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Pomegranate extract (POMx) decreases the atherogenicity of serum and of human monocyte-derived macrophages (HMDM) in simvastatin-treated hypercholesterolemic patients: A double-blinded, placebo-controlled, randomized, prospective pilot study

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ABSTRACT

Objective: To analyze pomegranate extract (POMx) effects on serum and on human HMDM atherogenicity in simvastatin − treated hypercholesterolemic patients.
Methods and results: Patients were randomly assigned to receive either simvastatin (20 mg/day) + vegan placebo pill ($n = 11$), or simvastatin (20 mg/day) + POMx pill (1g/day, $n = 12$). Fasting blood samples were collected at baseline and after 1 and 2 months of therapy. HMDM were collected from 3 patients in each group at baseline and after 2 months of therapy, as well as from 3 healthy subjects. After 2 months of therapy, serum LDL-cholesterol levels significantly decreased, by 23%, in the simvastatin + placebo group, and by 26% in the simvastatin + POMx group. Simvastatin + POMx therapy increased serum thiols concentration by 6%. Patients' HMDM reactive oxygen species (ROS) levels were significantly increased, by 69%, vs. healthy subjects HMDM. After 2 months of therapy, HMDM ROS levels decreased by 18% in the simvastatin + placebo group, whereas in the simvastatin + POMx group it decreased by up to 30%. A novel finding was the triglycerides levels in the patients' HMDM at baseline which were significantly higher, by 71%, vs. healthy subjects HMDM. The simvastatin + POMx, but not the simvastatin + placebo therapy, significantly reduced macrophage triglycerides content by 48%, vs. baseline levels. In addition, whereas the simvastatin + placebo therapy significantly decreased the patients' HMDM cholesterol biosynthesis rate by 33%, the simvastatin + POMx therapy further decreased it, by 44%.
Conclusion: The addition of POMx to simvastatin therapy in hypercholesterolemic patients improved oxidative stress and lipid status in the patient's serum and in their HMDM. These anti-atherogenic effects could reduce the risk for atherosclerosis development.

1. Introduction

Hypercholesterolemic patients are characterized by high levels of cholesterol and by oxidative stress in serum and in HMDM, and these patients are prone to accelerated atherosclerosis [1–4]. Protection from oxidative stress may be achieved by endogenous antioxidants, including the paraoxonase enzymes (PON 1, 2, 3 [5]). Nutritional potent antioxidants include: tocopherols (vitamin E), carotenoids (beta carotene, lycopene), ascorbic acid (vitamin C), nicotinic acid [4] and polyphenols [5,6] which are found in abundance in red wine [7], and in pomegranate that is rich in ellagitannin compounds, such as punicalagin [8].

Previous studies have demonstrated that PJ consumption by healthy volunteers significantly decreased their LDL and HDL oxidation [9]. Moreover, consumption of PJ by patients with carotid artery stenosis significantly increased serum PON1 activity, decreased serum oxidative stress, lesion oxidative stress, and lesion

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cholesterol content, and even attenuated atherosclerotic plaque development in the carotid arteries [10]. Similarly, daily consumption of PJ improved stress-induced myocardial ischemia in patients with coronary artery disease [11].

Statin therapy is widely used in hypercholesterolemic patients to lower their serum LDL cholesterol levels, and the risk for atherosclerosis development, and cardiovascular events [12]. Statins are potent inhibitors of HMGCoA-reductase, the rate limiting enzyme in cholesterol biosynthesis [13,14], and they possess some minor anti-oxidative properties. Statins reduce superoxide anion formation in activated monocytes [13], and in endothelial cells [14]. Simvastatin was shown to inhibit LDL oxidation [15], and to improve the activities of endogenous antioxidant enzymes in rat myocardium [16]. Side effects of simvastatin [17] are uncommon upon using low dosages, but at high dosages, side effects include myopathy with or without elevation of creatine phosphokinase, as well as hepatotoxicity, with elevation in serum liver enzymes.

Macrophages play a major role in the pathogenesis of atherosclerosis, as they can take up oxidized-LDL at enhanced rate, leading to cholesterol and oxidized lipids accumulation, and foam cell formation, the hallmark of early atherogenesis [18,19]. Addition of PJ to simvastatin in a macrophage cell culture model system, improved the statin ability to inhibit cellular cholesterol biosynthesis, and to protect the cells from oxidative stress [20]. These effects could be related to punicalagin, and to β-sitosterol, both present in PJ [20]. The aim of the present research was to extend the above study in order to elucidate the anti-atherogenic effects of POMx on serum and on HMDM, ex vivo, in hypercholesterolemic patients treated with simvastatin. Based on our previous in vivo studies with PJ and with POMx pills, as well as our in vitro studies in J774A.1 cultured macrophages [20], we hypothesized that addition of POMx to simvastatin therapy in hypercholesterolemic patients will reduce their oxidative stress in serum and in HMDM, and will also attenuate the extent of macrophage cholesterol biosynthesis rate. The novelty of the current study is the observation that indeed POMx decreased the atherogenicity of serum and HMDM in the simvastatin treated patients. In addition, we have now demonstrated, for the first time, that HMDM from hypercholesterolemic patients contain substantial amount of triglycerides (in addition to cholesterol), and that simvastatin + POMx therapy, unlike simvastatin + placebo therapy significantly decreased cellular triglycerides accumulation.

2. Materials and methods

2.1. Materials

Dihydrocoumarin (DHC), phenyl acetate, 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). PBS, DMEM and RPMI 1640 medium, FCS, penicillin, streptomycin, nystatin, L-glutamine, and sodium pyruvate were purchased from Biological Industries (Beth Haemek, Israel). POMx was purchased from PomWonderful LLC, Los Angeles, CA 90064. POMx is stored in vegan capsules, and each capsule contains 1000 mg of natural pomegranate polyphenol extract, which constitutes 650 mg of gallic acid equivalents, which is equal, in polyphenol content, to one glass (8 ounces) PJ. The vegan placebo pills were matched by size and shape to the POMx pills and were kept in similar brown bottles at room temperature. Each placebo pill contains 520 mg lactose monohydrate. Placebo pills were provided by the pharmacy service at Rambam Health Care Campus.

2.2. Subjects

Twenty three healthy male adults with statin-naïve hypercholesterolemia were enrolled in the study. All the subjects were otherwise healthy, and were known from regular follow up in primary care clinics. They all have signed an informed consent prior to any study-related procedures. The clinical and demographic features of all patients were registered. The patients were randomly assigned to one of two groups; in the first group ($n = 12$), the patients were treated by simvastatin (20 mg/day) and 1 POMx pill/day, and in the second group ($n = 11$), the patients were treated by simvastatin (20 mg/day) and a vegan placebo pill. Randomization was performed with the use of sealed, opaque, serially numbered envelopes, each of them contained a box with 30 tablets of simvastatin 20 mg, together with a bottle containing 30 capsules of either POMx or matching placebo vegan capsules. Unblinding was performed only at the end of the treatment period. Fasting blood samples were drawn in the morning from all patients at baseline and after one and two months of treatment. Monocytes were isolated from the blood of three patients in each group, at baseline and after two months of treatment, and also from three healthy subjects. The study was approved by the local ethics committee of Rambam Health Care Campus, Haifa, Israel (approval number 0353/12).

2.3. Analyses in serum

2.3.1. Serum biochemical parameters

Biochemical analyses in serum were performed by commercially available diagnostic kits, and included measurement of glucose, kidney function [BUN, creatinine and electrolytes-sodium and potassium], liver function (AST and total bilirubin) and CRP.

2.3.2. Serum lipids

Serum lipids (total cholesterol, HDL cholesterol (HDL-C) and triglyceride) concentrations were measured using enzymatic colorimetric methods (CHOD/PAP) which include cholesterol esterase, cholesterol oxidase, and 4-aminoantipyrine.

LDL cholesterol (LDL-C) was calculated.

2.3.3. Serum oxidative stress

2.3.3.1. Basal serum oxidation status. The level of serum aldehydes was measured by the thiobarbituric acid reactive substances (TBARS) assay [21].

2.3.3.2. AAPH-induced serum lipid peroxidation. The diluted serum samples (×4 with PBS) were incubated with 100 mM of 2.2′-azobis, 2-amidinopropane hydrochloride (AAPH, Wako, Japan) for 2 h at 37 °C [22]. The extent of lipid peroxidation was measured by the TBARS assay [21], and by the lipid peroxides assay [23].

2.3.3.3. Serum SH groups. The assay procedure determines the amount of protein bound SH groups, as well as that of reduced glutathione [24].

2.3.3.4. Serum PON1 arylesterase activity. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm for 3 min. The assay mixture included 1 mM phenyl acetate in Tris buffer. The extinction coefficient is $E = 1310\ M^{-1}\ cm^{-1}$. One unit of arylesterase activity equals to 1 μmol of phenyl acetate hydrolyzed/min/ml [25].

2.4. Analyses in macrophages

2.4.1. J774A.1 murine macrophage cell line

The cell line was purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). The cells were grown in DMEM + 5% FCS.

2.4.2. Serum-mediated cholesterol efflux from macrophages

J774A.1 macrophages were pre-incubated with [^{3}H]-labeled cholesterol (2 μCi/ml) for 1 h at 37 °C, followed by cell wash in ice-cold PBS (×3) and a further incubation in the absence or presence of 20 μl/ml of the patient's serum, for 3 h at 37 °C. Serum-mediated cholesterol efflux was than calculated [26].

2.4.3. HMDM

Peripheral blood mononuclear cells were isolated from the patients' blood, by Ficoll density-gradient separation [27].

2.4.4. HMDM intracellular oxidative stress analysis by the DCFH assay

Cells were washed with PBS and then incubated with 10 μM DCFH-DA, in medium, for 30 min at 37 °C [28]. Adherent cells were detached by gentle scraping, and washed (×2) with PBS. Measurement of cellular fluorescence by FACS was performed at 510–540 nm after excitation of the cells at 488 nm with an argon ion laser. Cellular fluorescence was quantitated as mean fluorescence intensity (MFI).

2.4.5. HMDM triglyceride content

Cellular lipids were extracted with hexane:isopropanol (3:2, v:v). The lipid phase was evaporated under nitrogen, and the amount of triglycerides was determined using a kit from Sigma–Aldrich (St. Louis, MO, USA). After lipid extraction, NaOH 0.1 N was added to the cells for protein determination by the Lowry method [29].

2.4.6. HMDM cholesterol biosynthesis rate

The cells we incubated with [^{3}H]-acetate (1 mCi/L) for 3 h in DMEM + 0.2% BSA. After cell wash and lipid extraction, the cholesterol biosynthesis rate was assayed by a thin layer chromatography [TLC, [30]]. NaOH 0.1 N was added to the cells for protein determination [29].

2.5. Statistical analyses

Statistical analysis preformed with SPSS version18. The normality of the data was tested by Kolmogorov Smirnov test. Differences between the two groups were calculated using-t-test, Mann–Whitney *U* test, ANOVA, Kruskal_Wallis with multiple comparisons, and Fisher exact test. A two way ANOVA-Repeated Measure with Bonferroni adjustment was conducted to determine whether there was a statistical significance between two different types of treatment (POMx vs. Placebo) for improving several parameters of hypercholesterolemia (total cholesterol, LDL-C, HDL-C, and triglycerides). $p < 0.05$ is considers as significant.

The post hoc power calculation results are: total cholesterol −19%, LDL cholesterol-26%, HDL cholesterol-6%, triglycerides-8%.

Effect size: Eta-squared value for each effect and each parameter estimate.

The eta-squared statistic describes the proportion of total variability attributable to a factor. The higher is better.

Two pools of monocytes-macrophages were prepared from each patient or healthy subjects and each analysis was performed in triplicates. The serum oxidative stress parameters (Table 3) were analyzed in duplicates for each sample.

3. Experimental results

3.1. Serum

3.1.1. The effect of simvastatin + POMx vs. simvastatin + placebo therapy on the patients' serum biochemical parameters

Table 1 describes baseline serum lipids profile in the two groups of patients, and it includes also additional parameters such as age, smoking and alcohol consumption. Upon analyzing all these parameters we did not observe any statistical significant differences between these two groups. During the study period, there was nochange of lifestyle.

Table 1
Baseline characteristics of patients.

	Simvastatin + placebo (11 patients)	Simvastatin + POMx (12 patients)	*p*-value
Age (years ± SD)	45 ± 12	46 ± 8	0.5
Cholesterol (mg/dL)	237 ± 29	253 ± 41	0.1
LDL-C (mg/dL)	160 ± 23	175 ± 31	0.06
HDL-C (mg/dL)	46 ± 12	44 ± 12	0.87
Triglycerides (mg/dL)	153 ± 74	171 ± 74	0.69
Tobacco users (%)	20	30	1
Alcohol users (%)	10	10	1

Results are expressed as mean ± SD.

The effect of simvastatin + POMx vs. simvastatin + placebo therapies on serum lipid concentrations is shown in Table 2. Total cholesterol levels in the simvastatin + placebo group decreased by 16% or 19% after 1 and 2 months of therapy, respectively, as compared to the baseline levels ($p < 0.0001$). In the simvastatin + POMx group, serum total cholesterol levels were decreased, by 18% or 20%, after 1 month and 2 months of therapy, respectively, as compared to the baseline levels ($p < 0.0001$). The effect size was 0.699. In the simvastatin + placebo group, the LDL-C levels were similarly decreased by 23% after 1 month or 2 months of therapy vs. the baseline levels ($p < 0.0001$). In the simvastatin + POMx group, a reduction in LDL-C, by 23% or 26%, was noted after 1 and 2 months of therapy, respectively, as compared to baseline levels ($p < 0.0001$). The effect size was 0.767. In the serum total cholesterol and LDL-C results, there were no statistical significant differences between the two therapies ($p = 0.32$, $p = 0.14$ respectively, by repeated measure models). Both therapies did not affect the serum HDL-C levels ($p = 0.43$), effect size 0.120.

Serum triglyceride levels were decreased non significantly ($p = 0.39$) in the simvastatin + placebo treated patients by 6% and by 28% after 1 month or 2 months of therapy respectively, as compared to the baseline levels. Also in the simvastatin + POMx group, there was a non significant ($p = 0.13$) reduction in serum triglyceride levels by 25% only after 1 month of treatment. The effect size was 0.308. All other biochemical parameters (glucose, sodium, potasium, creatinine, BUN, AST, total bilirubin and CRP) showed no significant changes between the two studied groups during the treatment period (data not shown).

3.1.2. The effect of simvastatin + POMx vs. simvastatin + placebo therapy on the patients' serum oxidative stress

The effect of simvastatin + placebo vs. simvastatin + POMx therapy, on serum oxidative stress is summarized in Table 3. Both therapies did not affect the patients' basal serum oxidative status, and also AAPH-induced oxidation. In contrast, whereas the simvastatin + placebo therapy had almost no effect on serum SH groups' concentration, in the simvastatin + POMx group there was a non significant increment in serum SH groups, by 4% or 6% after 1 month or 2 months of therapy, respectively, as compared to the baseline levels.

Serum PON1 is HDL-associated [31], and it protects lipoproteins and macrophages from oxidative stress [32]. PON1 activity was shown to be decreased under oxidative stress [33] and to increase, following statin therapy [34,35], or PJ consumption [10]. However, the patients' serum PON1 arylesterase activity was not affected by both therapies (Table 3).

Table 2
The effects of simvastatin + POMx vs. simvastatin + placebo therapy in hypercholesterolemic patients on serum lipid concentrations.

Time	Simvastatin + placebo (11 patients)			Simvastatin + POMx (12 patients)		
	Baseline	1 Month	2 Months	Baseline	1 Month	2 Months
Total Cholesterol (mg/dL)	237 ± 30	198 ± 55[a]	192 ± 35[a]	253 ± 41	208 ± 40[a]	202 ± 29[a]
LDL-C (mg/dL)	160 ± 24	123 ± 38[a]	123 ± 27[a]	175 ± 31	135 ± 37[a]	129 ± 15[a]
HDL-C (mg/dL)	46 ± 19	46 ± 19	46 ± 12	44 ± 12	47 ± 13	45 ± 11
Triglycerides (mg/dL)	153 ± 74	144 ± 71	110 ± 19	171 ± 74	129 ± 43	187 ± 138

Results are expressed as mean ± SD.
[a] $p < 0.0001$.

Table 3
Effect of Simvastatin + POMx vs. Simvastatin + Placebo therapies in hypercholesterolemic patients on serum oxidative stress.

Therapy	Simvastatin + Placebo			Simvastatin + POMx		
Time of therapy	Baseline	1 month	2 months	Baseline	1 month	2 months
Aldehydes (nmol TBARS/ml) basal	2.0 ± 0.3	2.0 ± 0.3	1.9 ± 0.3	1.9 ± 0.3	2.1 ± 0.7	1.9 ± 0.3
Aldehydes (nmol TBARS/ml) AAPH-induced	14.4 ± 1.3	15.4 ± 1.9	15.0 ± 1.9	15.0 ± 1.9	15.7 ± 3.6	15.1 ± 1.3
Lipid peroxides (nmol/ml) AAPH-induced	620 ± 46	643 ± 40	597 ± 43	667 ± 52	667 ± 53	643 ± 46
Thiols (SH Groups) concentration (μM)	164 ± 42	157 ± 41	177 ± 41	179 ± 31	175 ± 32	167 ± 45
PON1 arylesterase activity (Units/ml)	427 ± 71	430 ± 80	385 ± 114	442 ± 111	438 ± 70	417 ± 93

Results are expressed as mean ± SD.

3.2. HMDM

3.2.1. The effect of simvastatin + POMx vs. simvastatin + placebo therapy on macrophage oxidative stress

In all studied patients, the level of ROS was significantly increased ($p < 0.001$), by 69%, vs. healthy subjects HMDM. After 2 months of therapy, in the simvastatin + placebo group there was a non significant reduction in the patients' HMDM ability to produce ROS, by 18%, whereas in the simvastatin + POMx group there was as much as a 30% reduction (Fig. 1A). Next, we analyzed the effect of the serum from three patients in each group before and after 2 months of therapy, on ROS formation in J774A.1 cultured macrophages. In the cells treated with the serum samples from 3 patients on simvastatin + placebo, there was a minimal but significant ($p = 0.029$) reduction in ROS formation, by 6% compared to ROS levels in cells treated with baseline serum samples of these patients (Fig. 2B). However, in cells treated with serum samples after simvastatin + POMx therapy, there was a significant reduction ($p = 0.029$) in ROS, by 33%, as compared to cells treated with baseline serum samples of these patients (Fig. 1B). There were no statistical significant differences between therapies.

3.2.2. The effect of simvastatin + POMx vs. simvastatin + placebo therapy on macrophage triglyceride and cholesterol accumulation

Macrophage foam cells isolated from atherosclerotic lesions were shown to be lipids—rich, as they contain both triglycerides and cholesterol [18,19]. Interestingly, we observed in the hypercholesterolemic patients' HMDM vs. healthy subjects HMDM the accumulation of remarkable amounts of triglycerides (Fig. 2A). We thus measured the baseline triglyceride content in the patient's HMDM from both groups vs. HMDM from healthy subjects, as well as the macrophage triglyceride levels after 2 months of therapy (Fig. 2B). The triglyceride levels in HMDM from the simvastatin + placebo, or from the simvastatin + POMx groups at baseline were significantly (p〈0.001) higher, by 71%, or 63%, respectively, vs. healthy subjects HMDM. The simvastatin + placebo therapy had no effect on the patients' HMDM triglyceride content (Fig. 2B). In contrast, the simvastatin + POMx therapy resulted in a significant ($p = 0.019$) reduction, by 48%, in the patients' HMDM triglyceride content, as compared to the baseline levels (Fig. 2B).

The cholesterol biosynthesis rate in HMDM from the simvastatin + placebo group at baseline, was significantly ($p = 0.034$) higher by 5.6 fold vs. healthy subjects HMDM (Fig 2C), and in the simvastatin + POMx group at baseline, it was significantly ($p = 0.006$) higher by 7 fold vs. healthy subjects HMDM (Fig 2C). In both treated groups there was a significant ($p < 0.0001$) reduction in HMDM cholesterol biosynthesis rate as compared to the baseline levels by 33% after simvastatin + placebo therapy, and by 44% after simvastatin + POMx therapy (Fig. 2C). There was no statistical significant differences between the effect of simvastatin + POMx, and that of simvastatin + placebo therapies ($p = 0.17$).

We next analyzed the ability of the patients' serum, before and after both therapies, to remove cholesterol from J774A.1 macrophages. The extent of cholesterol efflux by the serum samples before and after simvastatin + placebo therapy was similar (Fig. 2D). In contrast, the simvastatin + POMx therapy resulted in a non significant 5% increment in the serum ability to induce cholesterol efflux from the cells vs. the effect of serum obtained before therapy (Fig. 2D).

4. Discussion

The present study demonstrated, for the first time, that POMx addition to low dosage of simvastatin in hypercholesterolemic patient's improved anti-atherogenicity of their serum, and mostly of their HMDM. In serum there was a significant decrease in cholesterol levels, and in HMDM there was a decrease in oxidative stress, in cholesterol biosynthesis rate and in triglyceride content.

Pomegranate (unlike grapefruit) does not significantly affect statin serum levels, as it does not inhibit the P-450 enzymes, and thus, the combination of statin with pomegranate does not result in adverse accumulated drug effect [36]. Unlike grapefruit, pomegranate does not alter the clearance of intravenous or oral midazolam, a probe for cytochrome P450-3A activity. Similarly, despite inhibition of CYP2C9 in vitro, PJ and extract had no effect on CYP2C9 activity in human subjects, and can be consumed by patients taking CYP2C9 substrate drugs with negligible risk of pharmacokinetic interactions.

Hypercholesterolemic patients are characterized by increased oxidative stress in their serum and monocytes—macrophages [2–

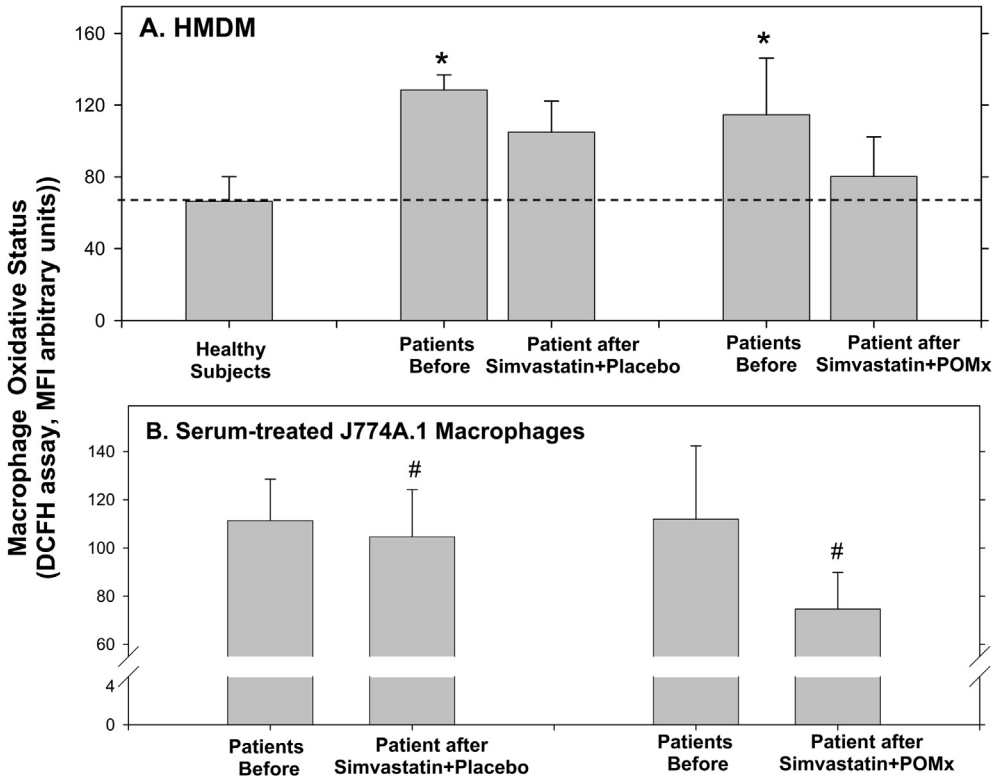

Fig. 1. The effect of simvastatin + POMx vs. simvastatin + placebo therapy in hypercholesterolemic patients on macrophage oxidative stress. (A) Monocytes were isolated from the blood of three patients in each group at baseline and after 2 months of therapy, as well as from three healthy subjects. Monocytes were differentiated into macrophages after 7 days in culture in the presence of 10% of the patients' autologous serum. (B) J774A.1 macrophages were incubated for 20 h with 20 μl/ml of the patients serum samples ($n = 3$), that were collected in each group at baseline and after 2 months of therapy. (A&B) After cell wash the extent of ROS production by the cells was determined by the DCFH assay. Results are expressed as mean ± S.D. $^{*}p$ Patients Before vs. Healthy Subjects. $^{\#}p$ After therapy vs. Before therapy

4]. Statins, or their metabolites are most potent hypocholesterolemic drugs, and they also act as modest antioxidants, either directly, or indirectly, by removing from the circulation of "aged LDL", which is more prone to oxidation. Pomegranate consumption, in contrast, modestly affects blood lipids levels, but it is a most potent nutritional antioxidant. As far as oxidative stress analyses, under the current protocol conditions, in serum, the simvastatin + POMx therapy (but not the simvastatin + placebo therapy) increased the concentration of the potent antioxidant marker—SH groups. In both cultured macrophages and HMDM the effects of the combination (simvastatin + POMx) were most impressive. The serum samples after both therapies decreased the extent of ROS formation in J774A.1 macrophages, as compared to the baseline serum samples, but the inhibitory effect after simvastation + POMx therapy was greater. Similarly, HMDM from the patients treated with simvastatin + POMx showed a more pronounced reduction in ROS formation, vs. HMDM from the simvastatin + placebo group. These results are in accordance with our recent in vitro cultured macrophage study [20], where we demonstrated that addition of PJ to simvastatin in a J774A.1 macrophage cell culture model system, improves the statin ability to protect the cells from oxidative stress. These effects could be related to the most potent polyphenolic antioxidant, the hydrolyzable tannin, punicalagin, which is present in POMx. The pomegranate polyphenols content in one pill of POMx equals that present in one glass (8 ounces) of PJ, and both the pill and the juice were shown to be cardio protective at these dosages [37].

Atherosclerosis is characterized by high oxidative stress in serum, blood cells, and arterial wall cells [5,32]. Atherogenesis also involves high serum and macrophage lipids (cholesterol and triglycerides) accumulation [19]. Both statins and nutritional phytosterols were shown to decrease cardiovascular risk in association with a reduction in serum cholesterol levels in dyslipidemic patients [12,38]. Indeed, we observed in the current research a significant reduction in serum total cholesterol and LDL-C concentrations by both therapies. Similarly, in the patients' HMDM, both therapies decreased significantly the cellular cholesterol biosynthesis rate, but the inhibitory effect of simvastatin + POMx therapy was greater. These results are in accordance with our recent in vitro study in J774.A1 cultured macrophages [20]. While the simvastatin inhibitory effect is mediated at the cholesterol biosynthesis rate limiting enzyme, HMGCoA-reductase, the pomegranate phytosterol – β-sitosterol, as well as the pomegranate polyphenol - punicalagin inhibitory effects on cellular cholesterol biosynthesis, are both downstream to the mevalonate metabolic point. In line with the above results, it was previously shown that pomegranate indeed inhibits macrophage cholesterol biosynthesis, downstream the HMGCoA-reductase step [39].

Both statins and pomegranate were shown to beneficially upregulate serum PON1 [34,35,40]. However, in our current study PON1 activity was not significantly affected by both therapies. This could be related to the low dosages used for simvastatin and POMx, as well as to the short period of therapy.

A novel observation in the present study is that HMDM from hypercholesterolemic patients accumulate not only cholesterol, but also substantial amounts of triglycerides (Fig. 2). Whereas simvastin + placebo therapy had no effect on the patients' HMDM triglyceride mass, the simvastatin + POMx therapy resulted in a significant reduction in the patients' HMDM triglyceride content. This effect could be attributed to POMx, as PJ and punicalagin were

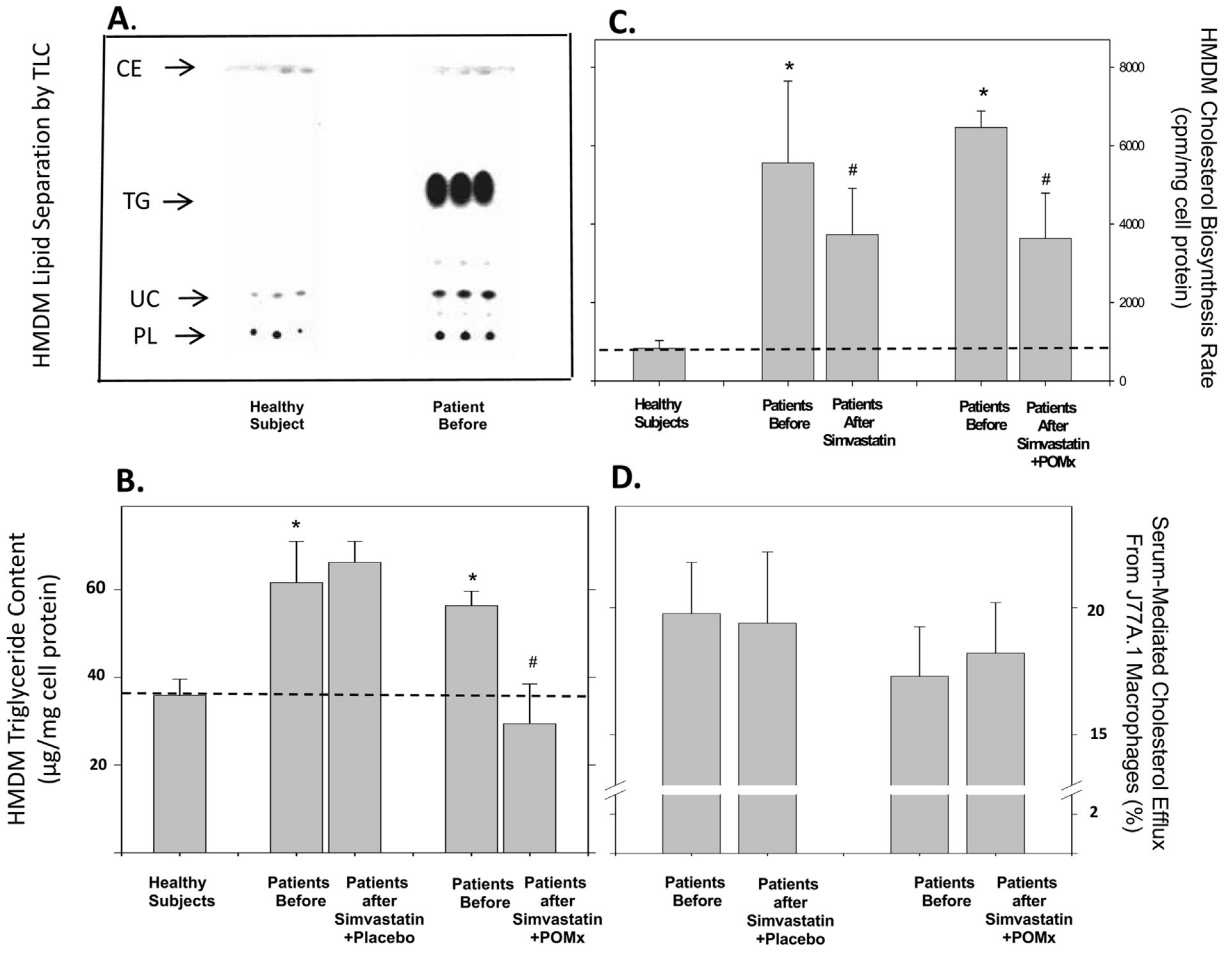

Fig. 2. The effect of simvastatin + POMx vs. simvastatin + placebo therapy in hypercholesterolemic patients, on macrophage triglyceride and cholesterol content. (A) Cellular lipids were extracted from HMDM of healthy subjects, and of hypercholesterolemic patients. Then, the lipids were separated by TLC, as described under the Methods section. A representative picture is shown. PL-phospholipids, UC-unesterified cholesterol, TG-triglyceride, CE-cholesteryl ester. (B) HMDM triglyceride content was determined in the dried lipid extracts. (C) The cells were incubated with [^{3}H]-acetic acid for 3 h, followed by cell wash and lipid extraction. The cellular lipids were separated by TLC and the free cholesterol spots were scraped and counted for radioactivity. (D) J774A.1 macrophages were labeled for 1 h with [^{3}H]-cholesterol, followed by cell wash and a further incubation for 3 h with 20 µ/ml of the patients' serum samples (n = 3) which were collected at baseline and after 2 months of therapy. Collected at baseline and after 2 months of therapy. The extent of serum-mediated cholesterol efflux from the cultured macrophages was determined as described under the Methods section. Results are expressed as mean ± S.D. *p Patients Before vs. Healthy Subjects, #p After therapy vs. Before therapy.

both shown to inhibit triglyceride biosynthesis, via the rate limiting enzyme DGAT1 [41].

Although the power calculation values are low, we still received significant differences in total cholesterol and in LDL-C cholesterol after therapies, and the effect size for these parameters was quite good (about 70%).

It should be noted that there was no difference between the two groups with regards to most of the outcomes in serum. Regarding the HMDM results, although the number of the patients in each group is small, increasing the risk for error, we clearly observed that the effects with the addition of POMx to statin were greater than those of statin + placebo. The present study is a pilot one, and larger clinical studies are now needed.

5. Conclusions

The current study demonstrates, for the first time, that addition of pomegranate to low dosage of simvastatin in hypercholesterolemic patients, significantly improves the patients serum and macrophage atherogenicity. These results may also suggest the use of low statin dosage together with pomegranate extract pills in order to reduce the risk for atherosclerosis development and for cardiovascular events in hypercholesterolemic patients.

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References

[1] Mizuno K, Nakaya N, Teramoto T, et al. Usefulness of LDL-C-related parameters to predict cardiovascular risk and effect of pravastatin in mild-to-moderate hypercholesterolemia. J Atheroscler Thromb 2012;19:176–85.
[2] da Silva Pereira R, Tatsch E, Bochi GV, et al. Assessment of oxidative, inflammatory, and fibrinolytic biomarkers and DNA strand breakage in hypercholesterolemia. Inflammation 2013;36:869–77.
[3] Rosenblat M, Hayek T, Hussein K, Aviram M. Decreased macrophage paraoxonase 2 expression in patients with hypercholesterolemia is the result of their increased cellular cholesterol content: effect of atorvastatin therapy. Arterioscler Thromb Vasc Biol 2004;24:175–80.
[4] Hamoud S, Kaplan M, Meilin E, et al. Niacin administration significantly reduced oxidative stress in patients with hypercholesterolemia and low high density lipoprotein. Am J Med Sci 2013;345:195–9.
[5] Aviram M, Kaplan M, Rosenblat M, Fuhrman B. Dietary antioxidants and paraoxonases against LDL oxidation and atherosclerosis development. Handb Exp Pharmacol 2005;170:263–300.
[6] Dragsted LO. Antioxidant actions of polyphenols in humans. Int J Vitam Nutr Res 2003;73:112–9.
[7] Aviram M, Fuhrman B. Wine flavonoids protect against LDL oxidation and atherosclerosis. Ann N Y Acad Sci 2002;957:146–61.

[8] Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agric Food Chem 2000;48:4581–9.
[9] Aviram M, Dornfeld L, Rosenblat M, et al. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. Am J Clin Nutr 2000;71:1062–76.
[10] Aviram M, Rosenblat M, Gaitini D, et al. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. Clin Nutr 2004;23:423–33. Erratum in: Clin Nutr. 2008; 27:671.
[11] Sumner MD, Elliott-Eller M, Weidner G, et al. Effects of pomegranate juice consumption on myocardial perfusion in patients with coronary heart disease. Am J Cardiol 2005;96:810–4.
[12] Bozentowicz-Wikarek M, Koce1ak P, Smertka M, Olszanecka-Glinianowicz M, Chudek J. Effectiveness of lipid-lowering therapy with statins for secondary prevention of atherosclerosis- guidelines vs. reality. Pharmacol Rep 2012;64:377–85.
[13] Delbosc S, Morena M, Djouad F, et al. Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are able to reduce superoxide anion production by NADPH oxidase in THP-1derived monocytes. J Cardiovasc Pharmacol 2002;40:611–7.
[14] Wagner AH, Köhler T, Rückschloss U, et al. Improvement of nitric oxide-dependent vasodilatation by HMG-CoA reductase inhibitors through attenuation of endothelial superoxide anion formation. Arterioscler Thromb Vasc Biol 2000;20:61–9.
[15] Sobal G, Sinzinger H. Effect of simvastatin on the oxidation of native and modified lipoproteins. Biochem Pharmacol 2005;70:1185–91.
[16] Luo JD, Zhang WW, Zhang GP, et al. Effects of simvastatin on activities of endogenous antioxidant enzymes and angiotensin-converting enzyme in rat myocardium with pressure-overload cardiac hypertrophy. Acta Pharmacol Sin 2002;23:124–8.
[17] Goldfine AB. Statins: is it really time to reassess benefits and risks? N Engl J Med 2012;366:1752–5.
[18] Tiwari RL, Singh Y, Barthwal MK. Macrophages: an elusive yet emerging therapeutic target of atherosclerosis. Med Res Rev 2008;28:483–544.
[19] Lusis AJ. Atherosclerosis. Nature 2000;407:233–41.
[20] Rosenblat M, Volkova N, Aviram M. Pomegranate phytosterol (β-sitosterol) and polyphenolic antioxidant (punicalagin) addition to statin, significantly protected against macrophage foam cell formation. Atherosclerosis 2013;226:110–7.
[21] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–10.
[22] Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. Proc Natl Acad Sci U S A 1988;85:9748–52.
[23] El-Saadani M, Esterbauer N, El-Sayed M, Goher M, Nassar AY, Jurgens G. Spectrophotometric assay for lipid peroxides in serum lipoproteins using commercially available reagent. J Lipid Res 1989;30:627–30.
[24] Hu M-L, Dillard CJ, Tappel AL. In vivo effects of aurothioglucose and sodium thioglucose on rat tissue sulfhydryl levels and plasma sulfhydryl reactivity. Agents Actions 1988;25:132–8.
[25] Gaidukov L, Tawfik DS. The development of human sera tests for HDL-bound serum PON1 and its lipolactonase activity. J Lipid Res 2007;48:1637–46.
[26] Gu X, Kozarsky K, Krieger M. Scavenger receptor class B, type I-mediated ^{3}[H] cholesterol efflux to high and low density lipoproteins is dependent on lipoprotein binding to the receptor. J Biol Chem 2000;275:29993–30001.
[27] Eierman DF, Johnson CE, Haskill SJ. Human monocyte inflammatory mediator gene expression is selectively regulated by adherence substrates. J Immunol 1989;142:1970–6.
[28] LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 1992;5:227–31.
[29] Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.
[30] Rozenberg O, Shih DM, Aviram M. Human serum paraoxonase 1 decreases macrophage cholesterol biosynthesis: a possible role for its phospholipase A2-like activity and lysophosphatidylcholine formation. Arterioscler Thromb Vasc Biol 2003;23:461–7.
[31] Gaidukov L, Tawfik DS. High affinity, stability, and lactonase activity of serum paraoxonase PON1 anchored on HDL with ApoA-I. Biochemistry 2005;44:11843–54.
[32] Aviram M, Rosenblat M. Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. Free Radic Biol Med 2004;37:1304–16.
[33] Nguyen SD, Sok DE. Oxidative inactivation of paraoxonase1, an antioxidant protein and its effect on antioxidant action. Free Radic Res 2003;37:1319–30.
[34] Deakin S, Leviev I, Guernier S, James RW. Simvastatin modulates expression of the PON1 gene and increases serum paraoxonase: a role for sterol regulatory element-binding protein-2. Arterioscler Thromb Vasc Biol 2003;23:2083–9.
[35] Nagila A, Permpongpaiboon T, Tantrarongroj S, et al. Effect of atorvastatin on paraoxonase1 (PON1) and oxidative status. Pharmacol Rep 2009;61:892–8.
[36] Farkas D, Oleson LE, Zhao Y, et al. Pomegranate juice does not impair clearance of oral or intravenous midazolam, a probe for cytochrome P450-3A activity: comparison with grapefruit juice. J Clin Pharmacol 2007;47:286–94.
[37] Rock W, Rosenblat M, Miller-Lotan R, Levy AP, Elias M, Aviram M. Consumption of wonderful variety pomegranate juice and extract by diabetic patients increases paraoxonase 1 association with high-density lipoprotein and stimulates its catalytic activities. J Agric Food Chem 2008;56:8704–13.
[38] Marangoni F, Poli A. Phytosterols and cardiovascular health. Pharmacol Res 2010;61:193–9.
[39] Fuhrman B, Volkova N, Aviram M. Pomegranate juice inhibits oxidized LDL uptake and cholesterol biosynthesis in macrophages. J Nutr Biochem 2005;16:570–6.
[40] Khateeb J, Gantman A, Kreitenberg AJ, Aviram M, Fuhrman B. Paraoxonase 1 (PON1) expression in hepatocytes is upregulated by pomegranate polyphenols: a role for PPAR-gamma pathway. Atherosclerosis 2010;208:119–25.
[41] Rosenblat M, Aviram M. Pomegranate juice protects macrophages from triglyceride accumulation: inhibitory effect on DGAT1 activity and on triglyceride biosynthesis. Ann Nutr Metab 2011;58:1–9.