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RESEARCH ARTICLE

Consumption of a grape extract supplement containing resveratrol decreases oxidized LDL and ApoB in patients undergoing primary prevention of cardiovascular disease: A triple-blind, 6-month follow-up, placebo-controlled, randomized trial

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Scope: The cardioprotective role of resveratrol as part of the human diet is not yet clear. Our aim was to investigate the effect of a grape supplement containing 8 mg resveratrol in oxidized LDL (LDLox), apolipoprotein-B (ApoB), and serum lipids on statin-treated patients in primary cardiovascular disease prevention (PCP).

Methods and results: A triple-blind, randomized, placebo-controlled trial was conducted. Seventy-five patients (three parallel arms) consumed one capsule (350 mg) daily for 6 months containing resveratrol-enriched grape extract (GE-RES, Stilvid®), grape extract (GE, similar polyphenolic content but no resveratrol), or placebo (maltodextrin). After 6 months, no changes were observed in the placebo group and only LDL cholesterol (LDLc) decreased by 2.9% ($p = 0.013$) in the GE group. In contrast, LDLc (−4.5%, $p = 0.04$), ApoB (−9.8%, $p = 0.014$), LDLox (−20%, $p = 0.001$), and LDLox/ApoB (−12.5%, $p = 0.000$) decreased in the Stilvid® group, whereas the ratio non-HDLc (total atherogenic cholesterol load)/ApoB increased (8.5%, $p = 0.046$). No changes were observed in hepatic, thyroid, and renal function. No adverse effects were observed in any of the patients.

Conclusion: This GE-RES reduced atherogenic markers and might exert additional cardioprotection beyond the gold-standard medication in patients from PCP. The presence of resveratrol in the GE was necessary to achieve these effects.

Keywords:
Cardiovascular / Clinical trial / Nutraceutical / Polyphenol / Resveratrol

1 Introduction

Cardiovascular diseases (CVDs) are the leading cause of death globally. According to the World Health Organization (WHO), an estimated 17.1 million people died from CVDs in 2004 (29% of all global deaths). Unfortunately, these estimations will be even worse since by 2030 almost 24 million people will die from CVDs according to WHO. However, despite these data, results of long-term prospective studies identify low risk factors in people having healthy lifestyles

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Abbreviations: **ApoB**, apolipoprotein B; **GE**, grape extract; **GE-RES**, resveratrol-enriched grape extract (Stilvid®); **HED**, human equivalent dose; **LDLox**, oxidized low-density lipoprotein; **non-HDLc**, whole atherogenic fraction of cholesterol (Tchol–LDLc); **PCP**, primary cardiovascular disease prevention; **Tchol**, total cholesterol

such as regular physical activity, not smoking, moderate alcohol intake, and following healthy diets (high fruit and vegetable consumption and low fat, red meat, and refined sugars intake). Therefore, the evidence that many CVDs are preventable continues to grow [1]. In this context, it is essential the assessment, management, and follow-up of people who may be at risk for CVDs (i.e. hypertensives, diabetics, familial hyperlipidemia, or strong family history of CVD) but who have not yet manifested any type of CVD event. These patients belong to the so-called primary prevention of CVD (primary cardiovascular disease prevention, PCP). A number of guides have arisen to assist primary care [1, 2]. These guides deal with risk intervention and goals mainly related to smoking, blood pressure, dietary intake, physical activity as well as blood lipid, weight, and diabetes management [1, 3]. These risk factors should not be considered in isolation as they are used to estimate 10-year CVD risk according to different risk-charts such as the American Farmighan or the European SCORE (Systematic Coronary Risk Evaluation).

In PCP, a primary goal is blood lipid management since the evidence showing that reducing circulating total cholesterol (Tchol) and LDL cholesterol (LDLc) reduces cardiovascular risk is well documented [3, 4]. Therefore, management of Tchol and LDLc levels is the primary target of therapy. Besides Tchol and LDLc, the total number of atherogenic particles in plasma, i.e. the non-HDLc load, (VLDL + IDL + LDL), has been suggested as a better risk estimation parameter compared with LDLc [5]. In addition, apolipoprotein B (ApoB), the major lipoprotein of the atherogenic families VLDL, IDL, and LDL, is a good estimate of the number of these particles in plasma and has been reported to be a better risk marker and treatment target than LDLc [6] or non-HDLc [7]. Moreover, small LDL particles are easily oxidized to yield the atherogenic oxidized LDL (LDLox) particles that can accumulate in the foam cells of atherosclerotic lesions [8] and can trigger a number of effects including endothelial activation and smooth muscle cell proliferation [9]. Although LDLox particles can be detected in healthy people [10], elevated levels of LDLox particles in blood stream have been reported to be associated with cardiovascular risk [11, 12].

Statins, inhibitors of hydroxyl-3-methyl-glutaryl-CoA (HMG-CoA), are the lipid-lowering drugs of first choice [3]. In addition, statins can also affect ApoB and LDLox levels but with discrepant results [13, 14]. Nevertheless, the action of statins on serum ApoB or LDLox is not as remarkable as their reduction effect on LDLc or non-HDLc levels. Therefore, patients with reduced LDLc concentration may still have high levels of small LDL particles, leaving them with potential residual risk [15]. Bearing all this in mind, the question is: can a dietary intervention improve serum lipid markers such as LDLox and ApoB beyond statins action in PCP patients?

The grape and wine polyphenol resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) has been reported to exert cardioprotective effects among many other health-promoting properties [16]. However, the current evidence is based on rather pharmacological approaches due to the use of quite unrealistic doses (hundreds of milligrams or grams) that cannot be reached in a common diet [17]. In addition, although the use of high resveratrol doses has been reported to be safe in short-term trials with low number of individuals [18], the safety of long-term consumption of high resveratrol doses has not yet been assessed.

The presence of resveratrol in the general population diet is almost negligible [19], mainly due to the highly variable content of resveratrol in red wine, the main dietary source of this molecule [20]. Taking into account that resveratrol is a phytoalexin (i.e. a stress-inducible metabolite synthesized by the plant to face unfavorable environmental conditions), its content can be increased in grapes under controlled ultraviolet illumination [21], which results in low (mg/g) but safe resveratrol-enriched grape extracts with standardized contents [22].

Randomized human clinical trials with resveratrol are very scarce, including those looking into cardiovascular effects [16], and especially with low resveratrol doses [23]. Therefore, there is a need to ascertain the relevance of resveratrol from a dietary point of view as cardioprotective compound. Whether a low resveratrol dose exerts further beneficial health effects on statin-medicated patients at high cardiovascular risk is not known and involves a real challenge.

Our study was a 6 months follow-up, randomized, triple-blind, placebo-controlled trial with three parallel arms in which the aim was to evaluate the effect of a resveratrol-enriched grape supplement (Stilvid®) on serum ApoB and LDLox, as primary outcomes, in statin-treated patients from PCP ($n = 75$).

2 Materials and methods

2.1 Study products

The resveratrol-enriched grape extract (GE-RES, Stilvid®) used in this study was obtained from grapes after postharvest controlled UV illumination [20] following a patented procedure (WO 02/085137, ES 2177465). The conventional grape extract (GE) was also obtained from the same grapes but without UV treatment. Both extracts, GE and GE-RES, were kindly provided by Actafarma S.L. (Pozuelo de Alarcon, Madrid, Spain). Stilvid® is the most relevant ingredient included in the formulation of the commercially available nutraceutical Revidox® (Actafarma S.L.). Both extracts, GE and GE-RES, contained approximately similar polyphenolic content, i.e. ∼110 mg/g procyanidins, ∼70 mg/g anthocyanins, ∼3 mg/g flavonols, and ∼2 mg/g hydroxycinnamic acids. However, GE-RES were obtained from UVC-treated grapes to induce resveratrol and allowing an enrichment of resveratrol in the extract depending on the purification grade (approximately from 4 to 80 mg/g RES; communication from Actafarma S.L.). The specific GE-RES extract used in this

Table 1. Demographic characteristics and laboratory values of patients in primary cardiovascular disease prevention (PCP) at the inclusion

	Values			*p* values at baseline		
	A ($n = 25$)	B ($n = 25$)	C ($n = 25$)	A versus B	A versus C	B versus C
Age	63±9	56±11	62±9	$\mathbf{p = 0.04}$	$p = 0.99$	$p = 0.06$
Gender (Male %)	52	56	28	$p = 0.84$	$p = 0.10$	$p = 0.07$
BMI (kg/m^2)	29.4±3.4	31.0±5.2	32.1±8.7	$p = 0.11$	$p = 0.67$	$p = 0.31$
Systolic blood pressure (mm Hg)	129±18	132±18	130±16	$p = 0.99$	$p = 0.73$	$p = 0.71$
Diastolic blood pressure (mm Hg)	76±10	77±11	76±9	$p = 0.66$	$p = 0.85$	$p = 0.54$
Heart rate (beats per min)	68±10	67±9	66±8	$p = 0.90$	$p = 0.52$	$p = 0.60$
Diabetes mellitus (%)	36	48	44	$p = 0.51$	$p = 0.67$	$p = 0.82$
Hypertension (%)	92	80	87	$p = 0.55$	$p = 0.55$	$p = 1.00$
Smoking (%)	52	64	48	$p = 0.71$	$p = 0.55$	$p = 0.34$
Antiaggregants (%)	40	56	44	$p = 0.69$	$p = 0.83$	$p = 0.55$
ASA	36	52	40	$p = 0.68$	$p = 0.83$	$p = 0.53$
Clopidogrel	4	4	4	$p = 1.00$	$p = 1.00$	$p = 1.00$
Statins (%)	100	100	100	$p = 1.00$	$p = 1.00$	$p = 1.00$

A, placebo group (maltodextrin); **B**, grape extract group (GE); **C**, resveratrol-enriched grape extract group (Stilvid®). ASA, acetyl salicylic acid. Baseline values of lipid markers are included in Table 2. Values are expressed as mean ± SD. Significant differences ($p < 0.05$) are boldfaced.

trial contained ~23 mg/g resveratrol as well as other minor stilbenes such as astringin, piceatannol, δ-viniferin, and *trans*-piceid that were present at trace levels. Both extracts were analyzed using HPLC-MS/MS and their qualitative/quantitative composition as well as the analytical procedure is detailed in the Supporting Information Table 1. The placebo consisted of maltodextrin (Amylum Slovakia, Boleráz, Slovakia).

All the products were encapsulated in similar hard gelatin capsules. The capsules (370 mg) contained 350 mg of either placebo (maltodextrin), GE, or GE-RES, plus magnesium stearate and SiO_2 as excipients. The capsule blisters were labeled with the codes A, B, or C and packed in blank boxes with the corresponding codes. The patients, cardiologists, and scientists in charge of determinations were blind to the treatments along the trial.

2.2 Subjects and study design

The study was a 6 months follow-up randomized, triple-blind, placebo-controlled trial with three parallel arms. This trial is registered at clinicaltrials.gov as NCT01449110. The study was included in the Spanish National Research Project BFU2007-60576 and was carried out according to the principles outlined in the Declaration of Helsinki and its amendments. 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 Bioethics Committee-CSIC (Madrid, Spain). The trial (recruiting and follow-up) was conducted between April 2009 and October 2010.

Patients undergoing PCP were recruited for the study in the Cardiology Service from the Morales Meseguer University Hospital. All patients signed a written consent in which all the information concerning the trial was explained. The inclusion criteria for participating were Diabetes mellitus (DM) or hypercholesterolemia under statin treatment with at least one of the following risk factors: active tobacco smoking, arterial hypertension (≥140/90 mm Hg), and/or overweight obesity (body mass index [BMI], >30 kg/m^2). Exclusion criteria included age below 18 or above 80 years, pregnancy, known grape allergy, use of food complements, documented CVD (coronary acute syndrome, stable ischemic cardiopathy, periferic arteriopathy, and cerebrovascular diseases), or other known chronic pathology.

Group size was based on power calculations using a power of 80% and a two-sided test with an α of 0.05 on the variable ApoB. To show a difference of 10% for ApoB, we needed a sample size of 20 patients. Seventy-five eligible patients were randomly distributed in three groups (25 patients each, to compensate any possible dropout), i.e. A (placebo), B (GE), and C (GE-RES, Stilvid®). Table 1 shows the baseline characteristics of the 75 patients. All patients completed the study.

2.3 Study procedures

The participants were requested to consume daily one capsule of the study products as well as to keep their medication (Table 1), habitual lifestyle, and diet throughout the 6 months trial. Study products provision was calculated for the whole study. Three boxes of 60 capsules were provided to each patient. Compliance checks and possible incidences (adverse effects, nonclinical outcomes, etc.), possible changes in their dietary habits, etc. were monitored with questionnaires and through phone calls along the study, and at the intermediate and at the final citation. The subjects were instructed to fast overnight before each blood collection.

2.4 Sampling procedure

Blood samples were collected before (baseline) and after the 6 months intervention period. Biochemical parameters were determined in serum using an automated biochemical auto-analyzer (Advia Systems, Siemens Healthcare Diagnostic Inc., Deerfield, IL). The tests included the measurement of glucose, creatinine, albumin, bilirubin, Tchol, HDL-cholesterol (HDLc), LDLc, triglycerides, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), creatine phosphokinase (CPK), and urate. Non-HDLc was calculated as Tchol-LDLc. Coagulation parameters were determined in serum using an ACL TOP 700 analyzer (Instrumentation laboratory, Lexinton, MA,USA).

Thyroxine (T4) and stimulant hormone of the thyroid gland (TSH) were measured in an Advia Centaur XP system (Siemens Healthcare Diagnostic).

Hematological parameters were determined using an automated hematological analyzer (LH 780 Beckman Coulter, Fullerton, CA,USA). The parameters analyzed were: red blood cells, globular sedimentation velocity, hemoglobin concentration, glycated hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, mean platelet volume, leucocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

ApoB was determined by immunoturbidimetry (Siemens Healthcare Diagnostics, Barcelona, Spain) and LDLox by ELISA, using the mAb-4E6 antibody (Mercodia AB, Uppsala, Sweden), according to manufacturers' specifications.

Plasma (300 μL) and LDL particles (500 μL) were obtained and processed according to Azorín-Ortuño et al. [22] to detect phenolics (including resveratrol) and derived metabolites. Detection was achieved using LC-DAD-ESI ion trap and UHPLC-triple quadrupole (QqQ) MS detection.

The LC-DAD-ESI ion trap system was a 1100 series HPLC-DAD device (Agilent Technologies, Waldbronn, Germany) equipped with an ion-trap mass spectrometer (Agilent). This equipment was used to detect flavonol, anthocyanin, flavan-3-ol metabolites, as well as their gut microbiota-derived phenolic acids. Conditions were those previously described by Tomás-Barberán et al. [24].

The UHPLC-MS triple quadrupole system (QqQ) consisted of a 1290 Infinity HPLC series (Agilent) equipped with a 6460 series triple quadrupole mass spectrometer (Agilent). Grape-derived metabolites were not assayed in the QqQ due to the lack of suitable standards to optimize the detection of transitions. Conditions for detecting resveratrol and its derived metabolites as well as their identification and quantification were optimized according to Azorín-Ortuño et al. [22] using authentic standards. Twelve resveratrol and seven dihydroresveratrol metabolites were searched [22].

2.5 Statistical analysis

All analyses were performed with the statistical package SPSS v. 19.0 (SPSS Inc, Chicago, USA). Parameters with skewed distribution were transformed to their logarithm for analysis (glucose, creatinin, CPK, AST, ALT, GGT, ALP, TSH, T4, D-dimer, triglycerides, HDLc, non-HDL, ApoB, LDLc/LDLox, and LDLox/ApoB). Comparisons between baseline and 6 month changes, within and between groups, were carried out by a covariance model (ANCOVA) for repeated measures with the Bonferroni post hoc test. BMI, waist circumference, arterial hypertension, smoking, DM, antiaggregants, diagnosed hypercholesterolemia were considered as covariates (Supporting Information Table 2). Baseline comparisons for categorical variables were made with the chi-square test. The strength of association between two variables was measured with the Pearson's r correlation and was used for the pairs LDLox versus ApoB, LDLc versus LDLox, LDLc versus ApoB, and non-HDLc versus ApoB. Statistical significance was considered when $p < 0.05$.

3 Results

3.1 Serum lipid profile, ApoB, and LDLox levels

The patients recruited in the present trial were randomly distributed in three groups (Table 1). In general, the three groups were quite homogenous except in the case of group C in which a higher proportion of females was found, and in group B in which no patients were treated with the antiaggregant clopidrogel. All the patients were treated with statins. The possible influence of different covariates, including gender, was evaluated and included in the statistical analysis to adjust any possible effect due to the demographic characteristics or medical treatments of the patients (Supporting Information Table 2).

The baseline values of the parameters evaluated did not show significant differences among groups (Table 2). No changes were observed in the placebo group (A) after 6 months in any of the parameters. In the group B, in which patients consumed GE, a slight decrease of −3.3 mg/dL (95% confident interval [CI] −10.6, 4; $p = 0.013$) in LDLc was observed after 6 months. In the group that consumed the GERES (group C, Stilvid®), a clear decrease was observed in ApoB (−9.3 mg/dL, 95% CI −16.4, −2; $p = 0.014$) and LDLox (−14.5 U/L, 95% CI −19.5, −9.5; $p = 0.001$) levels after 6 months (Table 2). Figures 1 and 2 illustrate the individual and global group changes in both ApoB and LDLox, respectively, after 6 months of intervention. In addition, a decrease in LDLc (−5 mg/dL, 95% CI −14, 4; $p = 0.04$) and LDLox/ApoB (−0.01 U/mg, 95% CI −0.015, −0.005; $p = 0.000$) as well as an increase of non-HDLc/ApoB (0.12, 95% CI 0.04, 0.13; $p = 0.046$) was also observed (Table 2). The ratios LDLc/ApoB (6%, $p = 0.114$) and LDLc/LDLox (11%, $p = 0.094$) tended

Table 2. Changes in serum lipid markers in patients undergoing primary cardiovascular disease prevention (PCP) after consumption of one daily capsule of placebo (group A, $n = 25$), grape extract (group B, $n = 25$), or resveratrol-enriched grape extract (Stilvid®, group C, $n = 25$) for 6 months

	Values at baseline			Values after 6 months			*p* value after 6 months (among groups)		
Parameter	A	B	C	A	B	C	A versus B	A versus C	B versus C
TChol (mg/dL)	187.5 ± 31.2	193.1 ± 54.5	187.4 ± 23.6	191.4 ± 34.7 $p = 0.482$	185.6 ± 44.5 $p = 0.264$	184.8 ± 33.9 $p = 0.656$	$p = 1$	$p = 1$	$p = 1$
Triglycerides (mg/dL)	121.0 ± 47.8	121.1 ± 65.1	123.9 ± 52.4	131.8 ± 65.4 $p = 0.154$	120.5 ± 58.7 $p = 0.952$	127.1 ± 64.8 $p = 0.730$	$p = 1$	$p = 1$	$p = 1$
LDLc (mg/dL)	106.1 ± 26.8	114.2 ± 42.8	110.1 ± 15.7	104.4 ± 27.1 $p = 0.673$	110.9 ± 39.9 **$p = 0.013$ ↓2.9%**	105.1 ± 22.1 **$p = 0.040$ ↓4.5%**	$p = 0.215$	$p = 1$	$p = 0.655$
HDLc (mg/dL)	55.0 ± 12.6	52.7 ± 11.6	54.0 ± 11.5	55.5 ± 11.2 $p = 0.713$	52.3 ± 11.9 $p = 0.827$	54.5 ± 13.2 $p = 0.788$	$p = 1$	$p = 1$	$p = 1$
Non-HDLc (mg/dL)	132.5 ± 28.8	140.4 ± 50.3	133.4 ± 19.8	135.9 ± 34.3 $p = 0.482$	133.3 ± 42.7 $p = 0.231$	130.3 ± 22.6 $p = 0.669$	$p = 1$	$p = 1$	$p = 1$
ApoB (mg/dL)	94.2 ± 16.2	94.6 ± 20.3	94.5 ± 18.4	92.3 ± 17.5 $p = 0.472$	90.9 ± 17.4 $p = 0.231$	85.2 ± 18.0 **$p = 0.014$ ↓9.8%**	$p = 1$	$p = 0.473$	$p = 0.644$
LDLox (U/L)	77.7 ± 20.1	72.1 ± 19.6	72.8 ± 17.8	75.1 ± 22.0 $p = 0.381$	68.9 ± 19.5 $p = 0.822$	58.3 ± 16.2 **$p = 0.001$ ↓20%**	$p = 0.984$	$p = 0.103$	$p = 0.578$
LDLc/ApoB	1.13 ± 0.23	1.21 ± 0.26	1.16 ± 0.14	1.15 ± 0.23 $p = 0.694$	1.22 ± 0.27 $p = 0.901$	1.23 ± 0.25 $p = 0.114$	$p = 1$	$p = 0.662$	$p = 0.896$
LDLc/HDLc	1.93 ± 0.58	2.17 ± 0.74	2.04 ± 0.39	1.88 ± 0.61 $p = 0.252$	2.12 ± 0.80 $p = 0.064$	1.93 ± 0.43 $p = 0.924$	$p = 0.165$	$p = 0.328$	$p = 1$
LDLc/LDLox (mg/U)	14.6 ± 4.6	16.0 ± 5.1	16.1 ± 4.1	15.1 ± 5.0 $p = 0.987$	16.1 ± 7.1 $p = 0.363$	18.1 ± 4.5 $p = 0.094$	**$p = 0.028$**	**$p = 0.004$**	$p = 1$
LDLox/ApoB (U/mg)	0.08 ± 0.01	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.02 $p = 0.926$	0.08 ± 0.02 $p = 0.791$	0.07 ± 0.01 **$p = 0.000$ ↓12.5%**	$p = 1$	**$p = 0.040$**	$p = 0.439$
Non-HDL/ApoB	1.41 ± 0.25	1.49 ± 0.20	1.41 ± 0.14	1.48 ± 0.21 $p = 0.082$	1.46 ± 0.20 $p = 0.401$	1.53 ± 0.16 **$p = 0.046$ ↑8.5%**	$p = 1$	$p = 1$	$p = 0.562$

Values are expressed as mean ± SD. Significant differences ($p < 0.05$) after 6 months within and among groups are boldfaced. The arrows designate the percent of increase/decrease with respect to their corresponding baseline value. All the patients completed the study ($n = 25$ in each group after 6 months). Numeric changes with 95% CI are shown in the text. No significant differences were found among groups at baseline. Covariates used in the analysis are shown in Supporting Information Table 2.
TChol, total cholesterol; LDLc, LDL-cholesterol; HDLc, HDL-cholesterol; non-HDLc, TChol-HDLc; ApoB, apolipoprotein B; LDLox, oxidized LDL.

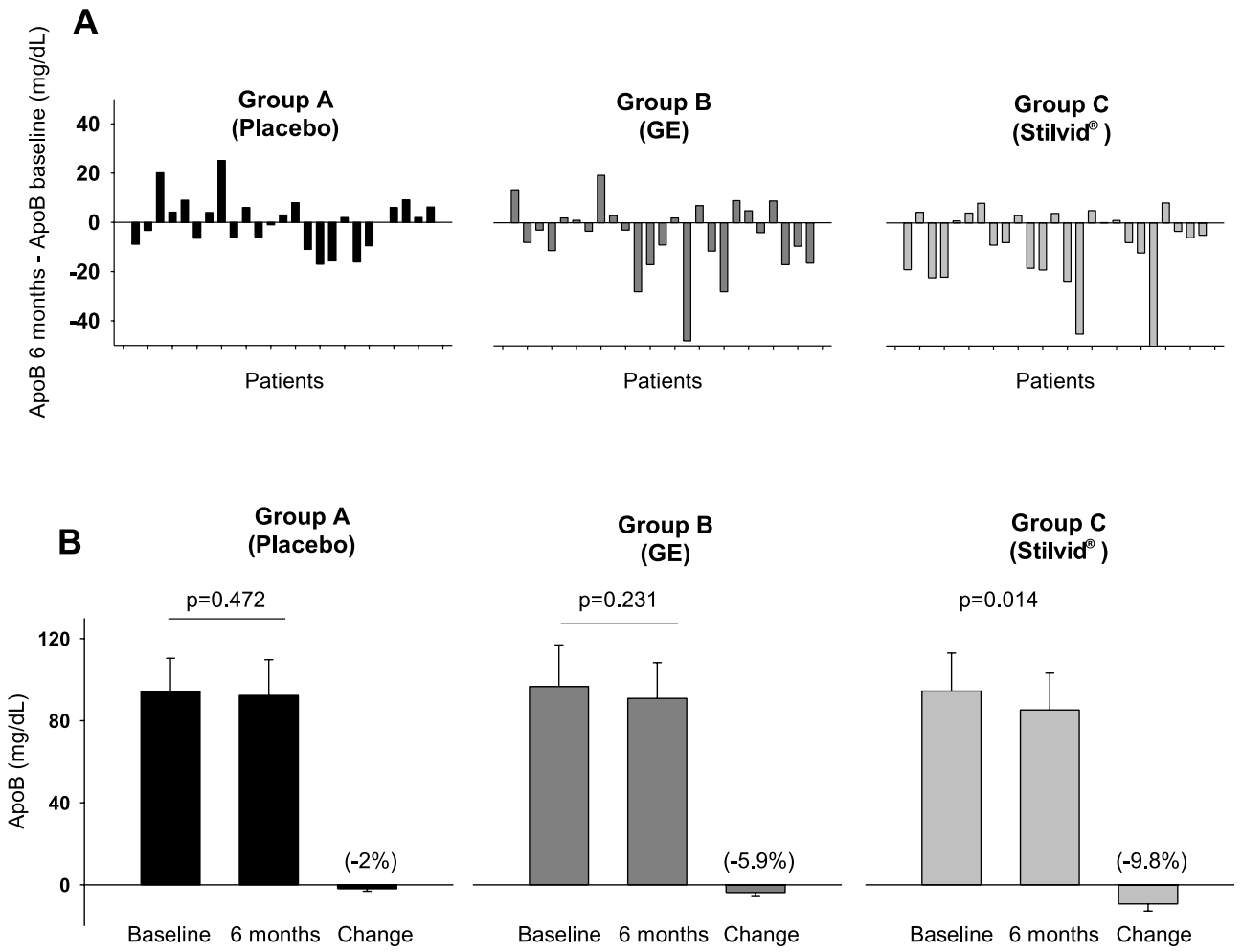

Figure 1. (A) Change of ApoB values in each patient within each group after 6 months of intervention. The ordinate with value 0 means no change. Positive values indicate ApoB increase and negative values, ApoB decrease. (B) Mean ApoB values in each group before and after 6 months of intervention, and the corresponding mean change. Results are expressed as mean ± SD.

to be higher in group C (Table 2). Among groups, after 6 months of intervention, significant differences were observed in the comparison of A versus B and A versus C in the ratio LDLc/LDLox, as well as A versus C in the ratio LDLox/ApoB. Moreover, the groups A and C tended to be different for LDLox ($p = 0.103$).

In general, a good correlation was obtained for the pairs LDLox versus ApoB, LDLc versus LDLox, LDLc versus ApoB, and non-HDLc versus ApoB (Fig. 3), which indicated that the two markers considered within each pair changed correspondingly. Pearson's coefficients were 0.75 and $p < 0.001$ for the pairs LDLox versus ApoB (Fig. 3A) and LDLc versus ApoB (Fig. 3C). The correlation for the pair non-HDLc versus ApoB was slightly worse (Fig. 3D, $r = 0.62$, $p < 0.001$) and the poorest strength of association was observed for the LDLc versus LDLox pair (Fig. 3B; $r = 0.3$, $p < 0.001$).

3.2 Evaluation of hepatic, thyroid, and renal function

In order to evaluate the safety of the trial, a number of markers involved in the hepatic, thyroid, and renal function were measured (Table 3). Only slight changes were observed in the parameters evaluated. The small decrease observed in AST, ALP, and albumin, depending on the groups (Table 3), was always within the reference values of the hospital and with no clinical significance. Furthermore, routine hematological parameters were also evaluated without significant changes being found after 6 months of intervention in any patient (results not shown).

3.3 Phenolic-derived metabolites

No metabolites derived from grape phenolics or resveratrol were detected in plasma or LDL particles (groups B and C)

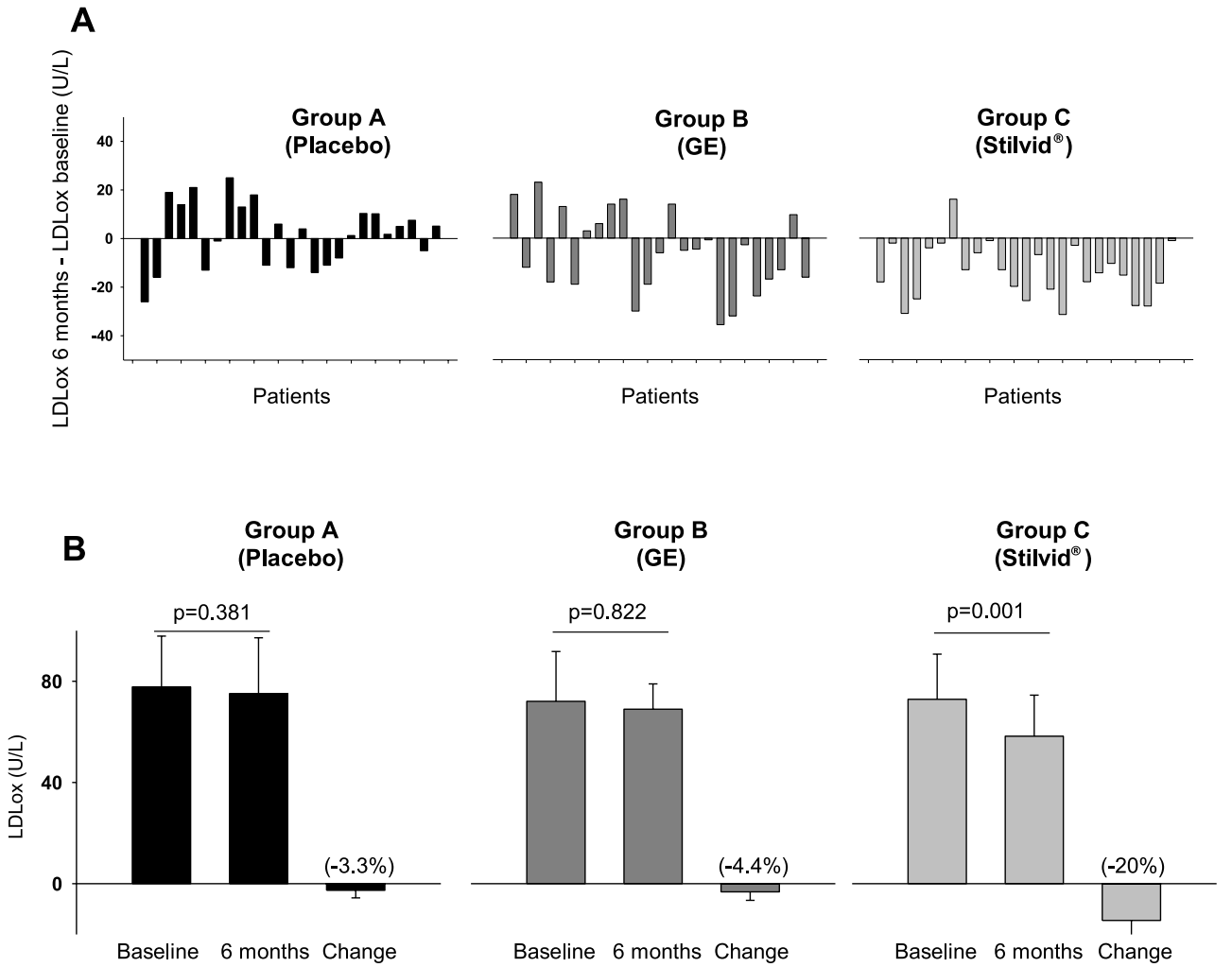

Figure 2. (A) Change of LDLox values in each patient within each group after 6 months of intervention. The ordinate with value 0 means no change. Positive values indicate LDLox increase and negative values, LDLox decrease. (B) Mean LDLox values in each group before and after 6 months of intervention, and the corresponding mean change. Results are expressed as mean ± SD.

using UPLC-QqQ and HPLC-MS/MS equipments (results not shown).

4 Discussion

The main finding of the present study is that a specific grape food complement containing resveratrol was able to improve a number of CVD risk lipid markers in patients from PCP. This is especially relevant because the patients were fully controlled depending on their cardiovascular risk and according to the guidelines for the management of dyslipemias [25] and primary CVD prevention in clinical practice [2, 3]. All the patients were treated with statins and many of them also with antiaggregants (mainly acetyl salicylic acid).

The use of dietary supplements in lowering LDLc and other lipid markers have been previously approached although only a few studies withstood the rigors of randomized controlled human trials [26]. A number of supplements including coenzyme Q10, sterols/stanols, soluble dietary fiber, and *n*-3 polyunsaturated fatty acids (PUFA) have been previously assayed in different clinical trials to support statin lipid-lowering therapy. At the moment, there is substantial evidence that consumption of various daily grams of plant sterols/stanols or n-3 PUFA to statin therapy further reduces LDL-c and Tchol. However, data are not conclusive in the case of soluble fiber or coenzyme Q10 [27].

To the best of our knowledge, the effect of grape-derived products on lipid serum values from statin-treated patients has not been previously reported, which makes difficult to establish a direct comparison with other studies. In the present study, the decrease of LDLc values was discrete upon consumption of GE (group B) or GE-RES (Stilvid©, group C) (Table 2). However, this finding is very positive in hypercholesterolemic patients whose statin treatment has been optimized to reach a LDLc goal. Other human placebo-controlled

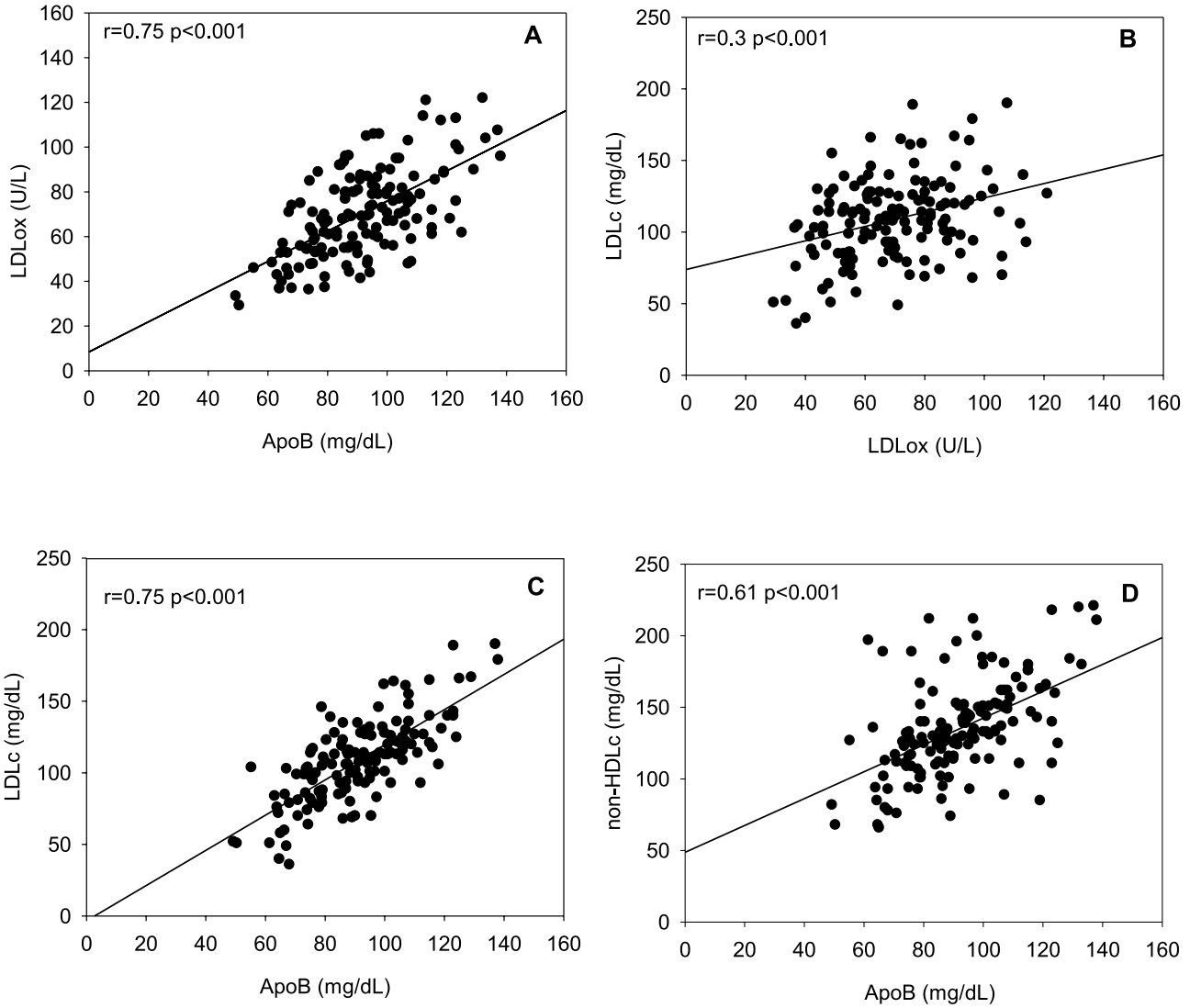

Figure 3. Correlation obtained for the pairs LDLox versus ApoB (A), LDLc versus LDLox (B), LDLc versus ApoB (C), and non-HDLc versus ApoB (D). Baseline and 6-month values, for each pair and patient, are plotted.

trials with grape-derived products in healthy pre- and postmenopausal women [28] as well as in dialysis patients without statin treatment [29] have reported similar or slightly higher effectiveness, but with much higher grape polyphenols intake than in the present study.

Although LDLc is a very important target in primary CVD care, some gold-standard medicated patients with optimized LDLc levels still experience adverse CVD outcomes that involve the presence of a nonfully controlled residual risk [30]. Increasing evidence highlights the limitation of using LDLc and non-HDLc alone as markers of individual's residual CVD risk in lipid-lowering therapy [30, 31].

In the present study, the group C showed a significant decrease in ApoB and LDLox levels despite the slight modification of LDLc values (Table 2, Figs. 1 and 2). In this context, ApoB has been suggested as a better indicator of CVD risk and target treatment than LDLc [6, 30] or non-HDLc [7]. These observations arise from the established linear correlation between high ApoB levels and small size of LDLc particles that are easily oxidized to render atherogenic LDLox particles, associated with cardiovascular risk [10, 11]. This is in agreement with the strong correlation observed in the present study for the pair LDLox versus ApoB (Fig. 3). In contrast, the correlation between LDLc and LDLox was much poorer (Fig. 3). In this case, total LDLc contains a mixture of heterogeneous large and small particles with different susceptibility to oxidation, which justified the poor correlation between LDLc and LDLox values (Fig. 3). Therefore, our results suggest that the effects of the grape complement containing resveratrol (Stilvid®) are mainly related to the decrease of the atherogenicity of LDLc particles, and associated to the decrease of LDLox and ApoB, which is in the line of future

Table 3. Parameters related to the hepatic, thyroid, and renal function in patients undergoing primary cardiovascular disease prevention (PCP) after consumption of one daily capsule of placebo (group A, $n = 25$), grape extract (group B, $n = 25$), or resveratrol-enriched grape extract (Stilvid®, group C, $n = 25$) for 6 months

	Values at baseline			Values after 6 months			p value after 6 months (among groups)		
Parameter	A	B	C	A	B	C	A versus B	A versus C	B versus C
GGT (U/L) (1–24)	28.0 ± 13.5	30.1 ± 21.7	30.0 ± 25.6	31.5 ± 15.6 $p = 0.126$	26.5 ± 14.8 $p = 0.259$	39.1 ± 35.2 $p = 0.194$	$p = 0.359$	$p = 0.658$	**$p = 0.027$**
AST (U/L) (8–30)	25.0 ± 5.3	27.1 ± 11.1	26.8 ± 9.2	25.8 ± 9.8 $p = 0.936$	23.0 ± 5.8 **$p = 0.025$ ↓15%**	25.4 ± 6.3 **$p = 0.018$ ↓5.2%**	$p = 0.396$	$p = 1$	$p = 1$
ALT (U/L) (7–35)	27.6 ± 10.1	33.7 ± 20.1	28.7 ± 15.1	30.2 ± 13.9 $p = 0.874$	27.6 ± 13.4 $p = 0.090$	27.1 ± 11.2 **$p = 0.026$↓**	$p = 0.358$	$p = 0.750$	$p = 1$
LDH (U/L) (208–378)	333 ± 51	325 ± 43	336 ± 33	324 ± 48 $p = 0.280$	329 ± 34 $p = 0.619$	313 ± 36 $p = 0.102$	$p = 1$	$p = 1$	$p = 1$
ALP (U/L) (70–290)	197 ± 58	168 ± 43	205 ± 85	173 ± 45 **$p = 0.009$ ↓12%**	142 ± 33 **$p = 0.002$ ↓15%**	171 ± 58 **$p = 0.001$ ↓16%**	**$p = 0.044$**	$p = 1$	$p = 0.075$
CPK (U/L) (26–140)	129 ± 69	119 ± 44	129 ± 83	128 ± 67 $p = 0.927$	115 ± 28 $p = 0.63$↓6	103 ± 31 $p = 0.261$	$p = 1$	$p = 0.620$	$p = 1$
Glucose (mg/dL) (74–100)	121 ± 33	119 ± 33	115 ± 26	119 ± 34 $p = 0.691$	128 ± 50 $p = 0.149$	113 ± 20 $p = 0.529$	$p = 1$	$p = 0.483$	$p = 0.229$
TSH (mU/L) (0.35–5.5)	2.1 ± 1.2	2.2 ± 1.4	2.2 ± 1.0	2.0 ± 1.2 $p = 0.454$	2.2 ± 1.4 $p = 0.936$	1.9 ± 0.9 $p = 0.328$	$p = 1$	$p = 1$	$p = 1$
T4 (ng/dL) (0.9–1.77)	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.1 $p = 0.935$	1.1 ± 0.2 $p = 0.220$	1.1 ± 0.2 $p = 0.438$	$p = 1$	$p = 0.669$	$p = 0.614$
Bilirubin (mg/dL) (0.3–1.24)	0.64 ± 0.17	0.59 ± 0.24	0.54 ± 0.19	0.66 ± 0.23 $p = 0.556$	0.59 ± 0.21 $p = 0.880$	0.63 ± 0.24 $p = 0.068$	$p = 0.774$	$p = 1$	$p = 1$
Creatinin (mg/dL) (0.5–1.1)	0.79 ± 0.19	0.76 ± 0.16	0.74 ± 0.16	0.80 ± 0.20 $p = 0.582$	0.76 ± 0.18 $p = 0.984$	0.74 ± 0.22 $p = 0.875$	$p = 1$	$p = 0.685$	$p = 0.532$
Urate (mg/dL) (2.6–6.1)	5.3 ± 1.5	5.8 ± 1.2	5.7 ± 1.3	5.3 ± 1.9 $p = 0.971$	6.0 ± 1.6 $p = 0.215$	5.7 ± 1.6 $p = 0.706$	$p = 0.610$	$p = 0.203$	$p = 1$
Albumin (g/L) (34–48)	44.3 ± 2.4	44.8 ± 3.0	43.9 ± 3.7	43.9 ± 3.3 $p = 0.795$	43.1 ± 3.3 **$p = 0.000$ ↓3.8%**	43.2 ± 2.7 $p = 0.873$	$p = 0.210$	$p = 0.658$	$p = 1$

Values are expressed as mean ± SD. Normal values range is shown below each parameter. All the patients completed the study ($n = 25$ in each group after 6 months). Significant differences ($p < 0.05$) after 6 months within and among groups are boldfaced. The arrows designate the percent of increase/decrease with respect to their corresponding baseline value. No significant differences were found among groups at baseline.

GGT, γ-glutamyl transpeptidase; AST, aspartate aminotransferase; ALT, alanine transaminase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; TSH, thyroid stimulating hormone; T4, thyroxine.

therapeutic goals in PCP [30]. The modification of these parameters is very relevant in patients from PCP with optimized medication, also supported by correlation observed in the non-HDLc/ApoB pair (Fig. 3). In fact, the daily consumption of one capsule of Stilvid® moved the patients from group C to optimum ApoB values (<90 mg/dL) proposed by the Canadian Cardiovascular Society as primary target therapy [32, 33]. However, despite the emerging value of ApoB and LDLox in clinical care, unfortunately, they are not included in the guidelines for calculating global risk [3], which is a current debate among cardiologists [7].

We have recently observed that the specific formulation of Stilvid® was able to prevent vascular oxidative stress and early atherosclerotic lesions in the aorta of pigs fed with an atherogenic diet (Azorín-Ortuño et al., unpublished data). The effects seemed to be mediated, at least in part, by a reduction in vascular oxidative stress and the regulation of the expression of the suppressors of cytokine signaling 1 and 3 (SOCS1 and 3). These effects were less significant in pigs supplemented with either control grape or reveratrol alone in which the effects were rather discrete. Our results obtained in that pig study suggested that the antioxidant effect was mainly due to the grape polyphenols and that SOCS1 and 3 regulation was mainly governed by resveratrol.

In the present study, the specific association of grape polyphenols and resveratrol in Stilvid® seems to exert beneficial effects beyond the gold-standard medication for patients from PCP. In contrast, the effect of the grape complement (group B), similar in polyphenolic content except for resveratrol, was rather discrete. The lipid-lowering underlying mechanism for resveratrol seems to be similar to that of statins since the down-regulation of the enzyme HMG-CoA by resveratrol has been reported in a hypercholesteloremic hamster model [34]. In this context, a combination of statin (pravastatin, human equivalent dose [HED] of 11 mg/day for a 70-kg person) and resveratrol (HED 225 mg/day for a 70-kg person) has been previously shown to be more effective than statin alone against myocardial infarction in hypercholesterolemic rats [35].

The safety in a human trial, even in a dietary intervention trial, must be monitored. The combination of statins with other drugs such as fibrates (fenofibrate, bezafribate, or ciprofribate), nicotinic acid (currently under debate), or especially with gemfibrozil, is known to cause adverse effects and should be avoided [25]. These negative interactions can be due to interference with the metabolism pathway of statins that mainly undergo significant hepatic metabolism via liver and intestine cytochrome P450 isoenzymes (CYPs). In this context, the search for coadjuvant compounds with no statin interaction is required. In the present study, no adverse effects were observed in any patient which was supported by the normal hepatic, renal, and thyroid function (Table 3). Our dietary prevention approach, with a low dose of food bioactives, is illustrated by the nondetection of phenolic-derived metabolites in plasma or LDL samples from the patients. These results are not surprising due to the low dose of resveratrol assayed and the rapid clearance of this molecule. In a recent study, resveratrol metabolites, below quantification limit, were barely found in LDL particles from pigs after oral administration of a HED of 500 mg [22]. In addition, previous studies in rodents support systemic effects after orally administration of HED of 10 mg in which circulating metabolites were not detected [36]. Peak plasma nanomolar to low micromolar concentration of resveratrol metabolites were described in human pharmacokinetic assays upon oral intake of 0.5–5 g resveratrol [37]. All of this focus the attention on the so-called "resveratrol paradox" in which the low bioavailability of resveratrol versus its pleiotropic effects is still under research and debate [22].

In the present trial, 34 males and 41 females were finally enrolled. No stratification by gender was planned in each group and, at random, a higher proportion of females were allocated in the group C (Table 1). In this regard, estrogenic activity but also antiestrogenic activity by resveratrol has been previously reported [38]. However, the clinical relevance of these observations has not yet been determined [16]. Therefore, whether the higher effects observed in the group C are somehow related to the weak estrogenic/antiestrogenic properties described for resveratrol cannot be ruled out and deserves further research.

In summary, the intake of one capsule/day of Stilvid® (containing grape polyphenols including 8 mg resveratrol) significantly reduced LDLox and ApoB in patients undergoing primary prevention of cardiovascular disease with gold-standard medication. This food complement reduces these cardiovascular risk markers levels beyond statin effects with no drug interactions or adverse effects. Our results suggest that the presence of a low resveratrol dose in the grape supplement was essential to exert these effects. A synergistic effect of resveratrol with the rest of grape polyphenols and/or statins cannot be ruled out and deserves further research.

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