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Consumption of Wonderful Variety Pomegranate Juice and Extract by Diabetic Patients Increases Paraoxonase 1 Association with High-Density Lipoprotein and Stimulates Its Catalytic Activities

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Association of paraoxonase 1 (PON1) with high-density lipoprotein (HDL) stabilizes the enzyme. In diabetic patients, PON1 dissociates from HDL and, as a consequence, is less biologically active. Our aim was to investigate the effects of Wonderful variety pomegranate juice (WPJ) and pomegranate polyphenol extract (WPOMxl) consumption on PON1 association with HDL in diabetic patients. Thirty patients with type 2 diabetes mellitus participated in the study. Ten male patients and 10 female patients received concentrated WPJ (50 mL/day for 4 weeks), while another group of 10 male patients received WPOMxl (5 mL/day for 6 weeks). There were no significant effects of WPJ or WPOMxl consumption on fasting blood glucose or hemoglobin A1c levels. After 4 weeks of WPJ consumption by male patients, basal serum oxidative stress was significantly decreased by 35%, whereas serum concentrations of thiol groups significantly increased by 25%. Moreover, HDL-associated PON1 arylesterase, paraoxonase, and lactonase activities increased significantly after WPJ consumption by 34−45%, as compared to the baseline levels. PON1 protein binding to HDL was significantly increased by 30% following WPJ consumption, and the enzyme became more stable. In male patients that consumed WPOMxl and in female patients that consumed PJ, a similar pattern was observed, although to a lesser extent. We conclude that WPJ as well as WPOMxl consumption by diabetic patients does not worsen their diabetic parameters. Furthermore, WPJ as well as WPOMxl consumption contribute to PON1 stabilization, increased association with HDL, and enhanced catalytic activities. These beneficial effects of pomegranate consumption on serum PON1 stability and activity could lead to retardation of atherosclerosis development in diabetic patients.

KEYWORDS: Pomegranate; polyphenol; ellagitannins; punicalagin; HDL; paraoxonase 1; diabetes mellitus

INTRODUCTION

Paraoxonase 1 (PON1) is a high-density lipoprotein (HDL) associated lipo-lactonase (*1*) that has been shown to protect against the development of atherosclerosis. Overexpression of human PON1 in mice with combined leptin and low-density lipoprotein (LDL) receptor deficiency, a model of metabolic syndrome, significantly reduces the volume of atherosclerotic plaque, the number of plaque macrophages, and the amount of oxidized LDL in the lesion (*2*). PON1 antiatherogenic properties include the breakdown of oxidized lipids in oxidized lipoproteins and macrophages, inhibition of oxidized LDL uptake by the cells (*3*), decrease in macrophage cholesterol biosynthesis (*4*), and stimulation of HDL-mediated cholesterol efflux from macrophages (*5*). PON1 activity has been shown to be reduced in patients with type 2 diabetes mellitus (DM), and in these patients, a more severe coronary artery disease, as measured by coronary angiography, was observed (*6*). Recently, studies in PON1-knockout or PON1-transgenic mice demonstrated that PON1 has a protective role against diabetes development, secondary to the unique antioxidant properties (*7*). Wonderful variety pomegranate juice (WPJ) was shown to be a most potent antioxidant, and this property is due to the polymolecular ellagitannin compounds such as punicalagin, which are potent polyphenolic hydrolyzable tannins (*8, 9*). These tannins are either converted (by bacteria in the large bowel) to ellagic acids or to other tannin derivatives and metabolites. Previous studies

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have demonstrated that PJ consumption by healthy volunteers significantly decreased LDL and HDL oxidation (*10*). Moreover, consumption of PJ for 3 years by patients with carotid artery stenosis significantly increased serum PON1 activity, decreased serum oxidative stress, and inhibited atherosclerotic plaque development in the carotid arteries (*11*). Similarly, consumption of PJ by DM male patients significantly decreased serum oxidative stress and the extent of oxidized LDL uptake by macrophages derived from these patients, with no harmful effects on their diabetic parameters (*12*). In vitro studies have demonstrated that WPJ sugar fraction, unlike white grape sugar fraction, reduced macrophage oxidative stress under normal and under diabetic conditions (*13*). In addition to the juice prepared from the pomegranate arils, other parts of the pomegranate fruit have also been studied for their antioxidant properties. Research using atherosclerotic apolipoprotein E-deficient (E0) mice, and also in cultured macrophages, has demonstrated beneficial antiatherogenic effects of Wonderful variety pomegranate fruit liquid extract (WPOMxl, a pomegranate polyphenol extract), on macrophage oxidative stress, on oxidized LDL uptake by the cells, and on atherosclerosis development (*14*).

In diabetic patients, PON1 was shown to partly dissociate from HDL to the lipoprotein-deficient serum (LPDS) fraction where it is present as a free enzyme. HDL-associated PON1, but not free PON1, is more potent in attenuation of lipid peroxidation and in stimulation of cholesterol efflux from macrophages (*15*). The high concentrations of glucose in diabetic serum could account for PON1 dissociation from HDL (*16*). In light of the above observations, the aim of the current study was to determine whether consumption of the potent antioxidant pomegranate (as WPJ or WPOMxl) by diabetic patients can beneficially affect serum HDL PON1 activities secondary to the ability to increase PON1 binding to HDL.

MATERIALS AND METHODS

Throughout the study, we used California-grown and processed Wonderful variety pomegranate (POM) extracts supplied by POM Wonderful (Los Angeles, CA).

WPJ. Pomegranates were picked by hand, washed, and stored in tanks. The fruit was crushed and squeezed, and the juice was filtered, pasteurized, concentrated, and stored at −18 °C. Each day during the study period, the concentrated PJ was diluted with water 1:5 (v: v) to obtain a single strength PJ. The antioxidant composition of the juice was 2600 ppm gallic acid equivalents (GAE), which includes a polymolecular mixture of hydrolyzable tannins such as ellagitannins or punicalagin, anthocyanins (delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, delphinidin-3-glucoside, cyanidin 3-glucoside, and pelargonidine 3-glucoside), and ellagic acids derivatives. The juice contained also 30 mg/L vitamin C.

WPOMxl. After most of the juice was expelled from the pomegranate fruit, the remaining fruit, which included aril residues, was collected and processed to remove the seeds before going through a screw press to produce a puree water extract. The polyphenols of the puree were concentrated via a membrane system, and the resulting cloudy POMxl was filtered. The extract was then concentrated after passing through an evaporator and pasteurized. The final product had a 65 Brix concentration and was stored at 4 °C. WPOMxL contained 130000 ppm GAE or 650 mg per 5 mL, which consisted of about 95% polymolecular mixture ellagitannins and 5% ellagic acid.

Subjects. Thirty patients with type 2 DM participated in the study. There were 10 males and 10 postmenopausal females that consumed WPJ [50 mL of concentrated PJ/day (which contained 2600 ppm GAE polyphenols) for a period of 4 weeks]. Then, there was a "washout" period of 4 weeks. Blood was collected from the patients after 12 h of fast and 2 and 4 weeks after WPJ consumption, as well as after the "washout" period. Another group of 10 diabetic male patients consumed WPOMxl (5 mL WPOMxl/day for a period of 6 weeks). Then, there was a period of "washout" for 4 weeks. Blood was collected before and 2, 4, and 6 weeks after WPOMxl consumption, as well as after the "washout" period. **Table 1** demonstrates the diabetic parameters of all patients before treatment. All of the above procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983. The patients served as their own control, as we compared all data along the pomegranate consumption to the baseline values. All patients were nonsmokers and were treated with statins for at least 2 weeks before the beginning of the study.

Table 1. Diabetic Parameters of the Three Groups of Patients

	age (years)	DM duration (years)	glucose (mg/dL)	hemoglobin A1c (HbA1c, %)	body mass index (BMI; kg/m^2)
WPJ males	59 ± 2	14 ± 3	199 ± 33	7.5–11.3	30 ± 3
WPJ females	55 ± 8	11 ± 7	167 ± 21	7.0–11.2	37 ± 3
WPOMxl males	54 ± 3	8 ± 2	170 ± 24	7.0–11.0	33 ± 2

Serum Biochemical Parameters. Serum glucose was measured using an automated enzymatic UV hexokinase test (Olympus, Medtechnica, Israel). Cholesterol, HDL cholesterol, and triglyceride concentrations in serum were measured using automated enzymatic color tests (Olympus, Medtechnica, Israel). Serum LDL was calculated. Hemoglobin A1c was measured using a whole blood application immunoturbidimetric-colorimetric test (Roche Integra, Roche Diagnostics, Germany).

Serum Oxidative Stress Parameters. *Serum Basal Oxidative Status.* Basal serum oxidative status was determined by the thiobarbituric acid reactive substances (TBARS) assay (*17*).

AAPH-Induced Serum Lipid Peroxidation. Serum samples were diluted 4× with phosphate-buffered saline (PBS) and were incubated with 100 mmol/L of 2,2′-azobis,2-amidinopropane hydrochloride (AAPH, Wako, Japan) for 2 h at 37 °C (*18*). The extent of lipid peroxidation was measured by the TBARS assay (*17*).

Serum Total Thiols (SH Groups). Serum (10 μL) was mixed with 200 μL of Tris-EDTA buffer, and the absorbance at 412 nm was measured. To these samples, 8 μL of 10 mmol/L DTNB was added, and after 15 min of incubation at room temperature, the absorbance was measured again together with a DTNB blank. Total SH groups were then calculated (*19*).

Ferric Reducing Antioxidant Power (FRAP) of Plasma. Serum (10 μL) was mixed with 30 μL of H_2O. Freshly prepared FRAP reagent (270 μL) was warmed to 37 °C and added to the diluted samples. Absorbance readings were taken after 0.5 s and 4 min of incubation. Blank (40 μL of H_2O) and Fe^{II} standard solutions were tested in parallel (*20*).

Isolation of Serum Lipoproteins by Discontinuous Density Gradient Ultracentrifugation. HDL and LPDS were isolated from serum obtained from fasted diabetic patients by density gradient ultracentrifugation (*21*). Solid KBr was added to 3 mL of serum + 1 mL of NaCl solution (d = 1.006 g/mL) to increase its density to 1.25 g/mL. This solution was overlayered with 4 mL of NaCl solution (d = 1.084 g/mL) followed by 4 mL of NaCl solution (d = 1.006 g/mL). All solutions contained 2 mmol/L of $CaCl_2$ and 100 μmol/L of diethylenetriaminepeta-acetic acid (DTPA) to preserve PON1 activity. The tubes were centrifuged in a SW41 rotor (Beckman Coulter Canada, Inc.) at 35000 rpm (100000g) for 48 h at 4 °C. The HDL and LPDS fractions were visualized, isolated, and stored at 4 °C. The volume of each fraction was measured.

Assays of Serum or HDL-PON1 Activities. All assays were performed in a 96 well plate, in a total reaction volume of 200 μL.

Paraoxonase Activity. Serum, HDL, or LPDS (10 μL, nondiluted samples) were analyzed. The basal assay mixture included 1.0 mmol/L paraoxon and 1.0 mmol/L $CaCl_2$ in 50 mmol/L glycine/NaOH buffer, pH 10.5. Paraoxon hydrolysis was kinetically monitored for 8 min (every 15 s) at 412 nm. Nonenzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. One unit of PON1 paraoxonase activity was equivalent to 1 nmol of paraoxon hydrolyzed/min/mL (*22*).

Arylesterase Activity. The serum samples were diluted 1:10 with "activity buffer" (1 mmol/L $CaCl_2$ in 50 mmol/L Tris HCl, pH 8.0),

and then, 5 μL was taken for a total reaction volume of 200 μL. Arylesterase activity in the HDL or LPDS fractions was measured using 5 μL (nondiluted). Initial rates of hydrolysis were determined spectrophotometrically at 270 nm for 3 min (every 15 s). The assay mixture included 1.0 mmol/L phenyl acetate in "activity buffer". One unit of arylesterase activity was equivalent to 1 μmol of phenyl acetate hydrolyzed/min/mL (*22*).

Lactonase Activity toward Dihydrocoumarin (DHC). The serum samples were diluted 1:10 with "activity buffer" (1 mM $CaCl_2$ in 50 mmol/L Tris HCl, pH 8.0), and 3 μL was then taken for the assay. From the HDL or LPDS fractions, 5 μL (nondiluted) was used. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm for 10 min (every 15 s). The assay mixture included 1 mmol/L DHC in "activity buffer". Nonenzymatic hydrolysis of DHC was subtracted from the total rate of hydrolysis. One unit of lactonase activity was equivalent to 1 μmol of DHC hydrolyzed/min/mL (*23*).

Lactonase Activity toward TBBL. Five microliters of nondiluted HDL was analyzed. Initial rates of hydrolysis were determined spectrophotometrically at 412 nm for 3 min (every 15 s). The assay mixture included 0.25 mmol/L TBBL and 1 mmol/L $CaCl_2$ in 50 mmol/L Tris HCl, pH 8.0. Nonenzymatic hydrolysis of TBBL was subtracted from the total rate of hydrolysis. One unit of lactonase activity was equivalent to 1 μmol of TBBL hydrolyzed/min/mL (*24*).

HDL Composition. The HDL fractions were dialyzed against 50 mmol/L Tris-HCL and 2 mmol/L $CaCl_2$, pH 7.4, and their protein content was determined using the Folin phenol reagent (*25*). Apolipoprotein A-I and cholesterol levels were determined using commercially available kits.

Serum PON1 Stability: Inactivation Assay. Serum samples of patients were diluted 10-fold with TBS (10 mmol/L Tris, 150 mmol/L NaCl, pH 8.0). To these diluted serum samples, an equal volume of inactivation buffer [TBS supplemented with 2 mmol/L nitrilotriacetic acid (NTA), which is a calcium chelator, and 5 mmol/L β-mercaptoethanol] was added. Then, the samples were incubated at 25 °C for 1 h. The arylesterase activity was measured at different time points along the incubation period, using 2 mmol/L of phenyl acetate. The serum PON1 activity was expressed as a percentage of residual activity (*22*).

HDL-Asscoiated PON1 Protein. Western blot analysis was performed using SDS- PAGE, 10% bis-acrylamide gels. From the HDL or LPDS fractions, 25 μg protein/mL or 20 μL, respectively, were loaded on the gel. Blocking of the gel was performed with 2% BSA for 2 h at room temperature. The primary antibody was mouse monoclonal antihuman PON1 [diluted 1:7500, v/v in TBS-T (5 mol/L NaCl, 2 mol/L Tris, pH 7.5, and Tween 20) with 1% BSA], and it was incubated with the nitrocellulose membrane at 4 °C overnight. The secondary antibody [antimouse horseradish peroxidase-conjugated antimouse IgG (Sigma-Aldrich), diluted 1:10000 in TBS-T] was incubated for 1 h at room temperature. The membranes were developed using the ECL Western blotting kit (Amersham). Two microliters (diluted 1:200) of purified human PON1 (3.8 mg/mL) was loaded as a positive control.

Haptoglobin (Hp) Phenotyping. Hp phenotyping was performed in all of the serum samples of patients that were collected before treatment. The serum (10 μL) was mixed with 2 μL of 10% hemoglobin (Hb) solution, and the samples were then incubated for 5 min at room temperature to allow the formation of Hp−Hb complexes. An equal volume (12 μL) of sample buffer [125 mmol/L TrisBase, pH 6.8, 20% (w/v) glycerol, and 0.001% (w/v) bromophenol blue] was added to each sample. The Hp−Hb complex was resolved by polyacrylameide gel electrophoresis [PAGE followed by their visualization by soaking the gel in freshly prepared staining solution (*26*)].

Statistics. For comparison of mean differences between paired groups, we used the Wilcoxon rank test, and a p value < 0.05 was considered significant. Results are given as means ± standard errors of the mean (SEM).

RESULTS

WPJ Males. *Effects of WPJ Consumption on Serum Oxidative Stress.* In diabetic males that consumed WPJ, the basal oxidative stress of the serum as measured by the TBARS assay decreased by 19 and 35% after 2 or 4 weeks of WPJ consumption, respectively, as compared to baseline and remained 26% below the baseline level at the end of the washout period (**Figure 1A**). Similarly, levels of thiol groups, which represent the antioxidant capacity of the serum, increased by 25% after 4 weeks of WPJ consumption as compared to baseline and remained 17% higher than baseline at the end of the "washout" period (**Figure 1B**). Serum FRAP concentration also increased by 35% (from 9 ± 1 to 13 ± 2 nmol/mL) after 4 weeks of WPJ consumption, as compared to baseline, and remained 59% (15 ± 3 nmol/mL) higher than baseline value at the end of the "washout" period.

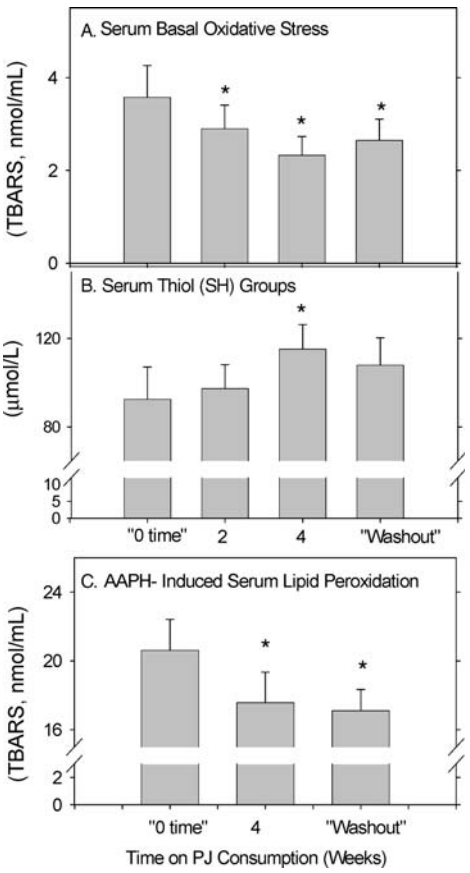

Figure 1. Effects of WPJ consumption by diabetic males on their serum basal oxidation. Blood samples were collected from WPJ males before (0 time), 2, and 4 weeks after PJ consumption, as well as after the "washout" period. The basal serum oxidative stress was measured by the TBARS assay (**A**), by the level of thiol (SH) groups (**B**), or by AAPH-induced serum lipid peroxidation as measured by the TBARS assay (**C**). Results are presented as means ± SEM ($n = 10$). *$p < 0.05$ (vs 0 time).

Furthermore, AAPH-induced serum lipid peroxidation, as measured by the TBARS assay, decreased by 15% after 4 weeks of WPJ consumption. At the end of the "washout" period, the level of TBARS was still 17% lower than that observed at baseline (**Figure 1C**). These results clearly indicate that WPJ consumption by diabetic patients significantly decreased their serum oxidative stress.

Effect of WPJ Consumption on HDL or LPDS PON1 Catalytic Activities. The composition of HDL isolated from the serum of patients following WPJ consumption (cholesterol/protein ratio) was similar to that observed at baseline (data not shown). In WPJ males, HDL-associated PON1 arylesterase (**Figure 2A**), paraoxonase (**Figure 2B**), lactonase toward DHC (**Figure 2C**), and lactonase toward TBBL (**Figure 2D**) activities significantly

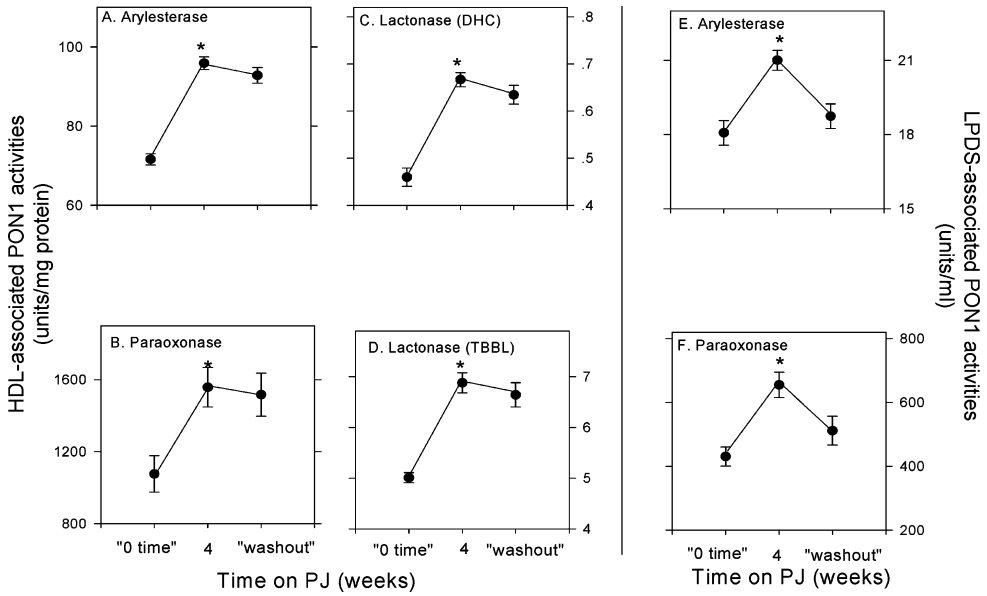

Figure 2. Effects of WPJ consumption by diabetic males on PON1 catalytic activities in HDL and in LPDS. Blood samples were collected from WPJ males before (0 time), 4 weeks after WPJ consumption, and after the "washout" period. The HDL and the LPDS fractions were isolated from the blood samples of four patients by ultracentrifugation. HDL-associated PON1 arylesterase activity (**A**), HDL-associated PON1 paraoxonase activity (**B**), HDL-associated PON1 lactonase activity toward DHC (**C**), HDL-associated PON1 lactonase activity toward thiobutyl-butyrolactone (TBBL, **D**), LPDS-associated PON1 arylesterase activity (**E**), and LPDS-associated PON1 paraoxonase activity (**F**) were determined as described under the Materials and Methods section. Results are given as means ± SEM ($n = 4$). *$p < 0.05$ (vs 0 time).

increased by 34, 45, 33, and 38%, respectively, after 4 weeks of treatment, as compared to baseline. At the end of the "washout" period, all of the above activities were still 30, 41, 27, and 33%, respectively, higher than baseline.

As in diabetic patients, PON1 was shown to be present also in its free form in LPDS (*15*); we next questioned whether WPJ consumption also affected LPDS PON1 catalytic activities. In WPJ males, LPDS PON1 arylesterase (**Figure 2E**) or paraoxonase (**Figure 2F**) activities significantly increased by 16% and by 52%, respectively, after 4 weeks of WPJ consumption. At the end of the "washout" period, LPDS arylesterase activities returned to baseline levels (**Figure 2E**), while LPDS paraoxonase activities were still 19% higher than baseline (**Figure 2F**).

Effect of WPJ Consumption on PON1 Binding to HDL and on Serum PON1 Stability. We next questioned whether the increment in HDL-associated PON1 catalytic activities could be attributed to increased PON1 binding to HDL. In WPJ males, PON1 binding to HDL, as measured by Western blot analysis, significantly increased by 30% after 4 weeks of WPJ consumption as compared to HDL PON1 protein levels at baseline (**Figure 3A**). In contrast, LPDS PON1 protein content significantly decreased by 62% after 4 weeks of WPJ treatment, as compared to baseline (**Figure 3A**), suggesting that WPJ consumption increased the binding of LPDS free PON1 to HDL.

The discrepancy between the increase in PON1 activity and the decrease in PON1 protein in LPDS could be related to an activation effect of WPJ on LPDS-PON1. The addition of WPJ or punicalagin (38 μg GAE/mL) to LPDS from diabetic patients (obtained before WPJ treatment) significantly increased LPDS-PON1 arylesterase activities by 22 and 32%, respectively, as compared to the activities observed in nontreated LPDS (20.9 ± 0.5 or 22.6 ± 1.5 units/mL, respectively, vs 17.1 ± 0.7 units/mL).

Because WPJ consumption by diabetic males increased PON1 protein content in HDL, we next questioned whether this phenomenon also stabilized PON1. As shown in **Figure 3B**, after 60 min of incubation with the calcium chelator NTA, serum PON1 residual arylesterase activity was 102% higher after 4 weeks of WPJ consumption than the activities observed at baseline (**Figure 3C**). These results indicate that after WPJ consumption, more PON1 is bound to HDL, and thus, it is more stable.

WPJ Females and WPOMxl Males as Compared to WPJ Males. *Effects of WPJ or WPOMxl Consumption on Serum Biochemical Parameters.* As shown in **Table 2**, WPJ consumption by diabetic patients (males or females) for 4 weeks, or WPOMxl consumption for 6 weeks by diabetic males, did not significantly affect serum lipids levels (LDL cholesterol, HDL cholesterol, or triglycerides) or fasting serum glucose and HbA1c levels.

Effects of WPJ or WPOMxl Consumption on Serum Oxidative Stress. Serum basal oxidative stress, as measured at baseline by the TBARS assay, in WPOMxl males or in WPJ females were 114 (7.65 ± 0.2 nmol/mL) and 115% (7.71 ± 0.3 nmol/mL) higher, respectively, than the basal oxidative stress measured at baseline in WPJ males (3.57 ± 0.68 nmol/mL). Similarly to WPJ males (**Figure 1C**), in WPOMxl males, AAPH-induced serum lipid peroxidation decreased by 14% (from 19.9 ± 2 to 17.2 ± 1 nmol/mL) after 4 weeks of WPOMxl treatment as compared to baseline and remained 15% lower than baseline at the end of the washout period. However, in WPJ females, AAPH-induced serum lipid peroxidation did not decrease after 4 weeks of treatment (data not shown). However, as observed in WPJ males (**Figure 1B**), also both in WPOMxl males and in WPJ females, the level of serum thiol groups significantly increased by 18% (from 122 ± 6 to 144 ± 11 μmol/L) and by 31% (from 92 ± 15 to 115 ± 22 μmol/L) after 4 weeks of treatment, as compared to baseline. At the end of the "washout" period, in WPOMxl males like in WPJ males (**Figure 1B**), serum thiol groups level remained 12% higher (137 ± 7 μmol/L) than baseline. In contrast, in WPJ females, the levels of serum thiol groups returned to baseline at the end of the "washout" period.

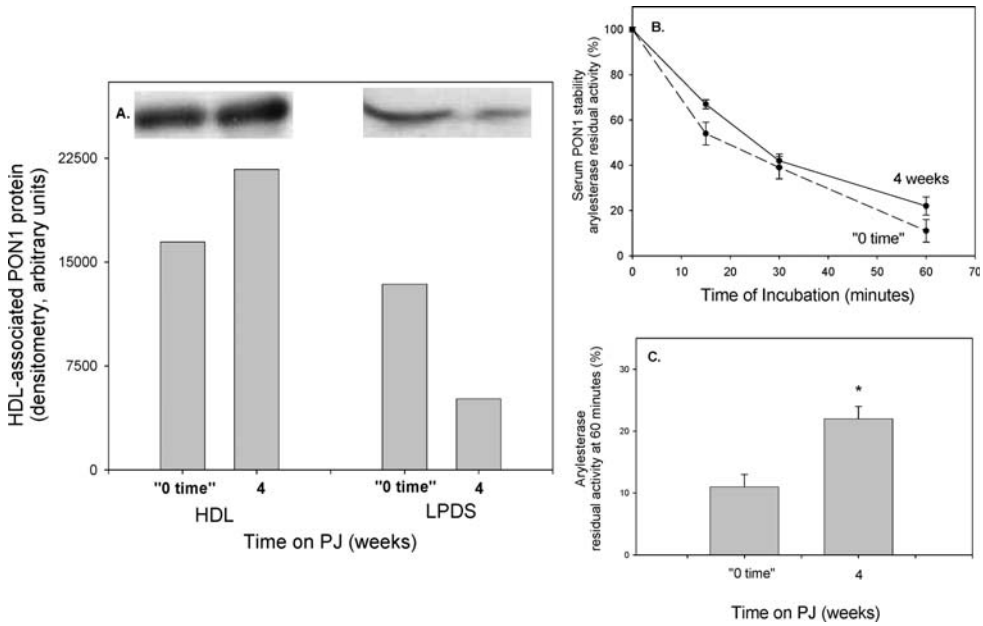

Figure 3. Effects of WPJ consumption by diabetic males on HDL- and LPDS-associated PON1 protein levels and on PON1 stability in serum. Blood samples were collected from WPJ males before (0 time) and 4 weeks after WPJ consumption. (**A**) The HDL or LPDS fractions were isolated from the blood samples of four patients by ultracentrifugation. The HDL fractions (25 μg of protein) or LPDS fractions (20 μL) were loaded on 10% acrylamide gel. The PON1 protein bands were visualized using mouse antihuman PON1 antibody, as described under the Materials and Methods section. Densitometric analysis of the PON1 bands as well as the picture of the bands is shown. This is a representative experiment out of four. (**B**) The serum samples of four patients were incubated with 2 mmol/L NTA for 1 h at 37 °C. The serum PON1 arylesterase residual activity was measured throughout the incubation period. (**C**) The serum PON1 residual activity at the end of the incubation period is shown. Results are given as means ± SEM ($n = 4$). *$p < 0.05$ (vs 0 time).

Table 2. Effects of WPJ and WPOMxl Consumption by DM Patients (Males or Females) on Serum Biochemical Parameters[a]

group	time	GLUC	HbA1c	HDL-C	LDL-C	TG
WPJ males	before	199 ± 33	9.0 ± 0.5	43 ± 3	83 ± 7	183 ± 23
	after	160 ± 16	9.0 ± 0.4	48 ± 4	100 ± 9	162 ± 30
WPOMxl males	before	167 ± 21	9.3 ± 0.6	42 ± 3	106 ± 13	167 ± 21
	after	179 ± 25	8.6 ± 0.5	40 ± 2	110 ± 12	179 ± 25
WPJ females	before	170 ± 24	10 ± 1.2	47 ± 4	96 ± 6	135 ± 15
	after	177 ± 22	9.0 ± 0.5	50 ± 4	97 ± 9	155 ± 23

[a] GLUC, glucose (mg/dL); HbA1c, hemoglobin A1c (%); HDL-C, HDL cholesterol (mg/dL); LDL-C, LDL cholesterol (mg/dL); and TG, triglycerides (mg/dL). All results are given as means ± SEM.

Effect of WPJ or WPOMxl Consumption on HDL and LPDS PON1 Catalytic Activities. Next, HDL fractions were isolated from the serum of WPJ females and WPOMxl males. Similarly to WPJ males, in WPJ females or in WPOMxl males, there were no significant changes in HDL composition (cholesterol/protein ratio) after weeks of treatment (data not shown). In WPJ males, HDL-associated PON1 arylesterase (**Figure 4A**), paraoxonase (**Figure 4B**), lactonase (toward DHC, **Figure 4C**), and lactonase (toward TBBL, **Figure 4D**) activities increased significantly by 34, 43, 47, and 40%, respectively, after 4 weeks of consumption as compared to baseline. Similarly, in WPOMxl males, the HDL-associated PON1 arylesterase (**Figure 4A**), paraoxonase (**Figure 4B**), lactonase (toward DHC, **Figure 4C**), and lactonase (toward TBBL, **Figure 4D**) activities increased by 33, 45, 29, and 37%, respectively, after 4 weeks of consumption as compared to baseline. The results in WPJ females showed a similar trend, but the increase in PON1 activities was less pronounced. In these patients, HDL-associated PON1 arylesterase (**Figure 4A**), paraoxonase (**Figure 4B**), lactonase (toward DHC, **Figure 4C**), and lactonase (toward TBBL, **Figure 4D**) activities increased by only 16, 9, 15, and 19%, respectively, as compared to baseline. In PJ males, the HDL-associated PON1 arylesterase, paraoxonase, lactonase (toward DHC), and lactonase (toward TBBL) at the end of the washout period remained 29, 30, 38, and 30% higher than baseline, respectively (**Figure 4**). In both WPOMxl males and in WPJ females, HDL-associated PON1 activities returned to baseline at the end of the "washout" period (**Figure 4**).

Finally, PON1 activities were analyzed also in the patients' LPDS fractions. Unlike WPJ males (**Figure 2E**,**F**), in WPOMxl males, LPDS PON1 arylesterase activity decreased by only 1% (from 36.4 ± 2.1 to 36.2 ± 2.5 units/mL) after 4 weeks of WPJ, and at the end of the "washout" period, the LPDS arylesterase activity was 14% lower than baseline (31.4 ± 3.8 vs 36.4 ± 2.1 units/mL). In contrast, in WPJ females, the LPDS arylesterase activity did not increase but decreased by 8% (from 38 ± 2 to 35 ± 1 units/mL) after 4 weeks of WPJ consumption, and at the end of the washout period, the arylesterase activity returned to baseline value.

Effects of WPJ or WPOMxl Consumption on PON1 Binding to HDL and on Serum PON1 Stability. A similar trend of increased PON1 protein association with HDL that was observed in WPJ males (**Figures 3A** and **5A**) was also observed in WPOMxl males (**Figure 5B**). After 4 weeks of WPOMxl consumption, HDL-bound PON1 protein increased by 32% (**Figure 5B**), as compared to baseline values. In contrast, in WPJ females, an opposite trend was seen, with a 9% reduction in the HDL-bound PON1 protein, as compared to baseline values (**Figure 5C**).

Incubation of the patients' serum samples (at baseline and after 4 weeks of WPJ or WPOMxl consumption) for 60 min with NTA revealed that serum PON1 arylesterase residual activity in WPJ males, WPOMxl males, or WPJ females after

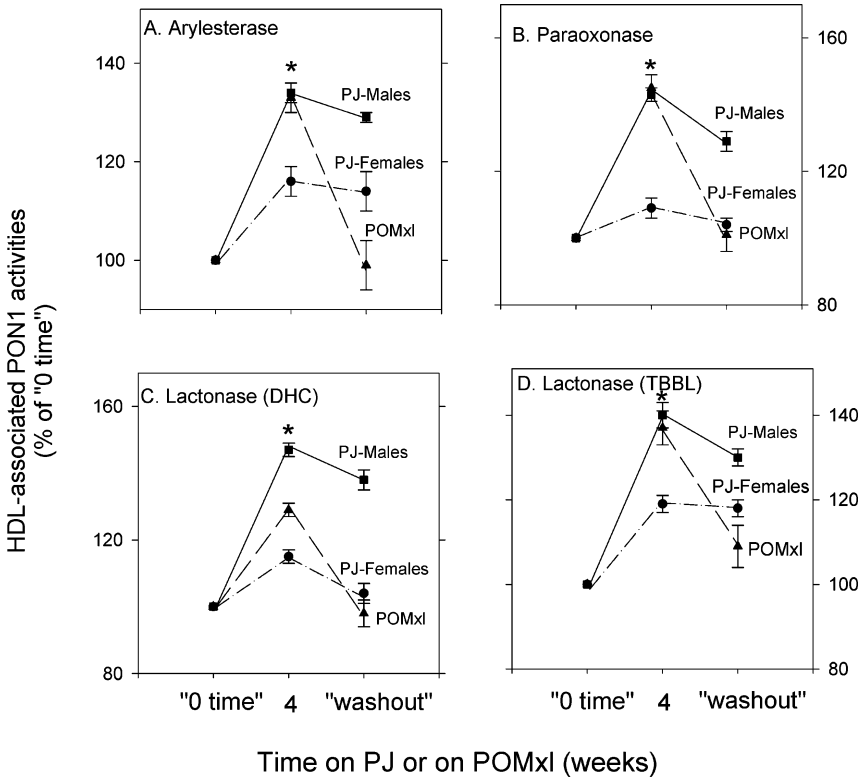

Figure 4. HDL-associated PON1 catalytic activities in WPJ females or WPOMxl males in comparison to WPJ males. Blood samples were collected from WPJ males, WPJ females, or WPOMxl males before (0 time) and 4 weeks after WPJ or WPOMxl consumption, as well as after the "washout" period. The HDL samples were isolated from the blood samples of four patients from each group by ultracentrifugation. The HDL-associated PON1 arylesterase (**A**), paraoxonase (**B**), lactonase (toward DHC, **C**), and lactonase (toward TBBL, **D**) activities were determined as described in the Materials and Methods section. The results are given as % of baseline (0 time) ± SEM ($n = 4$). *$p < 0.05$ (vs 0 time).

treatment were 100, 50, or 43% higher than the values observed at baseline, respectively (**Figure 5**). At the end of the "washout" period, in WPJ males, the residual arylesterase activity was still 87% higher than that observed at baseline, whereas in WPOMxl males or in WPJ females, these values were decreased toward those observed at baseline, respectively (**Figure 5**).

Direct Effect of WPJ and Its Major Polyphenol Punicalagin on Serum Oxidative Stress, PON1 Activity, and PON1 Binding to HDL. To find out whether the effects of pomegranate consumption on PON1 could be the result of a direct effect of WPJ constituents, we incubated serum from diabetic patients with WPJ or punicalagin (the major polyphenolic hydrolyzable tannin antioxidant in WPJ) (*8, 9*). After incubation of serum for 2 h with 18 μgGAE/mL of WPJ or punicalagin, serum oxidative stress decreased significantly by 35 and 38%, respectively, as compared to control serum (similarly incubated with no addition). Upon increasing the polyphenol concentration to 36 μg GAE/mL, no further reduction in serum oxidative stress was noted (**Figure 6A**).

Incubation of serum from diabetic patients with WPJ, WPOMxl, or punicalagin also resulted in a significant decrement in the extent of AAPH-induced serum lipid peroxidation as measured by the TBARS assay. After incubation with 18 or 36 μg GAE/mL of WPJ, the AAPH-induced serum lipid peroxidation decreased significantly by 30 and 41%, respectively, as compared to control serum. Similarly, incubation of serum with punicalagin (18 or 36 μg GAE/mL) resulted in a significant decrement in AAPH-induced serum lipid peroxidation by 21 and 32%, respectively (**Figure 6B**).

After serum incubation for 2 h with 18 μg GAE/mL of WPJ or punicalagin, serum PON1 arylesterase activities increased by 24 and 13%, respectively, as compared to control serum (incubated with no addition). After increasing the punicalagin concentration to 36 μg GAE/mL, serum PON1 arylesterase activity further increased by 19% as compared to control serum (**Figure 6C**).

As consumption of WPJ by diabetic males resulted in an increased HDL-bound PON1 protein, we questioned whether this is mediated directly by WPJ or specifically by the active phenolic compound punicalagin. Serum from diabetic patients was incubated with WPJ or punicalagin or with no addition (control) for 2 h at 37 °C. Then, HDL was isolated from the serum by ultracentrifugation, and Western blot analysis was performed. After serum incubation with18 μg GAE/mL of WPJ or punicalagin, the protein content of HDL-bound PON1 significantly increased by 36 and 14%, respectively, as compared to control serum. After increasing the concentration of WPJ or punicalagin to 36 μg GAE/mL, HDL-bound PON1 protein increased significantly, and it was 62 or 83% higher than in control serum, respectively (**Figure 6D**).

Effects of the Hp Phenotype of Diabetic Patients on the Extent of WPJ Stimulation of Serum PON1 Catalytic Activities in Male or Female Patients. Because the Hp phenotype was shown to affect the propensity of diabetic patients to develop vascular complications (*27*), we hypothesized that the Hp phenotype could possibly affect the response patterns of diabetic patients to treatment with WPJ.

In both WPJ males or WPJ females, after 4 weeks of treatment, the increase in serum PON1 arylesterase activity was more pronounced in patients with the Hp 2-1 phenotype (Hp2-1) than in patients with the Hp 2-2 (Hp2-2) phenotype (**Figure 7**). Males with the Hp2-1 phenotype had a 21% increase in serum PON1 arylesterase activity after 4 weeks of WPJ consumption, as compared to baseline values (**Figure 7A**), while

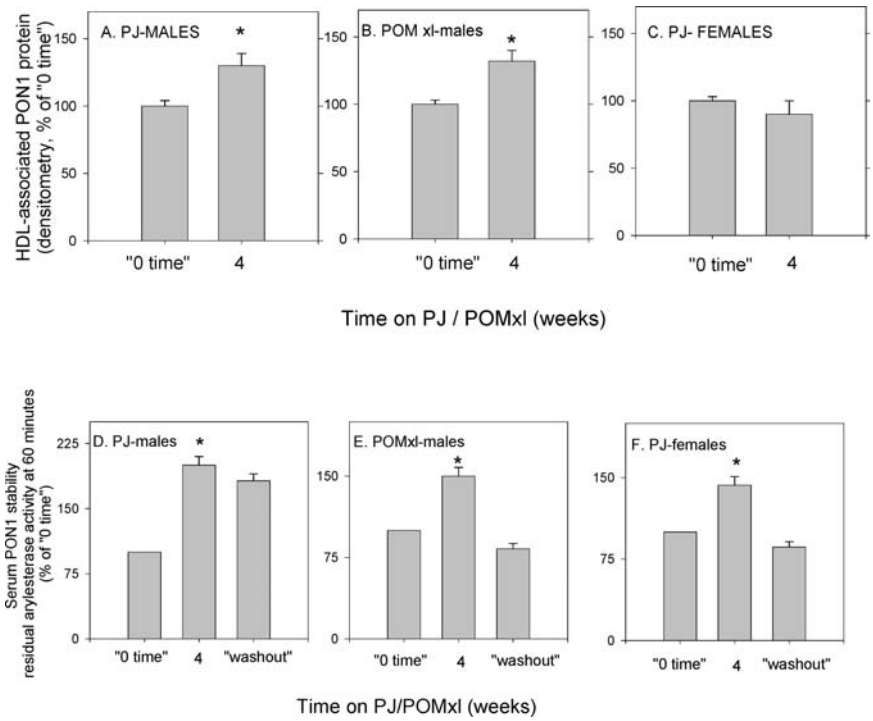

Figure 5. PON1 protein content in HDL and serum PON1 stability in WPJ females or WPOMxl males in comparison to WPJ males. Blood samples were collected from WPJ males, WPJ females, and WPOMxl males before (0 time) and 4 weeks after WPJ or WPOMxl consumption. (**A**−**C**) The HDL fractions were isolated from the blood samples of four patients from each group by ultracentrifugation. The HDL fractions (25 μg of protein) were loaded on 10% acrylamide gel. The PON1 protein bands were visualized using mouse antihuman PON1 antibody, as described under the Materials and Methods section. Densitometric analysis of the PON1 bands was performed. (**D**−**F**) The serum samples of four patients from each group were incubated with 2 mmol/L NTA for 1 h at 37 °C. The serum PON1 residual activity at the end of the incubation period is shown. Results are given as % of baseline (0 time) ± SEM ($n = 4$). *$p < 0.05$ (vs 0 time).

males with the Hp2-2 phenotype had only a 9% increase in activity (**Figure 7B**). Similarly, females with the Hp2-1 phenotype had a significant increase of 37% in serum PON1 arylesterase activities after 4 weeks of WPJ consumption (**Figure 7C**) as compared to baseline values, while females with the Hp2-2 phenotype had only a 14% increase in activity (**Figure 7D**).

DISCUSSION

The present study demonstrated, for the first time, that pomegranate consumption by diabetic patients (males or females) either as WPJ or as WPOMxl, increased PON1 association with HDL and serum PON1 stability, together with antioxidative effects in serum. WPJ consumption did not worsen the parameters in diabetic patients, in spite of the high content of total sugar (10%) in WPJ, similar to that of other fruit juices (*28*). These results are in accordance with our previous studies in diabetic patients (*12*). There are several possible explanations for this phenomenon. (i) Pomegranate fruit possesses α-glucosidase inhibitor activity, which could attenuate the postprandial elevation in blood glucose (*29*). (ii) WPJ activates the peroxisome proliferator activated receptor-γ (PPAR-γ), which plays an important regulatory role in insulin sensitivity (*30, 31*). (iii) Extract of pomegranate fruit was shown to have lasting hypoglycemic effects in diabetic rats (*32*). (iv) Pomegranate consumption decreases the high oxidative stress in diabetics (*33*), a phenomenon that was recently shown to be associated with attenuation of diabetes development (*7*). (v) In pomegranate, the sugars are attached to their unique polyphenols, forming a complex with beneficial properties against diabetic complications (*13*). The increased serum oxidative stress in DM could be the result of glycation and glycol-oxidation of LDL by glucose (*34*), and/or the decreased capability of the abnormal HDL in patients to protect LDL against oxidation (*35*). Our results demonstrate that WPJ consumption by diabetic males reduces their serum oxidative stress, and this effect lasted beyond the treatment period (even at the end of the washout period). The antioxidative properties of pomegranate are related to the high capability to scavenge free radicals and to inhibit LDL oxidation. These effects could be related to pomegranate increased content of unique polymolecular ellagitannins and to specific sugar composition, which involve glucose/fructose association with polyphenols (*8, 13*).

In diabetic patients, PON1 activity is reduced and PON1 dissociates from HDL to the LPDS fraction, as a free, unstable PON1 (*6, 15*). PON1 association with HDL protects lipoproteins from oxidation, and this could be related to PON1 ability to hydrolyze specific oxidized lipids (*3*). One mechanism by which pomegranate phenolics may have resulted in the increased association of PON1 to HDL is by decreasing serum oxidative stress (*12*). To check this hypothesis, we have incubated in vitro serum from diabetic patients with increasing concentrations of WPJ or punicalagin (a representative ellagitannin). This resulted in a significant decrease in basal and in AAPH- induced serum lipid peroxidation. Furthermore, serum PON1 activity and its binding to HDL significantly increased after WPJ or punicalagin treatment in a dose-dependent manner. Another possible mechanism is that WPJ mediates PON1 transfer from LPDS to HDL. A third possible mechanism is that WPJ or the active phenolic punicalagin could form a bridge between PON1 and HDL, thus stabilizing the enzyme. WPJ consumption by diabetic males resulted in a significant increment in PON1 catalytic activities not only in HDL-bound PON1 but also in LPDS free PON1. Similar effects were shown in vitro upon direct addition of WPJ

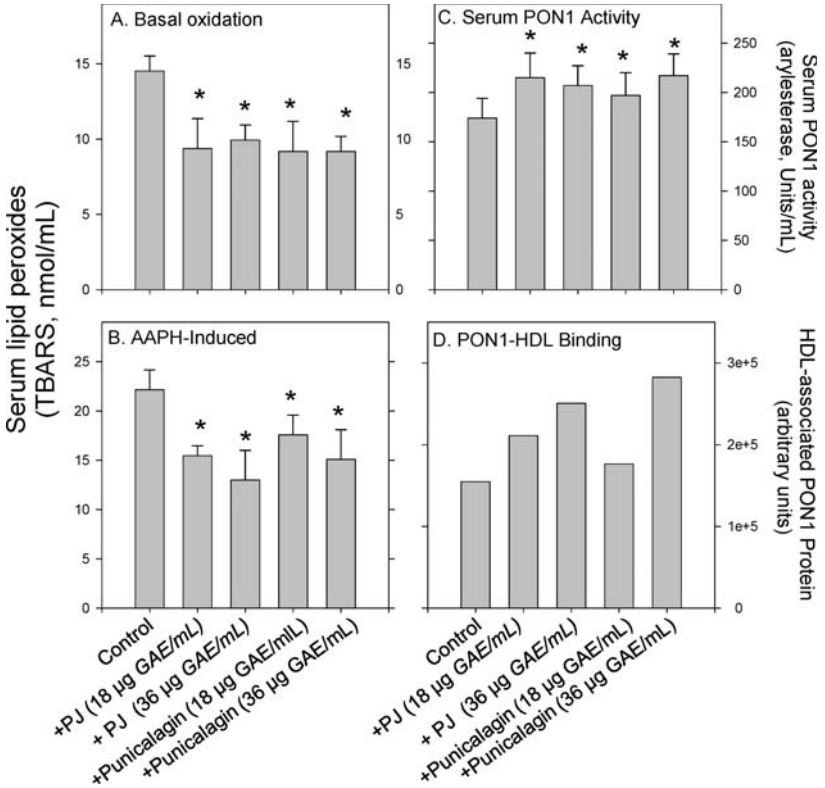

Figure 6. Direct effect of WPJ, WPOMxl, or punicalagin on serum oxidative stress, on HDL-associated PON1 arylesterase activity, and on HDL-associated PON1 protein content. The serum from diabetic patients was incubated with WPJ (18 or 36 μg GAE/mL), punicalagin (18 or 36 μg GAE/mL), or WPOMxl 36 μg GAE/mL for 2 h. Then, the HDL fraction was isolated by ultracentrifugation. (**A**) Basal serum oxidative stress, (**B**) AAPH-induced serum lipid peroxidation, (**C**) serum PON1 arylesterase activity, and (**D**) the amount of PON1 protein in HDL were determined as described in the Materials and Methods section. The results are given as means ± SEM of three different experements. *$p < 0.05$ (vs 0 time).

or punicalagin to LPDS obtained from diabetic patients (before WPJ treatment). It could be that specific polyphenols in WPJ, such as punicalagin, bind to the free PON1, change the enzyme conformation, and thus lead to increments in PON1 catalytic activities. These effects of WPJ might be important in protection against atherogenesis by affecting PON1, not only in its active conformation when present on HDL but also in its less active form, as a free PON1.

Studies using the atherosclerotic apolipoprotein E-deficient mice have demonstrated that WPOMxl consumption decreased oxidative stress and atherosclerotic lesion development, and these effects could be related to the high polyphenol content (*14, 36*). WPOMxl consumption by DM male patients resulted in a similar beneficial pattern, but the decrease in serum oxidative stress was to a lesser extent than that observed in patients that consumed WPJ. This phenomenon could be related to the fact that the WPOMxl males had 114% greater basal serum oxidative stress at the beginning of the study, in comparison to WPJ males, probably because they were smokers. Smoking is known to significantly increase serum oxidative stress and to decrease the response to antioxidant therapy (*37*). In addition, while WPOMxl contains the same polyphenol content as WPJ, it does not contain sugars. WPJ has been shown to reduce cellular oxidative stress more potently than its polyphenol fraction, and this was shown to be related to the unique sugar complex with WPJ phenolics (*13*). Incubation of serum from diabetic patients with WPJ resulted in a greater reduction in AAPH-induced serum lipid peroxidation in comparison to WPOMxl. WPOMxl consumption by DM males also resulted in an increase in HDL-associated PON1 activities similarly to that observed in WPJ males. In addition, consumption of WPOMxl resulted in increased PON1 protein binding to HDL, to a similar extent as that observed in WPJ males.

While oxidative stress in healthy premenopausal females was shown to be lower than in young healthy males (*38*), oxidative stress is increased in the postmenopausal state and this in turn increases the risk of cardiovascular disease (*39*). Nutritional antioxidants such as extra virgin olive oil and grape polyphenols were shown to reduce oxidative stress in postmenopausal females (*40, 41*). The basal oxidative stress in serum from WPJ females was 115% higher than that observed in WPJ males. This could be due to the postmenopausal state and also to the high BMI in the female vs male patients, as obesity has been shown to increase oxidative stress (*42*). In WPJ females, decreased oxidative stress and increased HDL-associated PON1 activity and serum PON1 stability were noted, but these effects were less pronounced in comparison to WPJ males.

In contrast to WPJ males and WPOMxl males, we could not demonstrate a significant increase in HDL-PON1 protein association in WPJ females. This phenomenon may be the result of the higher basal serum oxidative stress in the female group, or it could be related to hormonal differences related to their postmenopausal state. It may also be that WPJ polyphenols stimulated PON1 activities directly and also secondary to PON1 binding to the HDL particle.

Finally, Hp phenotype was found to be an independent risk factor for cardiovascular and microvascular complications in patients with type 2 DM (*27*). Patients with the phenotype 2-2 are under higher oxidative stress and have a greater risk for cardiovascular complications than patients with phenotype 2-1 (*27*). We thus hypothesized that patients with different Hp

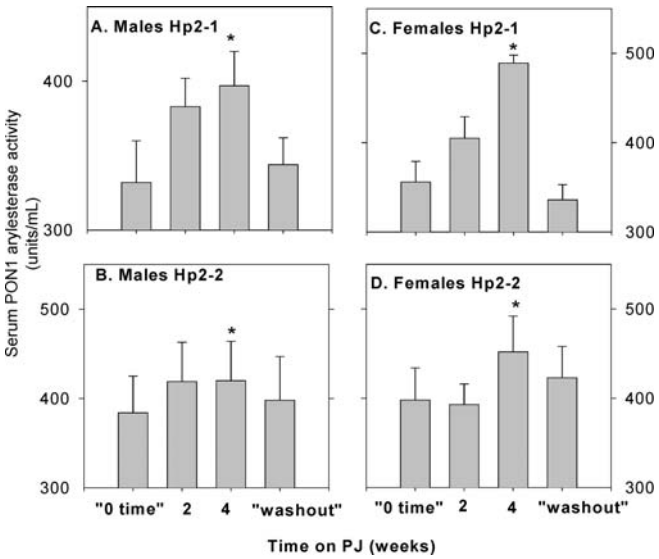

Figure 7. Effects of Hp phenotype of diabetic patients on the extent of WPJ stimulation of serum PON1 catalytic activities in male and female patients. Blood samples were collected from WPJ males or WPJ females before (0 time) and 2 and 4 weeks after WPJ consumption, as well as after the "washout" period. Hp phenotype and arylesterase activities were determined as described in the Materials and Methods section. In the male group, six patients had the 2-2 phenotype, and four patients had the 2-1 phenotype. In the female group, seven patients had the 2-2 phenotype, and three patients had the 2-1 phenotype. (**A**) Arylesterase activities in male DM patients with Hp phenotype 2-1 (HP2-1). (**B**) Arylesterase activities in male DM patients with Hp phenotype 2-2 (HP2-2). (**C**) Arylesterase activities in female DM patients with Hp phenotype 2-1 (HP2-1). (**D**) Arylesterase activities in female DM patients with Hp phenotype 2-2 (HP2-2). *$p < 0.05$ (vs 0 time).

phenotypes will react differently to WPJ consumption, as oxidative stress was shown to inactivate PON1 (*43*). Our results indeed clearly demonstrate that male and female patients with Hp2-1 phenotype reacted better to WPJ treatment than patients with Hp2-2 phenotype, as shown by a greater increase in PON1 arylesterase activity in their serum. Statin treatment reduces cardiovascular event rates by "only" 30%. Preventing the other 70% requires more than just control of hypercholesterolemia (*44*). The use of antioxidants, and anti-inflammatory agents (pomegranate juice and extracts), could be of major importance in the protection against the other 70% cardiovascular events.

We conclude that WPJ consumption by diabetic males and females and WPOMxl consumption by diabetic males not only did not worsen their diabetic parameters but in fact resulted in an increased PON1 association with HDL, a phenomenon that stabilized the enzyme and improved its' antiatherogenic properties.

ABBREVIATIONS USED

WPJ, Wonderful variety pomegranate juice; WPOMxl, Wonderful variety pomegranate fruit liquid extract; PON1, paraoxonase 1; HDL, high-density lipoprotein; LDL, low-density lipoprotein; DM, diabetes mellitus; LPDS, lipoprotein deficient serum; ACE, angiotensin converting enzyme; BMI, body mass index; Hp, haptoglobin; TBARS, thiobarbituric acid reactive substances; GAE, gallic acid equivalent; AAPH, 2,2′-azobis,2-amidinopropane hydrochloride; FRAP, ferric reducing antioxidant power; DHC, dihydrocoumarine; TBBL, thiobutyl-butyrolactone; NTA, nitriloacetate; SEM, standard error of the mean; HbA1c, hemoglobin A1c.

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W.R. and M.E. recruited subjects for the study and performed the clinical follow-up. W.R. and M.R. performed all of the in vivo and in vitro experiments. A.P.L. and R.M.-L. performed the Hp phenotyping studies. M.A. initiated the study and was supervisor and coordinator of the whole project and together with W.R. and M.R. wrote the manuscript.

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