SUPPLEMENTARY METHODS AND TABLES

Grape Resveratrol Increases Serum Adiponectin and Downregulates Inflammatory Genes in Peripheral Blood Mononuclear Cells: A Triple-Blind, Placebo-Controlled, One-Year Clinical Trial in Patients with Stable Coronary Artery Disease.

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Supplementary Methods

Peripheral blood mononuclear cells isolation

EDTA blood (BD Vacutainer, Franklin Lakes, NJ, USA) was 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. The total number of cells isolated ($11.1\pm3.9 \times 10^6$, n=54) and their viability (95-100%) were estimated by Trypan blue. Isolated PBMCs were also analyzed by flow cytometry (FACSort, BD, San José, CA, USA) using size and granularity to estimate the proportion of lymphocytes, monocytes and granulocytes. Cell types' percentage (mean ± SD) was: lymphocytes, 84.4±2.9; monocytes, 13.0±3.2 and granulocytes, 2.2±1.0. PBMCs cells were obtained at baseline, after 6 months and at the end of the intervention (12 months). Cells were washed (×2) with phosphate buffer solution (PBS), lysed in RLT buffer (Qiagen, Madrid, Spain) and stored at –80ºC for RNA extraction.

RNA extraction protocols

Total RNA was 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). Only samples with a ratio Abs_{260}/Abs_{280} between 1.8 and 2.1 were used in microarray experiments. The integrity of the ribosomal RNA was further checked using agarose gel electrophoresis (1%). Pure RNA samples were divided in aliquots and frozen at –80ºC until further analysis.

Human microarray analyses

A search for potential candidate genes expressed in the PBMCs for which transcription levels may have been modulated after the intake of GE or GE-RES was performed using 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. For microarray analyses a subpopulation of 18 male diabetic and hypertensive individuals (6 patients from each group) was selected from the total population of participants at the 3 different time points (baseline, after 6 months and after 12 months). Microarrays were performed on samples from individual patients (not pooled) for a total number of 54. 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 45ºC onto the chips. GeneChips were washed and stained in the Affymetrix Fluidics Station 450 and scanned using the GeneChip Scanner 3000.

Bioinformatic tools for statistical analysis

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)[1] implemented with Babelomics (http://babelomics.bioinfo.cipf.es/) which performs well for small n microarrays.[2] Significant changes were defined as those with an adjusted P-value <0.05 (FDR, false discovery rate: 5%) and with ratios >1.2 (up-regulation) and <-1.2 (down-regulation).

Identification of biological functions and pathway construction

Data sets (differentially expressed genes) containing gene identifiers (Affymetrix probe sets) and corresponding expression values were uploaded into Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, Redwood City, CA), a web-based biological data analysis application. Within IPA and based on prior knowledge stored in the Ingenuity Knowledge Base (IPKB), the Transcription Factor Analysis (TFA) identifies a number of putative upstream transcriptional regulators that may be activated or inactivated and implicated in the observed gene expression changes. The TFA tool provides a z-score that determines whether a transcriptional regulator has significantly more 'activated' predictions (z>0) or 'inhibited' predictions (z<0), where significance means that the observed number of 'activated' or 'inhibited' predictions are unlikely relative to randomly chosen predictions. In practice, z-scores greater than 2 or smaller than -2 are considered significant. IPA can visualize the networks of putative modulated regulators and their respective targets as well as their interactions to provide testable hypothesis for gene regulation in response to treatment. MIAME compliant data have been submitted to the Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE36930.

References for Supplementary Methods (these references are not cited in the main text)

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Supplementary Tables

Supplementary Table 1 Body mass index, blood pressure and serobiochemical variables after 12 months

	Experimental values after 12 months and intragroup *p*-values			Intergroup *p*-values (12 months)		
	A (Placebo)	B (GE)	C (GE-RES)	AvsB	AvsC	BvsC
Body mass index, kg/m^2	30.7±4.0 *p*=0.94	31.4±4.7 *p*=0.16	30.3±5.9 *p*=0.55	0.09	0.70	0.59
Systolic blood pressure, mmHg	130.6±19.5 *p*=0.33	135.7±22.9 *p*=0.07	134.9±22.6 *p*=0.43	0.38	0.21	0.64
Diastolic blood pressure, mmHg	73±10 *p*=0.78	72±9 *p*=0.51	74±12 *p*=0.24	0.57	0.69	0.30
Heart rate, beats per min	63±9 *p*=0.06	59±1 *p*=0.07	61±7 *p*=0.26	0.13	0.26	0.57
Total cholesterol, mg/dL (131-201)	168.8±38.7 *p*=0.29	157.1±33.5 *p*=0.20	161.0±37.2* *p*=0.02 ↓2.7%	0.01*	0.03*	0.48
HDLc, mg/dL (35-91)	41.3±7.9 *p*=0.22	42.7±8.1 *p*=0.68	42.4±8.1 *p*=0.11	0.85	0.78	0.60
LDLc, mg/dL (83-130)	89.9±37.1 *p*=0.82	83.0±25.3 *p*=0.56	91.4±29.5 *p*=0.07	0.45	0.87	0.34
LDLc/HDLc	2.3±0.2 *p*=0.70	2.0±0.1 *p*=0.65	2.2±0.1 *p*=0.32	0.25	0.77	0.36
Non-HDLc, mg/dL	127±8 *p*=0.31	114±7* *p*=0.01 ↓10.2 %	119±7* *p*=0.03 ↓13.4 %	0.22	0.39	0.68
Triglycerides, mg/dL (35-201)	155±85 *p*=0.28	142±51 *p*=0.65	124±52 *p*=0.96	0.45	0.17	0.53
Fibrinogen, g/L (2-4.5)	3.4±0.5 *p*=0.39	3.4±0.6 *p*=0.36	3.6±0.5 *p*=0.35	0.88	0.25	0.31
D-Dimer, mg/L (0-0.3)	0.14±0.08 *p*=0.73	0.13±0.08 *p*=0.95	0.12±0.07 *p*=0.52	0.93	0.69	0.75
GGT, U/L (1-24)	41±22 *p*=0.61	38±27 *p*=0.67	40±38 *p*=0.25	0.85	0.65	0.51
AST, U/L (8-30)	27±9 *p*=0.18	24±7 *p*=0.79	31±11 *p*=0.85	0.90	0.33	0.25
ALT, U/L (7-35)	30±12 *p*=0.53	31±15 *p*=0.36	32±14 *p*=0.72	0.73	0.44	0.65
LDH, U/L (208-378)	309±62 *p*=0.71	333±41 *p*=0.22	348±44 *p*=0.30	0.45	0.21	0.59
ALP, U/L (70-290)	162±51* *p*=0.01 ↓12%	164±40 *p*=0.15	161±45 *p*=0.05	0.28	0.21	0.91
CPK, U/L (26-140)	169±132 *p*=0.11	96±47 *p*=0.21	129±44 *p*=0.30	0.18	0.58	0.44
Glucose, mg/dL (74-100)	123.0±34.0* *p*=0.00 ↑18%	129.2±28.3 *p*=0.14	115.5±24.0 *p*=0.87	0.17	0.00*	0.09
TSH, mU/L (0.35-5.5)	2.2±0.8 *p*=0.35	1.9±1.1 *p*=0.15	1.7±1.1 *p*=0.59	0.52	0.17	0.51
GlHB, % (6-7)	6.8±1.6* *p*=0.00 ↑8.5%	6.6±0.6 *p*=0.10	6.6±0.8 *p*=0.32	0.01*	0.00*	0.51
T4, ng/dL (0.9-1.8)	1.2±0.1 *p*=0.08	1.2±0.1 *p*=0.97	1.2±0.2 *p*=0.63	0.56	0.83	0.72
Bilirubin, mg/dL (0.3-1.2)	0.5±0.1 *p*=0.33	0.6±0.2 *p*=0.251	0.5±0.2* *p*=0.04↓12.7%	0.28	0.49	0.08
Creatinin, mg/dL (0.5-1.1)	1.0±0.3 *p*=0.90	0.9±0.2 *p*=0.44	0.9±0.2 *p*=0.31	0.95	0.62	0.65
Urate, mg/dL (2.6-6.1)	6.8±2.1 *p*=0.35	6.0±1.3 *p*=0.61	5.9±1.5 *p*=0.41	0.64	0.50	0.70
Albumin, g/L (34-48)	42.2±2.8 *p*=0.34	46.4±3.2* *p*=0.00 ↑4.6%	45.1±2.4 *p*=0.13	0.03*	0.56	0.11

Values are expressed as mean±SD. GE, conventional grape extract; GE-RES, resveratrol-rich grape extract. *Significant differences ($p<0.05$). The arrows designate the % of increase/decrease with respect to baseline values. Statistical analysis was carried out with the covariates described in the Methods section. *HDLc* high density lipoprotein-cholesterol, *LDLc* low density lipoprotein-cholesterol, *GGT* gamma-glutamyl transferase, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *CPK* creatine phosphokinase, *GlHB* glycated hemoglobin, *TSH* thyroid stimulant hormone, *T4* thyroxin

Supplementary Table 2 Differentially expressed genes in the extracellular space as shown in Figure 4 and linked to regulation by the transcription factors listed in Table 4.

		Fold change					
		After 6 months			After 12 months		
Entrez Gene Name	**Symbol**	**A**	**B**	**C**	**A**	**B**	**C**
Interleukin 24	*IL24*	NC	NC	-1.4	-1.4	-1.3	**-1.5***
Natriuretic peptide A	*NPPA*	NC	NC	NC	-1.2	-1.24	**-1.4***
Cardiotrophin-like cytokine factor 1	*CLCF1*	NC	NC	NC	NC	NC	**-1.3***
Placental growth factor	*PGF*	NC	NC	NC	NC	NC	**-1.8***
Chemokine (C-C motif) ligand 22	*CCL22*	NC	NC	NC	-1.2	NC	**-1.2***
Chemokine (C-X3-C motif) ligand 1	*CX3CL1*	NC	NC	NC	NC	NC	**-1.4***
Interleukin 17C	*IL17C*	NC	NC	NC	NC	NC	**-1.4***
Insulin-like growth factor binding protein 4	*IGFBP4*	NC	NC	NC	-1.2	NC	**-1.3***
Gastrin	*GAST*	NC	**-1.4***	NC	-1.3	-1.3	**-1.3***
Chemokine (C-C motif) ligand 3	*CCL3*	-2.3	-1.5	**-5.3***	-2.9	-3.4	**-5.9***
Melanoma inhibitory activity	*MIA*	-1.2	NC	NC	NC	NC	**-1.4***
Wingless-type MMTV integration site family, member 10A	*WNT10A*	NC	NC	NC	NC	-1.2	**-1.2***
Surfactant protein B	*SFTPB*	NC	NC	NC	NC	NC	**-1.3***
Interleukin 1, beta	*IL-1β*	-1.9	NC	-2.3	-3.6	-2.9	**-3.5***
Collagen, type XVIII, alpha 1	*COL18α1*	NC	NC	NC	NC	NC	**-1.2***
Thyrotropin-releasing hormone	*TRH*	NC	NC	NC	NC	NC	**-1.2***
Interleukin 8	*IL-8*	-2.7	NC	-2.9	-3.0	-2.1	**-3.5***
Chemokine (C-X-C motif) ligand 6	*CXCL6*	NC	NC	NC	NC	NC	**-1.2***
Chemokine (C-X-C motif) ligand 2	*CXCL2*	-3.2	NC	-2.4	-3.4	-1.7	**-2.3***
Interleukin 3	*IL3*	NC	NC	NC	NC	NC	**-2.4***
Interleukin 13	*IL13*	NC	NC	NC	NC	NC	**-1.3***
Tumor necrosis factor	*TNF*	-1.3	NC	**-3.2***	-1.8	-2.0	**-4.1***
Interleukin 17A	*IL17A*	NC	NC	NC	NC	NC	**-1.3***
Connective tissue growth factor	*CTGF*	NC	NC	NC	NC	NC	**-1.2***
Sonic hedgehog	*SHH*	NC	NC	NC	NC	NC	**-1.3***
Interferon, beta 1, fibroblast	*IFNβ1*	NC	NC	NC	NC	NC	**-1.2***
Lymphotoxin alpha (TNF superfamily, member 1)	*LTA*	NC	NC	NC	NC	-1.3	**-1.3***

A Placebo group, B GE group (conventional grape extract), C GE-RES group (resveratrol-rich grape extract), NC no change. Fold change cut off > 1.2. *Adjusted $p<0.05$

Supplementary Table 3 Description of the extracellular space genes related to inflammatory transcription factors shown in Figure 4 and listed in Table 4.

Symbol	Description	References (Suppl. Table 3)
IL24	Cytokine involved in cell survival and proliferation	1
NPPA	Plays a key role in cardiovascular homeostasis	2
CLCF1	Cytokine from IL-6 family with B-cell stimulating capability. It induces IL1β and serum amyloid A.	3
PGF	Stimulates proliferation and migration of endothelial cells. It induces PAI-1 through AP1 and JUN pathways. Present in unstable plaque.	4
CCL22	Chemokine that may play a role in the trafficking of activated T lymphocytes to inflammatory sites	5
CX3CL1	Fractalkine. Chemokine that elicits leukocyte adhesive and migratory functions. It is overexpressed in unstable angina and promotes unstable plaque	6
IL17C	Cytokine that stimulates the release of TNFα and IL1β from the monocytic cell line THP-1	7
IGFBP4	Related to Wnt/β-catenin pathway in cancer promotion. Stimulates circulating human hematopoietic stem and progenitor cells CD34/CD133.	8
GAST	Acts as a potent cell-growth factor	9
CCL3	Overexpressed in AMI and unstable angina with poor prognosis. Recruitment and activation of polymorphonuclear leukocytes	10
MIA	Growth regulating protein involved in cell proliferation	11
WNT10A	Involved in the cellular response to transforming growth factor β stimulus	12
SFTPB	Circulating SFTPB levels are increased in patients with Chronic Heart Failure	13
IL-1β	Cytokine involved in cell proliferation, differentiation and apoptosis. Important mediator of the inflammatory response	14
COL18A1	Endostatin precursor, a potent antiangiogenic protein leads to increased leukocyte-vessel wall interactions. Higher levels in diabetics with CAD.	15
TRH	Among other roles, evidence supports a critical role in the T-cell dependent immune response	16
IL-8	Chemoattractant cytokine that is also a potent angiogenic factor. Important mediator of the inflammatory response	17
CXCL6	Chemoattractant chemokine for neutrophilic granulocytes	18
CXCL2	Chemotactic chemokine for polymorphonuclear leukocytes and hematopoietic stem cells	19
IL3	Cytokine that could stimulate colony formation by macrophages, granulocytes, eosinophils, mast cells, among others	20
IL13	Cytokine with a potent immunoregulatory role	21
TNF	Cytokine involved in the regulation of several biological processes including cell proliferation, differentiation and apoptosis	22
IL17A	Cytokine that modulates immune cell trafficking and may contribute to atherosclerosis and plaque instability	23
CTGF	Growth factor that can promote endothelial cell growth, migration, adhesion and survival	24
SHH	Potent chemoattractant for monocytes. Involved in inflammation via NF-κB. Promotes vascular smooth muscle cells proliferation.	25
IFNβ1	Type I IFNs, among other effects, activate macrophages and NK cells and promote T cell survival	26
LTA	Cytokine that mediates a large variety of inflammatory, immunostimulatory, and antiviral responses	27

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