reduce the number of false positives we used more stringent criteria and uploaded into the IPA software only those probe sets exhibiting an adjusted *P*-value < 0.05 and a fold change > 1.5 or < -1.5 (topmost regulated probes: 206). This analysis identified a significant effect on inflammatory processes mediated by the regulation of cytokines (Table 4). We found a general downregulation of the transcript levels of key pro-inflammatory mediators such as *CCL3*, *TNF-α*, *IL8* and *IL-1β* concomitant with the inhibition of NF-κB-mediated transcription regulation. We also detected the upregulation of other genes such as *LRRFIP-1*, a transcriptional repressor that regulates Toll-like receptor signaling, a pathway that culminates with the production of cytokines such as TNF-α. In addition, some *miRs* such as *miR-21* and *miR-181* with a regulatory role in inflammation and NF-κB signaling were activated whereas others, such as *miR-155* and *miR-34a* were found to be downregulated.

3.3. RT-PCR analyses of expression changes in selected gene targets and *miRs*

We further analysed by RT-PCR the changes in the transcript levels for three key cytokines involved in the inflammatory response: *CCL3*, *IL-1β* and *TNF-α* as well as for two important transcriptional regulators, *LRRFIP-1* and the *NFκB* complex, more specifically, for the *NFκB-1* unit (p105, precursor to the p50 subunit) and for *NFκBIA* (inhibitor to NFκB). In addition, we analysed the changes in several *miRs* implicated in inflammation. Baseline transcription

levels for all these genes and *miRs* are shown in Fig. 1. Based on the dCt values, the quantitative order for the expression levels of the genes was: *NFκBIA* ! *IL-1β* > *LRRFIP-1* > *NFκB-1* > *TNF-α* > *CCL3*. Data distribution indicated that the variability was slightly higher for the cytokines *IL-1β* (coefficient of variation, CV = 7.9 %) and TNF-α (CV = 5.6 %) than for *CCL3* and the transcription factors (CV = 3.2–3.6 %) (Fig. 1a). The *miR* exhibiting the highest expression level in PBMCs was *miR-663* whereas *miR-21* and *miR-30c-2* showed the lowest values. The variability in the expression levels for the *miRs* ranged from CV = 4.0 % for *miR-663* to CV = 7.0 % for *miR-21* (Fig. 1b).

The most significant intra-group gene expression changes were found after 12 months of intervention, mainly, after supplementation with GE-RES (Table 5 and Fig. 2). We confirmed that the transcript levels of *CCL3*, *IL-1β* and *TNF-α* were most significantly downregulated and *LRRFIP-1* upregulated in the GE-RES group and that the expression of *IL-1β* was also significantly downregulated in the GE group. The levels of *NFκB-1* were not altered except for the placebo group where a slight but significant downregulation was observed after 12 months. The inhibitor unit *NFκBIA* exhibited a decrease trend in the three groups which reached significance only in the volunteers who took the GE. We additionally calculated the inter-group differences at the end of the study (12 months) (Fig. 2). The mRNA levels of *IL-1β* and *TNF-α* resulted lower in the group consuming the GE-RES than in the placebo group by 62% (P=0.037) and 46% (P=0.064), respectively. *IL-1β* was also significantly lower in the group consuming the GE than in the placebo group. *CCL3* and *LRRFIP-1* exhibited only a tendency to decrease (24 %, P=0.189) and to increase (32%, P=0.142), respectively, when compared with the placebo group. *NFκBIA* mRNA levels were significantly higher (45%, P<0.039) in the GE-RES group than in the placebo group.

Intra-group changes for the *miRs* are shown in Table 6 and Figure 3. The profile of change was similar for *miR-21*, *miR-181b*, *miR-663* and *miR-30c2*. These molecules displayed a general downregulation in the placebo and the GE groups whereas the trend was slightly

different in the group consuming the GE-RES extract, where the levels of these *miRs* remained constant or exhibited a tendency to increase, especially after 12 months of supplementation. Inter-groups comparison supported these results with the levels of *miR-21*, *miR-181b*, *miR-663* and *miR-30c2* being significantly higher in the GE-RES group than in the placebo group ($P < 0.05$) and, for *miR-21*, *miR-663* and *miR-30c2*, also significantly higher than in the GE group (Fig. 3). On the other hand, the levels of *miR-155* were most significantly downregulated after treatment with GE-RES and with GE but we did not find any inter-group significant differences. *Mir-34a* was not significantly altered with time in any of the three groups but at the end of the study the levels of this *miR* were significantly lower in the GE-RES and the GE groups in comparison with the placebo group. Using the miRWalk database [18] we found that *IL-1β and NFκB-1* are validated or predicted targets for *miR-21* and *miR-181b* and that *NFκB-1* is also a target for *miR-30c*. Of note, *LRRFIP-1* is a predicted target of *miR-155* and *miR-34a*, while *NFκBIA* is a target of *miR-34a* (www.targetscan.org).

3.4. TNF-α in PBMCs from the three groups of volunteers

In an attempt to find out whether some of the observed transcriptional changes were translated into protein changes we measured the levels of TNF-α in PBMCs from the three groups of volunteers. At baseline, the values found were: 0.7 ± 1.1 pg/mg total protein for the placebo group, 1.4 ± 1.5 pg/mg in the GE group and 1.7 ± 1.6 pg/mg in the GE-RES group. No significant inter-group differences were detected. After 12 months of supplementation, no significant intra-group or inter-group variations of the protein were found (final values of TNF-α: 2.2 ± 1.9 pg/mg in the placebo group, 1.2 ± 1.0 pg/mg in the GE group and 1.4 ± 1.0 pg/mg in the GE-RES group).

3.5. Correlations between *miRs* expression, genes expression and serum markers

In an attempt to understand the potential interplay between the investigated *miRs*, target genes and serum markers we applied correlation analyses. We found that, at baseline (Supplemental Table 3), most of the *miRs* investigated in the PBMCs exhibited a significant ($P < 0.05$) and high positive correlation. The maximum correlation was found between *miR-21* and *miR-181b* with *miR-30c2* whereas *miR-663* was also highly correlated to *miR-34a*, *miR-155* and *miR-181b*. Among the genes examined, *IL-1β*, *TNF-α* and *NF!BIA* also displayed a significant positive correlation. In addition, *NF!BIA* was found to be negatively correlated with *miR-30c2* and *miR-21* and *LRRFIP-1* exhibited a negative correlation with *miR-663*. Supplemental Table 3 also shows some significant correlations found between genes or *miRs* and specific markers. The levels of TNF-α in PBMCs displayed a significant and positive correlation with the mRNA levels of *TNF-α*, *IL-1β* and *NF!BIA* but not with serum TNF-α which was found negatively correlated to *NF!B1*. Other serum markers of interest such as serum IL-6 had a negative correlation with the expression of *IL-1β* in PBMCs and adiponectin was found to be positively correlated with *miR-663* and *CCL3*. We did not find any significant correlation between the basal levels of ALP and any of the genes or *miRs* investigated.

We next examined the overall correlation at the end of the one-year supplementation and also explored each group separately (Supplemental Table 4). After 12 months of treatment, we observed that the overall correlation values were, in general, lower and less or not significant in comparison to baseline indicating a general deregulation of the investigated molecules. We estimated the extent of differential correlation between the three groups by computing the variance of the separate correlation values [19] which ranged from 0.001 to 0.406. The lowest variance is associated to pairs of molecules that are still correlated after supplementation and have similar correlation values in each group. This was the case for some of the *miRs*, e.g. the pairs formed by *miR-21*/*miR-181b*, *miR-21*/*miR-30c2* or *miR-181b*/*miR-30c2*. As the variance increases differences in the correlation values between the groups become larger suggesting an

412 effect of the supplementation on the correlation between molecules, e.g., the pair *miR-*
413 *663/miR-34a* or *miR-21/miR-34a*. With regards to the investigated markers, we found that
414 most of the correlation values were not significant at the end of the study. The correlation
415 between the protein and mRNA levels of TNF-α in PBMCs remained significant only in the
416 group exposed to the GE-RES. In addition, serum ALP also displayed a negative correlation
417 with the mRNA levels of *TNF-α*.
418

419 **4. Discussion**

420 A plethora of cell and animal studies have indicated that RES exerts anti-inflammatory
421 effects in association with the modulation of key molecules involved the inflammatory
422 response. Instead, clinical trials addressing the anti-inflammatory effects of RES are limited
423 and only a few studies have looked at potential molecular mechanisms triggered in humans
424 following the oral intake of RES [1–3]. Furthermore, studies looking at long-term effects of
425 RES in medicated patients are only beginning to emerge [13, 14, 20]. In this context, the
426 present study adds to the knowledge on the effects of long-term consumption of low doses of
427 RES in medicated subjects with established heart disease. Our main findings were: i) one-year
428 supplementation with a GE alone or containing an additional small amount of RES did not
429 modify body weight, blood pressure, serum levels of glucose, HbAlc or lipids, beyond the
430 values regulated by gold standard medication in hypertensive T2DM male patients; ii)
431 treatment with GE or GE-RES did not cause significant changes on a range of serum markers
432 except for a small but significant reduction of ALP; importantly, GE-RES significantly
433 downregulated the levels of IL-6; iii) the expression of the pro-inflammatory cytokines *CCL3*,
434 *IL-1β* and *TNF-α* was most significantly downregulated and that of the transcriptional
435 repressor *LRRFIP-1* upregulated in PBMCs from patients taking the GE-RES extract; iv) in
436 addition, several *miRs* involved in the inflammatory response and expressed in PBMCs (*miR-*

437 *21*, *miR-181b*, *miR-663*, *miR-30c2*, *miR-155* and *miR-34a*) were highly correlated and
438 significantly altered in the group consuming GE-RES.

439 Various studies in rodents had reported a reducing effect of RES on blood pressure, serum
440 lipids, and glucose [3]. In pigs, an animal with physiological and genomic similarities to
441 humans [21], RES exhibited different effects. For example, in high-fat fed pigs, 100 mg
442 RES/kg b.w. (Human Equivalent Dose, HED: ~6400 mg/70 Kg person) for 7–11 weeks
443 reduced the levels of serum cholesterol, glucose, BMI and systolic blood pressure [22–24]
444 whereas a much smaller dose (8 mg/70 Kg person) for one year did not affect these variables
445 [25] suggesting that high pharmacological rather than low dietary doses of RES are needed to
446 cause significant effects in these models. In humans, results are limited and inconsistent and
447 not only depend on the dose and duration of the trial but also on the individuals evaluated, e.g.
448 medicated patients or healthy volunteers. In T2DM patients, a reduction in blood pressure,
449 glucose, HbAlc, and serum lipids has been reported at high doses of the compound (250 mg
450 for 3 months) [26] whereas smaller doses (10 mg for 4 weeks) only affected glucose levels
451 [27]. On the other hand, in overweight patients [28] or with metabolic syndrome [29] these
452 variables were not modified even at high doses of RES and, in healthy volunteers, only a small
453 reduction in systolic pressure [30] and in triglycerides [31] has been observed following the
454 intake of high doses of RES for 30 days. In our study, both the low dose of RES administered
455 and the fact that these variables were tightly controlled by the medication may contribute to
456 explaining the lack of effects on these variables. We detected a small but significant decrease
457 of serum ALP in volunteers consuming the GE or the GE-RES. These results are in agreement
458 with previous studies showing a significant reduction of ALP in pigs fed a

hypercholesterolemic diet and supplemented with RES [32]. The physiological significance of these results is not well understood but ALP activity has been associated with glucose regulation [33] and is moderately but significantly increased (by ! 17%) in diabetic patients [34]. Also, treatment with some antidiabetic drugs caused a decrease in serum ALP levels positively correlated with changes in the levels of IL-6 [35] and thus, a reduction in ALP may be associated to a decrease in the inflammatory conditions. In agreement with this and with previous cell and animal studies [3], we also report here a significant downregulation of the serum levels of IL-6 following the one-year intake of GE-RES. Nonetheless, other human trials using high doses of RES (75 mg – 1500 mg for 1 to 3 months) failed to show changes in serum IL-6 [36, 37] giving evidence, once more, of the variability in the effects of RES and its dependence on doses, duration of treatment and individuals.

In this study we also confirmed a significant downregulation of the expression of key pro-inflammatory cytokines involved in the pathogenesis of T2DM [38] in PBMCs isolated from the volunteers consuming the GE-RES extract for 12 months. PBMCs play a critical role in the inflammatory response and are considered suitable for comparative gene expression analyses after dietary interventions [5, 7] but, variability must be controlled by strict sample handling and processing protocols [4]. In our study, PBMCs were routinely isolated from fasted patients during the same time gap in the morning and processed within 1 hour. Cell analyses showed that the cell population remained quite homogeneous and was constituted mainly by lymphocytes (84.4 ± 2.9 %) and monocytes (13.0 ± 3.2 %) and also by a small percentage of granulocytes (2.2 ± 1.0 %). In consonance with our results, various cell and animal studies have reported that RES is associated with the downregulation of IL-1β and TNF-α [2, 3]. More specific studies looking at the effect of RES on gene expression regulation in cultured lymphocytes or monocytes or in PBMCs are scarce but, for example, RES has been shown to suppress *IL-8* transcription in cultured phorbol ester-induced human monocytes [39] and to reduce *TNF-α* and *IL-8* mRNA in cultured LPS/IFNγ-induced PBMCs [40]. Recent studies

485 have examined targeted gene expression changes *in vivo* in PBMCs following the
486 consumption of RES. In healthy subjects, the consumption of a plant (*Polygonum cuspidatum*)
487 extract containing RES (40 mg) for 3 to 6 weeks led to a significant downregulation of various
488 inflammation-related transcripts including, *TNF-α*, *IL-6* and *I! ! B* transcripts, as well as to a
489 reduction in NF-! B binding activity [41]. In a later study, healthy subjects fed a high-fat, high-
490 carbohydrate diet and supplemented with RES plus a muscadine grape extract displayed a
491 significant downregulation of the diet-induced mRNA levels of *IL-1β* but no effects on *TNF-α*
492 were reported [42]. Also, multiple-targeted analysis using microarrays has been implemented
493 to investigate the molecular changes induced in PBMCs in healthy volunteers after the intake
494 of RES, a RES-phosphate or a catechin-rich grape extract. These authors reported a general
495 effect on antioxidant, stress and anti-inflammatory related genes [43]. We had previously
496 shown that the one-year consumption of GE-RES improved the inflammatory and fibrinolytic
497 status of patients undergoing both primary [14, 20] and secondary [13] prevention of CVDs. In
498 a group of male diabetic hypertensive patients with stable CAD consuming GE-RES for one
499 year, several inflammation-related transcription factors were predicted to be inactivated and
500 various genes involved in inflammation downregulated in PBMCs [13]. These previous results
501 and the results presented here support that the consumption of RES is associated with a
502 moderate anti-inflammatory effect that is partially exposed by a transcription downregulation
503 of pro-inflammatory cytokines in circulating cells of the immune system. This effect might be
504 mediated through regulation of the NF-! B signaling system. Our functional analysis predicted
505 the inactivation of this transcription factor in PBMCs isolated from the patients consuming the
506 GE-RES extract (Table 4). We hypothesized that this inactivation might have been associated
507 to the downregulation of the expression of the subunit *NFKB-1* and (or) upregulation of the
508 inhibitor *NFKBIA*, however, our microarrays and RT-PCR results did not confirm this
509 hypothesis and thus, NF-! B signaling might be regulated through alternative mechanisms. Of

note, we found that GE-RES significantly upregulated the expression of *LRRFIP-1*, a gene that encodes for a transcriptional repressor that regulates Toll-like receptor signaling and whose overexpression has been reported to inhibit the expression levels of *IL-1β* and of *TNF-α* in monocytic cell lines [44].

With regards to the contents of TNF-α in PBMCs we did not detect a significant decrease of the protein in parallel to the mRNA downregulation. Although a lack of correlation between transcription and translation changes is not unusual due to possible differences between the half-life and stability of the mRNA and protein molecules [45], we cannot discard that protein data variability and the small number of participants per group may contribute to these results. In addition, we were not able to find a correlation between the levels of TNF-α in PBMCs and serum levels of the cytokine which may be explained by the fact that circulating levels of TNF-α do not originate only from peripheral leukocytes, but may also come from the vessel wall, other lymphoid tissues, or non-lymphoid cells such as hepatocytes and adipocytes. Also, circulating cytokines can bind to extracellular matrix proteins or to cognate receptors and may not represent the actual degree of immune activation or deactivation [46]. It has been suggested that gene expression in peripheral blood cells may be more easily and rapidly affected than systemic cytokine levels and may constitute an independent sign for modulation of systemic inflammation [47, 48].

Growing evidence suggests that *miRs* play a significant role in inflammatory diseases such as obesity and T2DM [10]. *MiR-155*, *miR-21*, *miR-181b* and *miR-34a* are among the most investigated *miRs* in relation to inflammation and are involved in the regulation of Toll-like receptor and NF-! B signaling [49] as well as in the regulation of the levels of inflammatory cytokines such as TNF-α, IL-1β and IL-6 [50, 51]. Our results indicate that in patients consuming the GE-RES for 12 months, the observed downregulation of the pro-inflammatory cytokines in PBMCs was concomitant with higher levels of *miR-21*, *miR-181b*, *miR-663* and *miR-30c2* and lower levels of *miR-155* and *miR-34a* in this group when compared to the

placebo or the GE group. The exact regulatory role of these molecules is difficult to determine since both an effect of the cytokines on the *miRs* levels and of the *miRs* on the levels of cytokines have been reported. Indeed, some *miRs* seems to reinforce an appropriately ’balanced’ pathway by targeting both positive and negative regulatory components and thus buffering gene expression against minor physiological variation [52]. For example, *miR-181b* is a potent positive regulator of NF-! B signaling with TNF-α reducing *miR-181b* expression and *miR-181b* suppressing TNF-α-induced pro-inflammatory gene expression in endothelial cells [53]. *MiR-21* has also been described to act as an anti-inflammatory agent with a negative regulatory loop: NF-! B activity is necessary for *miR-21* induction but *miR-21* also works to inhibit NF-! B and its pro-inflammatory transcriptional targets *TNF-α* and *IL-1β* [49, 50]. *Mir-34a* has been recently identified as a novel inflammation negative regulator in macrophages where inhibition of *miR-34a* increased the expression of *TNF-α* and *IL-6* [51] and, *miR-30c2* is also induced by NF-! B [54] with low levels of *miR-30c2* being associated to high levels of PAI-1 [55]. Of interest, *miR-21*, *miR-34a* and *mir-30c2* have all been connected with diabetes [10, 50]. *MiR-663* has also been implicated in the inflammatory response of human endothelial cells reducing TNF-α-induced monocyte adhesion [56] and *miR-155* is one of the most dynamically regulated *miRs* highly expressed in both activated B and T lymphocytes and in monocytes/macrophages. The levels of *miR-155* are upregulated during the immune response in correlation with NF-! B activation and increased *miR-155* contributes to TNF-α production [49, 57]. Conversely, inhibition of *miR-155* reduces TNF-α production [58]. Also, both TNF-α and IL-1β have been reported to upregulate *miR-155* [59]. In general, *miR-155* has been reported to function in the promotion of T-cell-dependent tissue inflammation and its repression has been considered as a therapeutic target for the treatment of immune diseases [60].

Importantly, it has been shown that nutritional doses of various dietary polyphenols can modulate *miRs* expression profiles in the liver of mice [61]. Of specific interest for our study, RES has also been shown to modulate *miRs* related to metabolic and chronic diseases [11]. For example, and in good agreement with some of our results, RES upregulates *miR-663* and donwregulates *miR-155* in human THP-1 monocytic cells as well as in human blood monocytes. *Mir-663* is known to target *JunB* and *JunD* transcripts which are components of the inflammation regulatory transcription complex AP-1 [12]. Our microarray results (Supplementary Table S1) show the downregulation of the expression of *JunD* as well as of *ATF7*, another component of AP-1 and suggest that the anti-inflammatory effect of GE-RES may be mediate through the regulation of transcription regulator AP-1 as it had been previously predicted [13]. In general, our results support the *in vivo* involvement of *miRs* regulation in the molecular anti-inflammatory response observed in PBMCs following the long-term consumption of GE-RES. Of note, we found high and significant correlations between most of the *miRs* investigated before and after supplementation (Supplementary Tables S3 and S4). Since multiple co-expressed *miRs* can cooperatively regulate a given biological or pathological process [62] our results suggest that the *miRs* investigated may be part of a modulatory network and cooperate in the regulation of the observed anti-inflammatory effects in PBMCs. The possibility of targeting different *miRs* that influence inflammatory pathways by dietary supplementation with RES adds new perspectives to the prevention or treatment of inflammatory-related disorders. Further research is needed before the link between RES and *miRs* can be incorporated into clinical care.

5. Conclusions

The main limitation of this study is the sample size since it has been conducted in a relatively small number of patients (n= 35; 9–13 per group) and thus, a larger sample size is needed to further confirm these results. Nevertheless, this study provides preliminary evidence

that long-term supplementation with a grape extract containing a small quantity of RES: i) downregulates the expression of several important cytokines and, ii) modifies the expression of a modulatory network of *miRs* involved in the inflammatory response, in circulating cells of the immune system from T2DM hypertensive patients with stable CAD and treated according to accepted guidelines for secondary prevention of CVDs. Since T2DM is recognized as an inflammatory disease where activation of PBMCs is a relevant additional aspect that may be caused by a deregulation of the production of cytokines and of the cross-talk between adipocytes and the circulating immune cells [63] our results support a beneficial immunomodulatory effect of the GE-RES that may contribute to T2DM treatment. We cannot, however, discard that the observed effects may result from the combined action of RES with other phenolic compounds present in the GE extract or with some of the specific medication administered to these patients.

Conflict of interest

F.T.B. and J.C.E. are co-inventors of the patent ES 2177465 that describes the process to obtain resveratrol-enriched grapes. The rest of the authors declare no conflict of interest. The products used in this study were provided by Actafarma S.L. (Madrid, Spain). This company did not fund the study and had no role in trial design, data collection and analysis, decision to publish or preparation of the manuscript.

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Figure Legend

Figure 1. Comparative baseline transcriptional levels of the selected investigated (a) gene targets and (b) miRNAs. Data are presented as dCt (Ct target- Ct reference).

Figure 2. Intra-group gene expression changes in PBMCs of selected targets involved in the regulation of the inflammatory response and Type-2 diabetes mellitus (T2DM). Values are presented as relative changes *vs.* baseline following 6 and 12 months of supplementation with placebo, GE (grape extract) or GE-RES (grape extract containing resveratrol). Significant intra-group gene expression relative changes are marked with asterisks: **, $P<0.05$; ***, $P<0.01$; ****, $P<0.001$. Significant differences between groups after 12 months of supplementation are also indicated (fold- change and *P* value); ! upregulated or ! downregulated in the GE-RES group with respect to the placebo group; *CCL3*, chemokine (C-C motif) ligand 3; *IL-1!*, interleukin 1 beta; *TNF-!* , tumor necrosis factor alpha; *LRRFIP-1*, leucine rich repeat flightless interacting protein 1; *NFκB-1*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105); *NFκBIA*, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (*IκBα*).

Figure 3. Intra-group *miRs* changes in PBMCs of selected molecules involved in the regulation of the inflammatory response and Type-2 diabetes mellitus (T2DM). Values are presented as relative changes *vs.* baseline following 6 and 12 months of supplementation with placebo, GE (grape extract) or GE-RES (grape extract containing resveratrol). Significant intra-group gene expression relative changes are marked with asterisks: **, $P<0.05$; ***, $P<0.01$; ****, $P<0.001$. Significant differences between groups after 12 months of supplementation are also indicated (fold- change and *P* value). ! upregulated or ! downregulated in the GE-RES group with respect to placebo or GE groups.

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672 **References**

673 [1] Timmers S, Auwerx J, Schrauwen P. The journey of resveratrol from yeast to human
674 Aging 2012;4:146-58.

675 [2] Švajger U, Jeras M. Anti-inflammatory effects of resveratrol and its potential use in
676 therapy of immune-mediated diseases. International Reviews of Immunology 2012;31:202-22.

677 [3] Tomé-Carneiro J, Larrosa M, González-Sarrías A, Tomás-Barberán FA, García-Conesa
678 MT, Espín JC. Resveratrol and Clinical Trials: The Crossroad from in vitro Studies to Human
679 Evidence. Current Pharmacological Design 2013. In press.

680 [4] Mesko B, Poliska S, Nagy L. Gene expression profiles in peripheral blood for the
681 diagnosis of autoimmune diseases. Trends in Molecular Medicine 2011;17:223-33.

682 [5] de Mello VD, Kolehmanien M, Schwab U, Pulkkinen L, Uusitupa M. Gene expression
683 of peripheral blood mononuclear cells as a tool in dietary intervention studies: What do we
684 know so far? Molecular Nutrition and Food Research 2012;56:1160-72.

685 [6] Benagiano M, Azzurri A, Ciervo A, Amedei A, Tamburini C, Ferrari M, et al. T helper
686 type 1 lymphocytes drive inflammation in human atherosclerotic lesions. Proceedings of the
687 National Academy of Sciences U S A 2003;100:6658-63.

688 [7] Milenkovic D, Deval C, Dubray C, Mazur A, Morand C. Hesperidin displays relevant
689 role in the nutrigenomic effect of orange juice on blood leukocytes in human volunteers: a
690 randomized controlled cross-over study. PLoS One 2011; 6:e26669.
691 [8] Bar-Joseph Z, Gitter A, Simon I. Studying and modelling dynamic biological processes
692 using time-series gene expression data. Nature Reviews Genetics 2012;13:552-64.
693 [9] Adeli K. Translational control mechanisms in metabolic regulation: critical role of
694 RNA binding proteins, microRNAs, and cytoplasmic RNA granules. American Journal of
695 Physiology - Endocrinology and Metabolism 2011;301:E1051-64.
696 [10] Dehwah MA, Xu A, Huang Q. MicroRNAs and type 2 diabetes/obesity. Journal of
697 Genetics and Genomics 2012;39:11-8.
698 [11] Bladé C, Baselga-Escudero L, Salvadó MJ, Arola-Arnal A. miRNAs, polyphenols, and
699 chronic disease. Molecular Nutrition and Food Research 2013;57:58-70.
700 [12] Tili E, Michaille JJ, Adair B, Alder H, Limagne E, Taccioli C, et al. Resveratrol
701 decreases the levels of miR-155 by upregulating miR-663, a microRNA targeting JunB and
702 JunD. Carcinogenesis 2010;31:1561-6.
703 [13] Tomé-Carneiro J, Gonzálvez M, Larrosa M, Yáñez-Gascón MJ, García-Almagro FJ,
704 Ruiz-Ros JA, et al. Grape resveratrol increases serum adiponectin and downregulates
705 inflammatory genes in peripheral blood mononuclear cells: a triple-blind, placebo-controlled,
706 one year clinical trial in patients with stable coronary artery disease. Cardiovascular Drugs and
707 Therapy 2013;27:37-48.
708 [14] Tomé-Carneiro J, Gonzálvez M, Larrosa M, García-Almagro FJ, Avilés-Plaza F, Parra
709 S, et al. Consumption of a grape extract supplement containing resveratrol decreases oxidized
710 LDL and ApoB in patients undergoing primary prevention of cardiovascular disease: a triple-
711 blind, 6-month follow-up, placebo-controlled, randomized trial. Molecular Nutrition and Food
712 Research 2012;56:810-21.

713 [15] Wettenhall JM, Smyth GK. limmaGUI: a graphical user interface for linear modeling
714 of microarray data. Bioinformatics 2004;20:3705-6.
715 [16] Murie C, Woody O, Lee AY, Nadon R. Comparison of small n statistical tests of
716 differential expression applied to microarrays. BMC Bioinformatics 2009;10:45.
717 [17] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-
718 PCR. Nucleic Acids Research 2001;29:e45.
719 [18] Dweep H, Sticht C, Pandey P, Gretz N. miRWalk-database: prediction of possible
720 miRNA binding sites by 'walking' the genes of three genomes. Journal of Biomedical
721 Information 2011;44:839-47.
722 [19] Stäehler CF, Keller A, Leidinger P, Backes C, Chandran A, Wischhusen J, et al. Whole
723 miRNome-wide differential co-expression of microRNAs. Genomics Proteomics
724 Bioinformatics 2012;10:285-94.
725 [20] Tomé-Carneiro J, Gonzálvez M, Larrosa M, Yáñez-Gascón MJ, García-Almagro FJ,
726 Ruiz-Ros JA, et al. One-year consumption of a grape nutraceutical containing resveratrol
727 improves the inflammatory and fibrinolytic status of patients in primary prevention of
728 cardiovascular disease. American Journal of Cardiology 2012;110:356-63.
729 [21] Kuzmuk KN, Schook LB. Pigs as a model for biomedical sciences. In: Rothchild MF,
730 Ruvinsky A, editors. The genetics of the pig, Wallingford: CAB International; 2011, p. 426-
731 44.
732 [22] Robich MP, Chu LM, Chaudray M, Nezafat R, Han Y, Clements RT, et al. Anti-
733 angiogenic effect of high-dose resveratrol in a swine model of metabolic syndrome. Surgery
734 2010;148:453-62.
735 [23] Robich MP, Osipov RM, Nezafat R, Feng J, Clements RT, Bianchi C, et al.
736 Resveratrol improves myocardial perfusion in a swine model of hypercholesterolemia and
737 chronic myocardial ischemia. Circulation 2010;122: S142-9.

738 [24] Robich MP, Osipov RM, Chu LM, Han Y, Feng J, Nezafat R, et al. Resveratrol
739 modifies risk factors for coronary artery disease in swine with metabolic syndrome and
740 myocardial ischemia.European Journal of Pharmacology 2011;664:45-53.
741 [25] Azorín-Ortuño M, Yáñez-Gascón MJ, González-Sarrías A, Larrosa M, Vallejo F,
742 Pallarés FJ, et al. Effects of long-term consumption of low doses of resveratrol on diet-induced
743 mild hypercholesterolemia in pigs: a transcriptomic approach to disease prevention. Journal of
744 Nutritional Biochemistry 2012;23:829-37.
745 [26] Bhatt JK, Thomas S, Nanjan MJ. Resveratrol supplementation improves glycemic
746 control in type 2 diabetes mellitus. Nutrition Research 2012;32:537-41.
747 [27] Brasnyó P, Molnár GA, Mohás M, Markó L, Laczy B, Cseh J, et al. Resveratrol
748 improves insulin sensitivity, reduces oxidative stress and activates the Akt pathway in type 2
749 diabetic patients. British Journal of Nutrition 2011;106:383-9.
750 [28] Crandall JP, Oram V, Trandafirescu G, Reid M, Kishore P, Hawkins M, et al. Pilot
751 Study of resveratrol in Older Adults With Impaired Glucose Tolerance. Journals of
752 Gerontology Series A: Biological Sciences and Medical Sciences 2012;67:1307-12.
753 [29] Fujitaka K, Otani H, Jo F, Jo H, Nomura E, Iwasaki M, et al. Modified resveratrol
754 Longevinex improves endothelial function in adults with metabolic syndrome receiving
755 standard treatment. Nutrition Research 2011;31:842-7.
756 [30] Timmers S, Konings E, Bilet L, Houtkooper RH, van de Weijer T, Goossens GH, et al.
757 Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism
758 and metabolic profile in obese humans. Cell Metabolism 2011;14:612-22.
759 [31] Bo S, Ciccone G, Castiglione A, Gambino R, De Michieli F, Villois P, et al. Anti-
760 inflammatory and Antioxidant Effects of Resveratrol in Healthy Smokers. Current Medical
761 Chemistry 2012. [Epub ahead of print].

[32] Burgess TA, Robich MP, Chu LM, Bianchi C, Sellke FW. Improving glucose metabolism with resveratrol in a swine model of metabolic syndrome through alteration of signaling pathways in the liver and skeletal muscle. Archives Surgery 2011;146:556-64.

[33] Evliyaoğlu O, Kibrisli E, Yildirim Y, Gökalp O, Colpan L. Routine enzymes in the monitoring of type 2 diabetes mellitus. Cell Biochemistry and Function 2011;29:506-12.

[34] Yassin MM, Altibi HI, Elshanti AF. Clinical and biochemical features of type 2 diabetic patients in Gaza Governorate, Gaza Strip. West African Journal of Medicine 2011;30:51-6.

[35] Berberoglu Z, Gursoy A, Bayraktar N, Yazici AC, Bascil Tutuncu N, Guvener Demirag N. Rosiglitazone decreases serum bone-specific alkaline phosphatase activity in postmenopausal diabetic women. Journal of Clinical Endocrinology and Metabolism 2007;92:3523-30.

[36] Yoshino J, Conte C, Fontana L, Mittendorfer B, Imai S, Schechtman KB, et al. Resveratrol supplementation does not improve metabolic function in nonobese women with normal glucose tolerance. Cell Metabolism 2012;16:658-64.

[37] Poulsen MM, Vestergaard PF, Clasen BF, Radko Y, Christensen LP, Stodkilde.Jorgensen H, et al. High-Dose Resveratrol Supplementation in Obese Men: An Investigator-Initiated, Randomized, Placebo-Controlled Clinical Trial of Substrate Metabolism, Insulin Sensitivity, and Body Composition. Diabetes 2012. Doi: 10.2337/db12-0975.

[38] Alexandraki K, Piperi C, Kalofoutis C, Singh J, Alaveras A, Kalofoutis A. Inflammatory process in type 2 diabetes: The role of cytokines. Annals of the New York Academy of Sciences 2006;1084:89-117.

[39] Shen F, Chen SJ, Dong XJ, Zhong H, Li YT, Cheng GF. Suppression of IL-8 gene transcription by resveratrol in phorbol ester treated human monocytic cells. Journal of Asian Natural Products Research 2003;5:151-7.

788 [40] Richard N, Porath D, Radspieler A, Schwager J. Effects of resveratrol, piceatannol, tri-
789 acetoxystilbene, and genistein on the inflammatory response of human peripheral blood
790 leukocytes. Molecular Nutrition and Food Research 2005;49:431-42.
791 [41] Ghanim H, Sia CL, Abuaysheh S, Korzeniewski K, Patnaik P, Marumganti A, et al. An
792 antiinflammatory and reactive oxygen species suppressive effects of an extract of Polygonum
793 cuspidatum containing resveratrol. Journal of Clinical Endocrinology and Metabolism
794 2010;95:E1-8.
795 [42] Ghanim H, Sia CL, Korzeniewski K, Lohano T,Abuaysheh S, Marumganti A, et al. A
796 resveratrol and polyphenol preparation suppresses oxidative and inflammatory stress response
797 to a high-fat, high-carbohydrate meal. Journal of Clinical Endocrinology and Metabolism
798 2011;96:1409-14.
799 [43] De Groote D, Van Belleghem K, Devière J, Van Brussel W, Mukaneza A, Amininejad
800 L. Effect of the intake of resveratrol, resveratrol phosphate, and catechin-rich grape seed
801 extract on markers of oxidative stress and gene expression in adult obese subjects. Annuals of
802 Nutrition and Metabolism 2012;61:15-24.
803 [44] Arakawa R, Bagashev A, Song L, Maurer K, Sullivan KE. Characterization of
804 LRRFIP1. Biochemistry and Cell Biology 2010;88:899-906.
805 [45] Vogel C, Marcotte EM. Insights into the regulation of protein abundance from
806 proteomic and transcriptomic analyses. Nature Reviews Genetics 2012;13:227-32.
807 [46] Füth R, Herder C, Förster S, Müller-Scholze S, Kruse N, Rieckmann P, et al.
808 Evaluation of diagnostic relevance of mRNA levels in peripheral blood: predictive value for
809 mortality in hemodialysis patients. Cytokine 2004;27:166-72.
810 [47] Kempf K, Haltern G, Füth R, Herder C, Müller-Scholze S, Gülker H, et al. Increased
811 TNF-alpha and decreased TGF-beta expression in peripheral blood leukocytes after acute
812 myocardial infarction. Hormone and Metabolic Research 2006;38:346-51.

[48] Navarro JF, Mora C, Gómez M, Muros M, López-Aguilar C, García J. Influence of renal involvement on peripheral blood mononuclear cell expression behaviour of tumour necrosis factor-alpha and interleukin-6 in type 2 diabetic patients. Nephrology Dialysis Transplantation 2008;23:919-26.

[49] Ma X, Becker Buscaglia LE, Barker JR, Li Y. MicroRNAs in NF-kappaB signaling. Journal of Molecular Cell Biology 2011;3:159-66.

[50] Roggli E, Britan A, Gattesco S, Lin-Marq N, Abderrahmani A, Meda P, et al. Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells. Diabetes 2010;59:978-86.

[51] Jiang P, Liu R, Zheng Y, Liu X, Chang L, Xiong S, et al. MiR-34a inhibits lipopolysaccharide-induced inflammatory response through targeting Notch1 in murine macrophages. Experimental Cell Research 2012;318:1175-84.

[52] Small EM, Olson EN. Prevasive roles of microRNAs in cardiovascular biology. Nature 2011;469:336-42.

[53] Sun X, Icli B, Wara AK, Belkin N, He S, Kobzik L, et al. MicroRNA-181b regulates NF-κB-mediated vascular inflammation. Journal of Clinical Investigation 2012;122:1973-90.

[54] Byrd AE, Aragon IV, Brewer JW. MicroRNA-30c-2* limits expression of proadaptive factor XBP1 in the unfolded protein response. Journal of Cell Biology 2012;196:689-98.

[55] Marchand A, Proust C, Morange PE, Lompré AM, Trégouët DA. miR-421 and miR-30c inhibit SERPINE 1 gene expression in human endothelial cells. PLoS One 2012;7:e44532.

[56] Ni CW, Qiu H, Jo H. MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammatory response of endothelial cells. American Journal of Physiology - Heart and Circulatory Physiology 2011;300:H1762-9.

[57] Tili E, Croce CM, Michaille JJ. miR-155: on the crosstalk between inflammation and cancer. International Reviews of Immunology 2009;28:264-84.

838 [58] Kurowska-Stolarska M, Alivernini S, Ballantine LE, Asquith DL, Millar NL, Gilchrist
839 DS, et al. MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis.
840 Proceedings of the National Academy of Sciences 2011;108:11193-8.
841 [59] Stanczyk J, Pedrioli DM, Brentano F, Sanchez-Pernaute O, Kolling C, Gay RE, et al.
842 Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid
843 arthritis. Arthritis and Rheumatism 2008;58:1001-9.
844 [60] O'Connell RM, Kahn D, Gibson WS, Round JL, Scholz RL, Chaudhuri AA, Kahn ME,
845 Rao DS, Baltimore D. MicroRNA-155 promotes autoimmune inflammation by enhancing
846 inflammatory T cell development. Immunity 2010;33:607-19.
847 [61] Milenkovic D, Deval C, Gouranton E, Landrier J-F, Scalbert A, Morand C, Mazur A.
848 Modulation of miRNA expression by dieatry polyphenols in apoE deficient mice: a new
849 mechanism of the action of polyphenols. PLoS One 2012;7: e29837.
850 [62] Xiao Y, Xu C, Guan J, Ping Y, Fan H, Li Y, et al. Discovering dysfunction of multiple
851 microRNAs cooperation in disease by a conserved microRNA co-expression network. PLoS
852 One 2012;7:e32201.
853 [63] Nikolajczyk BS, Jagannathan-Bogdan M, Shin H, Gyurko R. State of the union
854 between metabolism and the immune system in type 2 diabetes. Genes and Immunity
855 2011;12:239-50.
856

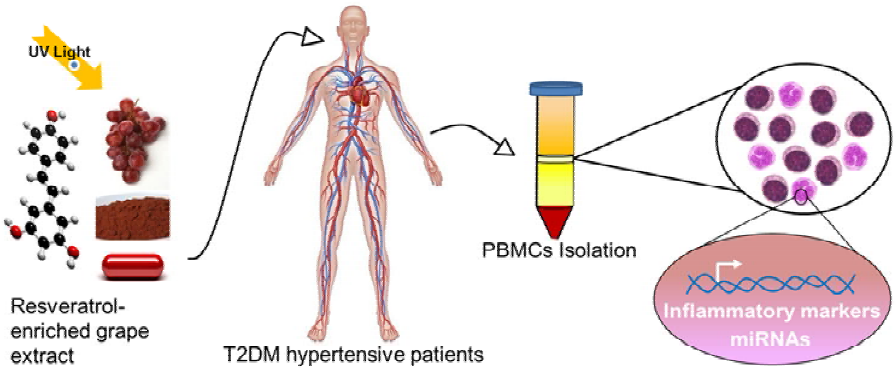

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Table 1. Baseline clinical characteristics of the hypertensive diabetic male patients taking part in the gene expression study.

Characteristics	Groups (at baseline)			
	Placebo (n=9)	GE (n=13)	GE-RES (n=13)	All (n=35)
Age, years	57 ! 10	60 ! 10	63 ! 12	60 ! 11
Weight, kg	86 ! 12	91 ! 19	84 ! 11	87 ! 14
BMI, kg/m^2	30.5 ! 3.8	32.2 ! 5.1	31.0 ! 5.1	31.3 ! 4.7
SBP, mmHg	129 ! 21	129 ! 20	130 ! 16	129 ! 18
DBP, mmHg	74 ! 13	73 ! 9	70 ! 10	72 ! 10
Heart rate, beats/min	63 ! 13	69 ! 9	64 ! 10	66 ! 10
LVEF (%)	55 ! 5	55 ! 5	55 ! 3	55 ! 4
Active smoking, n (%)	5 (56)	3 (33)	2 (22)	10 (29)
Diagnosed hypercholesterolemia, n (%)	9 (100)	10 (77)	12 (92)	31 (89)
Stable angina, n (%)	1 (11)	2 (15)	2 (15)	5 (14)
ST-segment elevation myocardial infarction, n (%)	3 (33)	6 (46)	5 (38)	14 (40)
non-ST-segment elevation acute coronary syndrome, n (%)	5 (56)	5 (38)	6 (46)	16 (46)
Myocardial revascularization, n (%)	7 (78)	11 (85)	12 (92)	30 (86)
Coronary stenting	5 (56)	8 (62)	9 (69)	22 (63)
Coronary artery bypass grafting	2 (22)	3 (23)	3 (23)	8 (23)
Ischemic ictus, n (%)	1 (11)	1 (8)	1 (8)	3 (9)
Intermittent claudication, n (%)	3 (33)	2 (15)	2 (15)	7 (20)
Family history of premature ischemic heart disease, n (%)	4 (44)	5 (38)	5 (38)	14 (40)
Antiplatelet therapy	9 (100)	13 (100)	13 (100)	35 (100)
Statins, n (%)	9 (100)	13 (100)	13 (100)	35 (100)
Atorvastatin	7 (20)	8 (23)	8 (23)	23 (66)
Rosuvastatin	1 (3)	4 (11)	-	5 (14)
Pravastatin	-	1 (3)	1 (3)	2 (6)
Fluvastatin	1 (11)	-	4 (11)	5 (14)
β-Blockers, n (%)	8 (89)	11 (85)	9 (69)	28 (80)
RAS-blockers, n (%)	9 (100)	13 (100)	13 (100)	35 (100)

Values are expressed as the mean value ± SD or n (%) (total number and percentage of volunteers per group). None of these characteristics presented statistically significant inter-group differences at baseline ($P < 0.05$). Placebo (maltodextrin); GE (grape extract); GE-RES (grape extract containing RES); BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVEF, left ventricular ejection fraction; RAS, renin-angiotensin system.

870 Table 2

871 Baseline serobiochemical variables, inflammatory and fibrinolytic markers of the recruited
872 hypertensive diabetic male patients taking part in the gene expression study.

Variables	Groups (at baseline)			
	Placebo	GE	GE-RES	All
Total cholesterol, mg/dL (131-	158 ! 39[a]	154 ! 36	165 ! 33	159 ! 35
HDL-cholesterol, mg/dL (35-67)	38 ! 6	42 ! 9	43 ! 6	41 ! 8
LDL-cholesterol, mg/dL (83-130)	86 ! 36	82 ! 29	90 ! 29	86 ! 30
Triglycerides, mg/dL (35-201)	191 ! 133	153 ! 52	166 ! 126	167 ! 105
Non-HDL-cholesterol, mg/dL	118 ! 20	114 ! 31	122 ! 31	118 ! 28
LDL-cholesterol/HDL-cholesterol	2.3! 0.9	2.0 ! 0.8	2.2 ! 0.5	2.1 ! 0.7
Glucose, mg/dL (73.8-100.1)	148 ! 69	132 ! 30	151 ! 54	143 ! 50
HbA1c, % (6.0-7.0)	7.0 ! 1.0	7.1 ! 1.3	7.4 ! 1.6	7.1 ! 1.3
GGT, U/L (2-30)	23 ! 7	23 ! 6	31 ! 14	26 ! 11
ALT, U/L (10-40)	44 ! 22	32 ! 20	62 ! 68	46 ! 46
AST, U/L (8-30)	30 ! 21	30 ! 12	38 ! 22	33 ! 18
ALP, U/L (90-360)	176 ! 27	174 ! 47	186 ! 62	180 ! 50
CPK, U/L (38-174)	125 ! 78	133 ! 109	160 ! 74	139 ! 118
Creatinin, mg/dL (0.68-1.26)	1.1 ! 0.3	1.1 ! 0.3	0.9 ! 0.2	1.0 ! 0.3
Albumin, g/dL (34-48)	45 ! 3	45 ! 2	45 ! 4	45 ! 3
Urate, mg/L (2.6-6.1)	6.6 ! 1.3	6.4 ! 1.7	5.6 ! 1.4	6.2 ! 1.5
TSH, mU/L (0.35-5.50)	2.3 ! 0.8	1.6 ! 0.6	2.1 ! 0.9	2.0 ! 0.8
T4, ng/dL (0.90-1.77)	1.3 ! 0.2	1.3 ! 0.2	1.2 ! 0.2	1.4 ! 0.2
Fibrinogen, g/L (2.0-4.0)	3.4 ! 0.4	3.6 ! 0.5	3.5 ! 0.6	3.5 ! 0.5
DD, mg/L (0.00-0.28)	0.14 ! 0.09	0.16 ! 0.09	0.19 ! 0.24	0.17 ! 0.16
hsCRP, mg/L (< 3 mg/L)	3.9 ! 2.4	3.3 ! 1.2	4.9 ! 4.5	4.0 ! 3.1
Adiponectin, µg/mL	11.2 ! 8.2	10.9 ! 5.2	10.3 ! 4.2	12.1 ! 6.0
PAI-1, ng/mL	26 ! 18	21 ! 18	17 ! 9	21 ! 14
TNF-α, pg/mL	12.5 ! 6.4	12.1 ! 6.6	11.7 ! 5.5	10.7 ! 5.6
IL-10, pg/mL	27 ! 12	16 ! 12	23 ! 16	21 ! 14
IL-6, pg/mL	2.4 ! 1.0	2.4 ! 2.0	3.0 ! 1.9	2.6 ! 1.8
IL-6/IL-10	0.11 ! 0.06	0.15 ! 0.12	0.16 ! 0.11	0.14 ! 0.11

873 [a]: Data are shown as the mean value ± SD. The established normal range of values is indicated
874 between brackets. None of these variables presented statistically significant inter-group
875 differences at baseline ($P < 0.05$). Placebo (maltodextrin); GE (grape extract); GE-RES (grape
876 extract containing RES); HDL, high density lipoprotein; LDL, low density lipoprotein; HbA1c,
877 glycated haemoglobin; GGT, γ-glutamyl transferase; AST, aspartate aminotransferase; ALT,
878 alanine aminotransferase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; TSH, thyroid

879 stimulating hormone; T4, thyroxine; DD, D-dimer; hsCRP, high-sensitivity C-reactive protein; PAI-

880 1, plasminogen activator inhibitor type-1; IL, interleukin; TNF-! , tumor necrosis factor alpha.

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883 Table 3

884 Serobiochemical variables, inflammatory and fibrinolytic markers of the hypertensive diabetic male

885 patients taking part in the gene expression study following the consumption of the placebo, GE or

Variables	Groups (after 12 months)					
	Placebo	*P*	GE	*P*	GE-RES	*P*
	(n=9)	(0-12m)	(n=13)	(0-12m)	(n=13)	(0-12m)
Total cholesterol, mg/dL (131-201)	165 ! 49[a]	0.47	154 ! 32	0.96	166 ! 38	0.89
HDL-cholesterol, mg/dL (35-67)	39 ! 5	0.30	41 ! 8	0.38	40 ! 7	0.13
LDL-cholesterol, mg/dL (83-130)	88 ! 43	0.88	81 ! 20	0.93	96 ! 28	0.50
Triglycerides, mg/dL (35-201)	196 ! 118	0.78	163 ! 84	0.55	148 ! 81	0.51
Non-HDL-cholesterol, mg/dL	123 ! 34	0.55	116 ! 26	0.80	121 ! 34	0.91
LDL-cholesterol/HDL-cholesterol	2.3 ! 1.1	0.92	2.0 ! 0.5	0.82	2.2 ! 0.5	0.97
Glucose, mg/dL (73.8-100.1)	140 ! 23	0.59	152 ! 65	0.21	148 ! 42	0.97
HbA1c, % (6.0-7.0)	7.4 ! 1.0	0.06	7.2 ! 1.2	0.69	7.6 ! 1.5	0.33
GGT, U/L (2-30)	25 ! 7	0.32	22 ! 7	0.64	29 ! 12	0.40
ALT, U/L (10-40)	28 ! 10	0.76	31 ! 14	0.81	34 ! 14	0.89
AST, U/L (8-30)	49 ! 23	0.33	31 ! 14	0.98	67 ! 71	0.36
ALP, U/L (90-360)	154 ! 38	0.15	146 ! 20	0.04	161 ! 69	0.02
CPK, U/L (38-174)	137 ! 91	0.37	92 ! 25	0.12	140 ! 70	0.20
Creatinin, mg/dL (0.68-1.26)	1.1 ! 0.4	0.76	1.1 ! 0.3	0.12	1.0 ! 0.2	0.32
Albumin, g/dL (34-48)	48 ! 3	0.73	46 ! 2	0.17	46 ! 2	0.59
Urate, mg/L (2.6-6.1)	6.7 ! 1.4	0.82	6.0 ! 1.4	0.22	6.2 ! 1.4	0.34
TSH, mU/L (0.35-5.50)	2.1 ! 0.6	0.32	1.8 ! 0.9	0.39	2.1 ! 1.0	0.83
T4, ng/dL (0.90-1.77)	1.3 ! 0.2	0.53	1.3 ! 0.2	0.72	1.2 ! 0.2	0.65
Fibrinogen, g/L (2.0-4.0)	3.5 ! 0.5	0.49	3.4 ! 0.9	0.26	3.5 ! 0.5	0.96
DD, mg/L (0.00-0.28)	0.14 ! 0.08	0.95	0.13 ! 0.08	0.31	0.13 ! 0.06	0.55
hsCRP, mg/L (< 3 mg/L)	4.5 ! 1.8	0.10	3.0 ! 1.2	0.46	3.7 ! 2.5	0.52
Adiponectin (! g/mL)	9.1 ! 5.9	0.03	11.1 ! 5.1	0.80	11.5 ! 4.9	0.13
PAI-1 (ng/mL)	32 ! 18	0.33	17 ! 10	0.82	16 ! 8	0.82
TNF-α, pg/mL	13.9 ! 6.9	0.16	11.9 ! 5.1	0.81	10.7 ! 5.8	0.22
IL-10, pg/mL	24 ! 14	0.04	15 ! 12	0.31	22 ! 16	0.92
IL-6, pg/mL	2.4 ! 1.0	0.89	2.4 ! 1.9	0.40	2.6 ! 1.9	0.00
IL-6/IL-10	0.14 ! 0.10	0.04	0.16 ! 0.11	0.13	0.15 ! 0.13	0.59

886 GE-RES for 12 months.

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888 [a]: Data are shown as the mean value ± SD. The established normal range of values is indicated

889 between brackets. Intra-group *P* values are also indicated (0-12m) and significant differences (*P* <

890 0.05) marked in bold. None of these variables presented statistically significant inter-group
891 differences after 12 months of supplementation ($P < 0.05$). Placebo (maltodextrin); GE (grape
892 extract); GE-RES (grape extract containing RES); HDL, high density lipoprotein; LDL, low density
893 lipoprotein; HbA1c, glycated haemoglobin; GGT, γ-glutamyl transferase; AST, aspartate
894 aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CPK, creatine
895 phosphokinase; TSH, thyroid stimulating hormone; T4, thyroxine; DD, D-dimer; hsCRP, high-
896 sensitivity C-reactive protein; PAI-1, plasminogen activator inhibitor type-1; IL, interleukin; TNF-! ,
897 tumor necrosis factor alpha.

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Table 4

Summary of the functional analysis performed with the IPA software and top regulated molecules with significant altered levels of expression in PBMCs from volunteers consuming GE-RES.

IPA Results	Genes most significantly modulated in PBMCs by GE-RES after 12 months (cut-off values: 1.5 and -1.5, adj. *P*-value < 0.05)
Top Bio Functions Diseases and Disorders Molecular and Cell Functions Physiological System Development and Function	 Inflammatory Response, Cardiovascular Disease, Inflammatory Disease Cellular Movement, Cell Death, Cell-to-Cell Signaling and Interaction Hematological System Development and Function, Immune Cell Trafficking, Cardiovascular System Development and Function
Top Canonical Pathways	Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F
Top downregulated molecules (fold- change by microarrays)	Cytokines involved in the inflammatory response: *CCL3* (- 5.93)[a] *TNF-α* (- 4.13) *IL-8* (- 3.503 *IL-1β* (- 3.46) *CXCL2* (- 2.29) MicroRNAs with a role in inflammation: *miR-155* (-1.75) *miR-34a* (-1.26) *EIF4B* (- 2.68) *RNASEK* (- 2.48) *BRK1* (- 2.48) *C14orf43* (- 2.38) *IER3* (- 2.11)
Top upregulated molecules (fold- change by microarrays)	MicroRNAs with a role in inflammation: *miR-21* (+ 2.24) *miR-181* (+ 2.14) *miR-186* (+ 1.91) *LRRFIP* (+ 1.85) *IFI44* (+ 1.79) *MT-ND6* (+ 2.11) RNA processing: *LUC7L3* (+ 1.86) *RNPC3* (+ 1.80) *SNRPA1* (+ 1.79)
Top Upstream regulators	NF-! B complex (predicted activation state: inhibited)
Target Molecules (genes that have expression direction consistent with inhibition of NF-! B complex)	*CCL3*, *TNF-α*, *IL-1β*, *IL-8*, *CXCL2*, *IER3*, *ZFP36* (all downregulated)

[a]: fold-change value, + upregulation; - downregulation. PBMCs, peripheral blood mononuclear cells; GE-RES, grape extract containing resveratrol; MT-ND6, NADH-ubiquinone

oxidoreductase chain 6; LUC7L3, cAMP regulatory element-associated protein 1; LRRFIP, Leucine-rich repeat flightless-interacting protein 2; RNPC3, RNA-binding protein 40; SNRPA1, U2 small nuclear ribonucleoprotein A; IFI44, Interferon-induced protein 44; CCL3, C-C motif chemokine 3; TNF-α, Tumor necrosis factor; IL-8, Interleukin-8; IL-1β, Interleukin-1 beta; CXCL2, C-X-C motif chemokine 2; EIF4B, Eukaryotic translation initiation factor 4B; RNASEK, Ribonuclease kappa; BRK1, Protein BRICK1; C14orf43, Uncharacterized protein C14orf43; IER3, Radiation-inducible immediate-early gene IEX-1; NF-! B, Nuclear factor NF-kappa-B; ZFP36, Zinc finger protein 823; miR, microRNA.

Table 5

Intra-group relative expression changes in PBMCs of selected genes involved in the regulation of the inflammatory response and Type-2 diabetes (T2D).

Gene	Biological function	Gene expression intra-group changes (ratio)								
		Placebo			GE			GE-RES		
		6 months *vs.* baseline	12 months *vs.* baseline	12 months *vs.* 6months	6 months *vs.* baseline	12 months *vs.* baseline	12 months *vs.* 6 months	6 months *vs.* baseline	12 months *vs.* baseline	12 months *vs.* 6 months
CCL3	Involved in chemotaxis of mononuclear cells.	0.86[a] (0.82-1.19)	1.02 (0.52-2.22)	1.29 (0.61-1.70)	1.11 (0.49-1.45)	0.58 (0.53-1.08)	0.67 (0.48-1.33)	0.96 (0.75-1.54)	0.61 (0.34-0.92)	0.63 (0.43-0.93)
		P=0.395	*P*=0.835	*P*=0.648	*P*=0.763	*P*=0.154	*P*=0.020	*P*=0.865	*P*=0.004	*P*=0.006
IL-1β	Critical early mediator of inflammation secreted by monocytes and macrophages. Regulates NFκB. Elevated levels in monocytes from T2D patients.	0.74 (0.07-1.50)	0.43 (0.21-1.04)	1.37 (0.33-4.83)	0.81 (0.16-2.11)	0.09 (0.04-0.84)	0.39 (0.08-1.06)	0.20 (0.04-2.71)	0.08 (0.03-0.31)	0.27 (0.06-1.16)
		P=0.134	*P*=0.070	*P*=0.551	*P*=0.326	*P*=0.003	*P*=0.037	*P*=0.170	*P*=0.000	*P*=0.008
TNF-α	Major adipocyte cytokine that can interfere with insulin signaling. Regulates NFκB. Elevated levels in monocytes from T2D patients.	0.83 (0.57-1.25)	0.80 (0.70-1.35)	1.14 (0.88-1.68)	0.78 (0.58-1.59)	0.86 (0.37-1.31)	0.62 (0.35-1.08)	0.39 (0.25-1.23)	0.65 (0.22-1.02)	0.71 (0.38-2.13)
		P=0.239	*P*=0.890	*P*=0.354	*P*=0.775	*P*=0.083	*P*=0.024	*P*=0.016	*P*=0.019	*P*=0.435
LRRFIP-1	Transcriptional regulator of Toll-like receptor signaling.	1.41 (0.65-2.00)	1.35 (0.69-2.27)	1.03 (0.96-1.14)	1.24 (0.96-1.74)	1.35 (0.97-1.77)	1.08 (0.72-1.47)	1.23 (0.89-1.59)	1.77 (0.98-2.27)	1.24 (1.16-1.62)
		P=0.358	*P*=0.261	*P*=0.277	*P*=0.326	*P*=0.116	*P*=0.585	*P*=0.125	*P*=0.009	*P*=0.002
NFκB-1	Major transcription regulator of inflammatory responses. Modulates the expression of cytokines such as TNF-α and IL-1β.	0.96 (0.70-1.06)	0.77 (0.48-1.01)	0.74 (0.52-1.30)	0.96 (0.73-1.09)	0.92 (0.61-1.16)	0.83 (0.74-1.31)	0.98 (0.80-1.20)	1.14 (0.69-1.38)	1.09 (0.94-1.37)
		P=0.137	*P*=0.045	*P*=0.245	*P*=0.249	*P*=0.122	*P*=0.428	*P*=0.769	*P*=0.698	*P*=0.355
NFκBIA	Inhibitor of dimeric NFκB/REL complexes. It degrades on cellular stimulation and allows the dimeric RELA to translocate to the nucleus.	0.90 (0.52-1.2)	0.77 (0.51-1.00)	1.18 (0.65-1.30)	0.78 (0.54-1.19)	0.63 (0.43-0.88)	0.77 (0.53-1.21)	0.97 (0.56-1.16)	0.74 (0.48-1.04)	0.88 (0.68-1.01)
		P=0.124	*P*=0.075	*P*=0.508	*P*=0.069	*P*=0.005	*P*=0.102	*P*=0.431	*P*=0.087	*P*=0.132

[a]: Results are displayed as the median (25^{th} to 75^{th} interquartile range); Placebo (maltodextrin, n=9); GE (grape extract, n=13); GE-RES (grape extract containing resveratrol, n=13); $P < 0.05$ was considered statistically significant (shaded cell); CCL3, chemokine (C-C motif) ligand 3; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor alpha; LRRFIP-1, leucine rich repeat flightless interacting protein 1; NFκB-1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105); NFκBIA, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα).

Table 6

Intra-group relative expression changes in PBMCs of selected microRNAs implicated in the regulation of the inflammatory response and Type-2 diabetes (T2D).

MicroRNA	Inflammatory related function	MicroRNA expression intra-group changes (ratio)								
		Placebo			GE			GE-RES		
		6 months *vs.* baseline	12 months *vs.* baseline	12 months *vs.* 6months	6 months *vs.* baseline	12 months *vs.* baseline	12 months *vs.* 6months	6 months *vs.* baseline	12 months *vs.* baseline	12 months *vs.* 6months
miR-21	Involved in the inflammatory response and the regulation of NF-κB and Toll-like receptors signaling, TNF-α and IL-1β.	1.52[a] (0.24-1.89) *P*=0.587	0.22 (0.17-0.37) *P*=0.000	0.15 (0.12-1.98) *P*=0.051	0.64 (0.26-1.13) *P*=0.041	0.61 (0.29-0.94) *P*=0.033	1.07 (0.70-1.95) *P*=0.736	0.82 (0.52-1.37) *P*=0.318	0.67 (0.22-3.34) *P*=0.652	1.27 (0.53-3.11) *P*=0.721
miR-181b	Involved in the regulation of NF-κB signaling and TNF-α.	0.36 (0.30-0.89) *P*=0.013	0.60 (0.37-1.07) *P*=0.179	1.37 (1.11-2.02) *P*=0.004	0.39 (0.28-1.02) *P*=0.012	0.69 (0.47-1.25) *P*=0.150	1.36 (0.66-3.21) *P*=0.128	0.49 (0.34-1.06) *P*=0.044	1.05 (0.51-1.95) *P*=0.645	1.71 (1.42-2.66) *P*=0.000
miR-155	Highly expressed in monocytes and macrophages. Involved in the regulation of NF-κB and Toll-like receptors signaling, TNF-α and IL-1β.	1.35 (0.73-2.11) *P*=0.709	0.86 (0.46-1.34) *P*=0.215	0.66 (0.44-1.05) *P*=0.060	0.48 (0.31-0.68) *P*=0.002	0.35 (0.23-0.72) *P*=0.001	0.65 (0.54-1.03) *P*=0.052	1.01 (0.47-1.21) *P*=0.325	0.80 (0.38-1.09) *P*=0.015	0.84 (0.50-1.30) *P*=0.113
miR-34a	Increased expression in T2D. Regulator of pancreatic cells function. Involved in the regulation of NF-κB signaling, TNF-α and IL-1β.	1.54 (0.40-3.89) *P*=0.568	0.70 (0.44-1.83) *P*=0.541	0.56 (0.49-1.23) *P*=0.130	1.03 (0.48-2.51) *P*=0.820	0.52 (0.35-1.80) *P*=0.327	0.75 (0.56-1.39) *P*=0.127	1.75 (0.83-2.18) *P*=0.056	0.61 (0.30-1.24) *P*=0.084	0.59 (0.21-0.88) *P*=0.004
miR-663	It may target transcripts encoding factors involved in the immune response known to activate cytokines.	1.61 (0.44-2.18) *P*=0.859	0.55 (0.21-1.31) *P*=0.086	0.46 (0.35-0.76) *P*=0.021	0.61 (0.42-0.88) *P*=0.006	0.66 (0.49-1.17) *P*=0.055	1.42 (0.60-1.84) *P*=0.753	0.74 (0.52-1.26) *P*=0.087	1.10 (0.49-2.19) *P*=0.861	1.15 (0.78-2.60) *P*=0.221
miR-30c2	Induced by NF-κB. Associated to diabetes.	0.62 (0.17-0.94) *P*=0.088	0.14 (0.11-0.43) *P*=0.000	0.27 (0.19-2.40) *P*=0.171	0.18 (0.12-0.63) *P*=0.000	0.30 (0.15-1.09) *P*=0.007	1.54 (0.61-2.95) *P*=0.293	0.36 (0.24-0.85) *P*=0.043	0.45 (0.21-4.06) *P*=0.761	1.42 (0.64-6.37) *P*=0.095

[a]: Results are displayed as the median (25th to 75th interquartile range); Placebo (maltodextrin, n=9); GE (grape extract, n=13); GE-RES (grape extract containing resveratrol, n=13); $P < 0.05$ was considered statistically significant (shaded cell); miR, microRNA.