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ORIGINAL ARTICLE

Exogenous (Pomegranate Juice) or Endogenous (Paraoxonase1) Antioxidants Decrease Triacylglycerol Accumulation in Mouse Cardiovascular Disease-Related Tissues

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Abstract The polyphenol-rich pomegranate juice (PJ) and the high-density lipoprotein (HDL)-associated paraoxonase1 (PON1) are known as potent atheroprotective antioxidants, but their effects on other tissues related to cardiovascular disease (CVD) remain unknown. The current study aimed to investigate the effects of treating mice with PJ or recombinant PON1 (rePON1) on the oxidation and lipid status of CVD-related tissues: serum, aorta, heart, liver, kidney, visceral, and subcutaneous adipose tissues (VAT and SAT). Both PJ consumption and rePON1 injection decreased the serum levels of thiobarbituric acid-reactive substances (16% and 19%) and triacylglycerols (TAG, 24% and 27%), while only rePON1 increased the levels of thiol groups (35%) and decreased serum cholesterol (15%). Both PJ and rePON1 significantly decreased aortic cholesterol (38% and 32%) and TAG (62% and 58%) contents in association with downregulation of the key TAG biosynthetic enzyme diacylglycerol O-acyltransferase 1 (DGAT1, 71% and 65%), while only PJ decreased aortic lipid peroxides (47%). Substantial TAG-lowering effects of both PJ and rePON1 were observed also in the heart (31% and 42%), liver (34% and 42%), and kidney (42% and 57%). In both VAT and SAT, rePON1 decreased the levels of lipid peroxides (28% and 25%), while PJ decreased the TAG content (22% and 18%). *Ex vivo* incubation of SAT with serum derived from mice that consumed PJ or injected with rePON1 decreased SAT lipid peroxides (35% or 28%) and TAG mass (12% or 10%). These novel findings highlight potent TAG-lowering properties of exogenous (PJ) and endogenous (PON1) antioxidants in tissues associated with CVD.

Keywords Antioxidants · Cardiovascular diseases · Lipids · Paraoxonase · Pomegranate juice · Triacylglycerols

Lipids (2018).

Abbreviations

CKD	chronic kidney disease
CVD	cardiovascular diseases
DGAT1	diacylglycerol O-acyltransferase 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDL	high-density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
LDL	low-density lipoprotein
NAFLD	nonalcoholic fatty liver disease
PJ	pomegranate juice
PON1	paraoxonase1
qPCR	quantitative polymerase chain reaction
rePON1	recombinant PON1
TAC	transverse aortic constriction
TAG	triacylglycerols
SAT	subcutaneous adipose tissues
SH	sulfhydryl
TBARS	thiobarbituric acid-reactive substances
VAT	visceral adipose tissues

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Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality worldwide, accounting for about 30% of all global deaths (Benjamin et al., 2017). Obesity, characterized by increased fat deposits in adipose tissues, is a major risk factor for CVD (Benjamin et al., 2017). Lipid accumulation in nonadipose tissues also plays important roles in the pathogenesis of CVD (Montani et al., 2004). For instance, dysregulated lipid metabolism in the heart can result in cardiomyocyte lipid accumulation, leading to cardiac lipotoxicity and dysfunction (Goldberg et al., 2012). In patients with pressure-overloaded heart and metabolic syndrome, increased myocardial lipid accumulation and overexpression of lipogenic genes are evident (Marfella et al., 2009). Moreover, nonalcoholic fatty liver disease (NAFLD), characterized by hepatic lipid accumulation (steatosis), as well as chronic kidney disease (CKD), are also known to increase the CVD risk (Bonora and Targher, 2012). Interestingly, renal lipid accumulation has also been suggested to be involved in CVD pathogenesis (Montani et al., 2004), and abnormal renal lipid metabolism and lipid accumulation are evident in patients with diabetic nephropathy (Herman-Edelstein et al., 2014).

The underlying cause of most CVD is atherosclerosis, an inflammatory disease of the arteries, which involves imbalanced lipid metabolism and increased oxidative stress (Rom and Aviram, 2016a). The antiatherogenic effects of various exogenous or endogenous antioxidants have been well recognized (Rom and Aviram, 2016a). Numerous studies from our laboratory and others have demonstrated potent atheroprotective effects of two specific antioxidants: the exogenous polyphenol-rich pomegranate juice (PJ) and the endogenous high-density lipoprotein (HDL)-associated enzyme—paraoxonase1 (PON1). Pomegranate (*Punica granatum*) is a major source of polyphenols including catechins, ellagic tannins, gallic acid, and ellagic acid (Gil et al., 2000). PJ has been shown for its potent antiatherogenic properties in cell cultures, animal models, and humans, which are mediated by inhibition of low-density lipoprotein (LDL) oxidation and its uptake by arterial macrophages, preventing macrophage-foam cell formation, a hallmark feature of early atherogenesis (Aviram et al., 2000a, 2004; Fuhrman et al., 2000, 2005; Kaplan et al., 2001; Rom et al., 2017b). In addition, PJ supplementation increases the hepatic expression of PON1 and induces its activity in the serum of mice and humans (Aviram et al., 2000a; Estrada-Luna et al., 2018; Rom et al., 2017b)

The PON gene family includes PON1, PON2, and PON3. All PON enzymes have been shown to possess antioxidative properties, but PON1 remains the most studied one (Rom and Aviram, 2017). PON1 is mainly synthesized in the liver, from which it is secreted to the circulation, where it is found in association with HDL (La Du, 1996; Rom and Aviram, 2017). PON1 catalyzes the hydrolysis of several compounds (e.g. paraoxon, arylesters, and lactones) and is known for its peroxidase-like activity demonstrated by its ability to hydrolyze hydrogen peroxides and lipid peroxides (Aviram and Vaya, 2013; Rom and Aviram, 2017). PON1 peroxidase-like activity contributes to the antioxidative and antiatherogenic properties of HDL, which inhibits lipoprotein oxidation, stabilizes vulnerable atherosclerotic plaques, and stimulates cholesterol efflux from arterial macrophages (Aviram et al., 1998, 2000b; Aviram and Vaya, 2013). While mice lacking PON1 are more susceptible to atherosclerosis, in PON1 transgenic mice atherosclerotic lesion formation is decreased in association with enhanced ability of their HDL to protect from LDL oxidation (Shih et al., 1998, 2000; Tward et al., 2002). The development of recombinant PON1 (rePON1) enabled research on the therapeutic potential of PON1 administration in attenuating atherogenesis (Aharoni et al., 2004). Indeed, injection of rePON1 to mice enhances their HDL ability to protect from LDL oxidation and to stimulate efflux of cholesterol from macrophages (Rosenblat et al., 2011).

Although the antioxidative and atheroprotective properties of PJ and PON1 are well established, especially in macrophages, their effects on other tissues associated with CVD remain unknown. The aim of the current study was to investigate the effects of 3-week PJ or rePON1 treatment on the oxidation and lipid status of CVD-related tissues from C57BL/6 mice: serum, aorta, heart, liver, kidney, visceral, and subcutaneous adipose tissues (VAT and SAT). Our findings reveal, for the first time, mostly potent triacylglycerols (TAG)-lowering properties for PJ and rePON1 in these tissues.

Materials and Methods

PJ and Polyphenol Quantification

Concentrated PJ (65° Brix concentrate) was obtained from POM Wonderful (Los Angeles, CA, USA) and diluted 1:5 (v/v) with water to obtain a single-strength PJ to be used in this study. PJ preparation and its polyphenol composition were described in our previous studies (Rom and Aviram, 2016b). Briefly, single-strength PJ contains 84% water, 6.6% glucose, 7.2% fructose, 0.15% protein, and 0.02% fat. The polyphenol composition is 2369 ppm phenolics, 2000 ppm hydrolyzable tannins, 251 ppm ellagitannins, and 1487 ppm punicalagin. The total polyphenol concentration of the single-strength PJ was determined spectrophotometrically with phospho-molybdicphosphotungstic acid reagent and gallic acid as a standard.

rePON1

rePON1 was generated by directed evolution as described previously (Aharoni et al., 2004). PON1 storage buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM CaCl2, and 0.1% v/v Tergitol) was supplemented with 0.02% (w/v) sodium azide and stored at 4 °C. Before experiments, rePON1 tergitol was removed by its treatment with Bio Beads (Bio Rad, Hercules, CA, USA, SM-2 Adsorbent 20–50 mesh, 30 mg/100 µl, 2 h at 4 °C), followed by centrifugation and collection of the supernatant. This procedure was repeated twice (Rosenblat et al., 2011).

Mouse Treatments

The experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH, USA. The protocol for the study was approved by the Committee for Supervision of Animal Experiments of the Technion—Israel Institute of Technology (Approval number IL045-04-16). Eight weeks old male mice (provided by Envigo, Jerusalem, Israel) were bred and housed under pathogen-free conditions at the Animal Care Facility of the Rappaport Faculty of Medicine, Technion. Mice were randomly divided into three experimental groups ($n = 6$ per group) and were treated for a period of 3 weeks as follows: (1) Control; (2) PJ supplementation (7 mg gallic acid equivalents/kg/day; and (3) intraperitoneal rePON1 injection (50 ug/mouse, every 48 h) (Rom et al., 2017b; Rosenblat et al., 2011). Throughout the study, the mice were allowed *ad libitum* access to standard chow.

Serum Analyses

Blood was collected from the retro-orbital plexus of mice under isoflurane anesthesia (*via* inhalation) following a 12 h fast. The serum was separated from the clotted blood by centrifugation (1000 g, 15 min) and kept at −80 °C. Analyses of serum thiobarbituric acid-reactive substances (TBARS assay), PON1 activity (determined spectrophotometrically at 412 nm with paraoxon as a substrate), thiol (sulfhydryl, SH) groups (determined spectrophotometrically at 412 nm using 5,5′-Dithiobis (2-nitrobenzoic acid), DTNB)) were conducted as previously described (Buege and Aust, 1978; Rom et al., 2017b; Rosenblat et al., 2006). Serum cholesterol and TAG were determined using commercially available kits: Cholesterol Determination Kit (Roche Diagnostics, Mannheim, Germany) and Triacylglycerol Determination Kit (T2449 Triacylglycerol Reagent and F6428 Free Glycerol Reagent, Sigma-Aldrich, St. Louis, MO, USA).

Tissue Lipid Extraction and Quantification of Lipid Peroxides, Cholesterol or TAG

Aortas, hearts, livers, kidneys, SAT (inguinal adipose tissue), and VAT (epididymal adipose tissue) were rapidly removed from the anesthetized mice and kept at −80 °C. Prior to lipid extraction, organs were cleared of adhering fat and connective tissue. Whole aortas (including the ascending aorta, the aortic arch, and the descending thoracic aorta), hearts, or kidneys as well as samples of liver (200 mg), VAT or SAT (30 mg), were homogenized in 1 mL PBS using a Polytron Homogenizer (Kinematica AG, Littau, Switzerland) at 60 W for 1 min. Homogenates were then centrifuged at 5000 g for 20 min and the supernatants were analyzed for protein levels by the Lowry assay. Tissue lipids were extracted with hexane: isopropanol (3:2, v:v), and the hexane phase was evaporated under nitrogen. The amount of tissue lipid peroxides was determined spectrophotometrically using a lipid peroxide assay, and were expressed as nmol lipid peroxides/mg protein (El-Saadani et al., 1989). The contents of cholesterol and TAG were determined using the commercially available kits mentioned above, and were expressed as µg cholesterol or TAG/mg protein (Grajeda-Iglesias et al., 2018; Rom et al., 2017a, b).

Quantitative Polymerase Chain Reaction (qPCR) Analysis

Total RNA was extracted from tissues with the MasterPure RNA purification kit (Epicenter, Madison, WI, USA). complementary DNA (cDNA) was generated from 1 ug of total RNA with the ThermoScientific cDNA kit (ThermoScientific, Waltham, MA, USA). Using Absolute Blue qPCR ROX mix (ThermoScientific, Waltham, MA, USA), products of the reverse transcription were subjected to qPCR using TaqMan gene expression assays with a Rotor-Gene 6000 amplification detection system (Corbett Life Science, Sydney, Australia). Diacylglycerol O-acyltransferase 1 (DGAT1), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), and PON2 messenger RNA (mRNA) data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers and probes were designed by Primer Design (Southampton, UK):

Pon2 Sense Primer: CGAGCTCCTTCCAAGTGTGAAT

Pon2 Antisense Primer: CTTCTGCCACCAGTTTAACTTCTT

Dgat1 Sense Primer: TGGTGGAATGCTGAGTCTGT

Dgat1 Antisense Primer: GGTCAAAAATACTCCTGTCCTG

Hmgcr Sense Primer: CCGAATTGTATGTGGCACTGT

Hmgcr Antisense Primer: TTATCTTTGATCTGTTGTGAACCAT

Ex vivo Subcutaneous Adipose Tissue Experiments

SAT (20 mg of inguinal adipose tissue) isolated from control mice was incubated in 100 µL of serum (diluted 1:5 in PBS) derived from mice treated with PJ or rePON1 as indicated above. Following incubation for 16 h, levels of lipid peroxides or TAG were determined as described above.

Statistics

All statistics were performed with SPSS 24.0 software (SPSS Inc. IBM, Chicago, IL, USA). Results are presented as box-plots or bars (mean ± SEM) of at least three independent observations. One-way ANOVA followed by Tukey *post hoc* tests was used to compare the means between the groups. $p < 0.05$ was considered statistically significant.

Results

Effects of PJ Consumption or rePON1 Injection on Serum Oxidation and Lipid Status in Mice

Following 3 weeks of PJ consumption or rePON1 injection, serum samples were collected from the mice and analyzed for their oxidation (TBARS, SH groups and PON1 activity) and lipid (cholesterol and TAG) status. Both PJ consumption and rePON1 injection significantly decreased serum TBARS levels (by 16%, $p = 0.014$ and 19%, $p = 0.004$, respectively, Fig. 1a). However, whereas rePON1 injection significantly increased serum SH groups (by 35%, $p = 0.045$), but not PON1 activity, PJ consumption significantly increased serum PON1 activity (by 24%, $p = 0.046$), but not SH groups (Fig. 1b, c). As for serum lipids, a minor (15%, $p = 0.048$), but significant, reduction in serum cholesterol was noted by rePON1 injection, but not by PJ consumption (Fig. 1d), whereas serum TAG were significantly decreased by both antioxidants (by 24%, $p = 0.034$ and 27%, $p = 0.016$, Fig. 1e).

PJ Consumption or rePON1 Injection Decrease Aortic TAG in Mice

The effects of PJ consumption or rePON1 injection on aortic lipids were determined next. Whereas PJ, but not rePON1, significantly decreased aortic lipid peroxidation (by 47%, $p = 0.014$, Fig. 2a), rePON1, but not PJ, significantly increased the expression of the endogenous antioxidant *Pon2* (by 2.3-fold, $p = 0.047$, Fig. 2b). Nevertheless, both PJ and rePON1 had marked lipid-lowering effects in the aorta, decreasing aortic cholesterol by 38% ($p = 0.001$) and 32%, ($p = 0.002$), respectively (Fig. 2c), with nonsignificant trends toward a compensatory increase in *Hmgcr* expression (Fig. 2d). Also, both PJ and rePON1 significantly reduced aortic TAG by 62% ($p = 0.001$) and 52% ($p = 0.003$), respectively (Fig. 2e), in association with

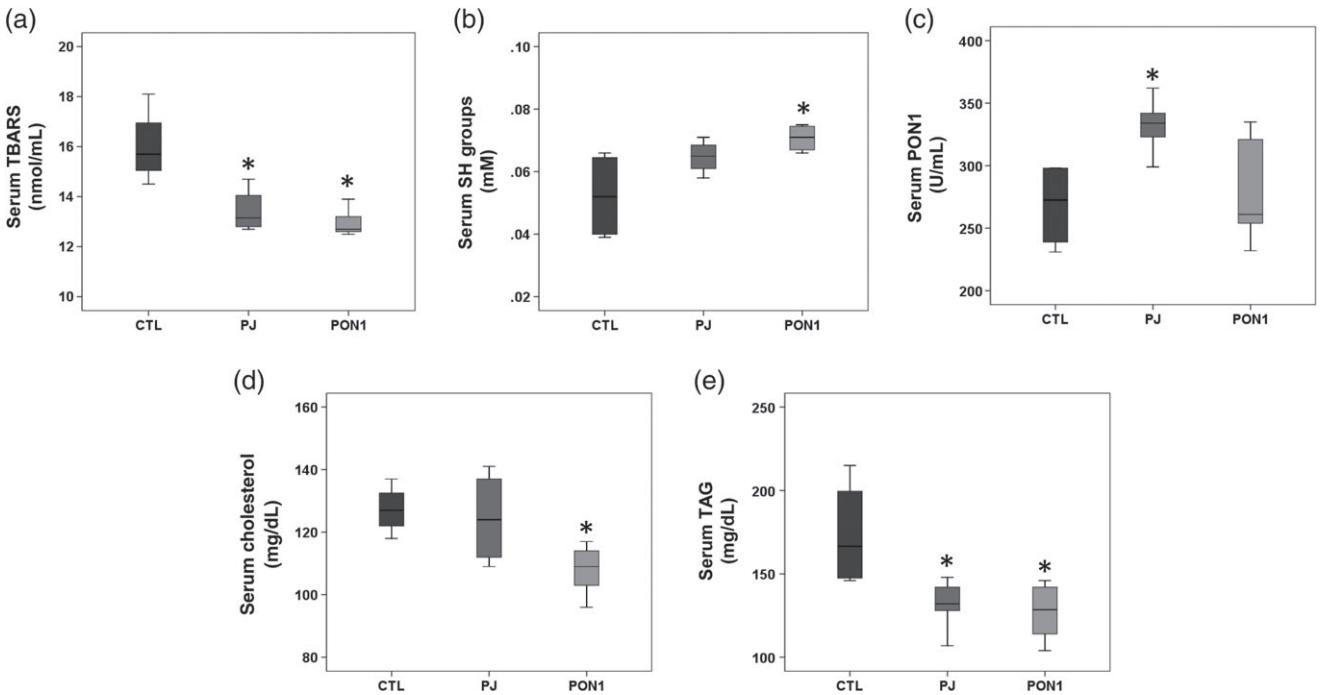

Fig. 1 Effects of pomegranate juice (PJ) or recombinant PON1 (rePON1) on serum oxidation and lipid status. Serum levels of (a) thiobarbituric acid-reactive substances (TBARS), (b) sulfhydryl (SH) groups, (c) paraoxonase1 (PON1) activity, (d) cholesterol, and (e) triacylglycerols (TAG), following 3 weeks of PJ consumption or rePON1 injection to C57BL/6 mice ($n = 6$). *$p < 0.05$ versus control (CTL) mice

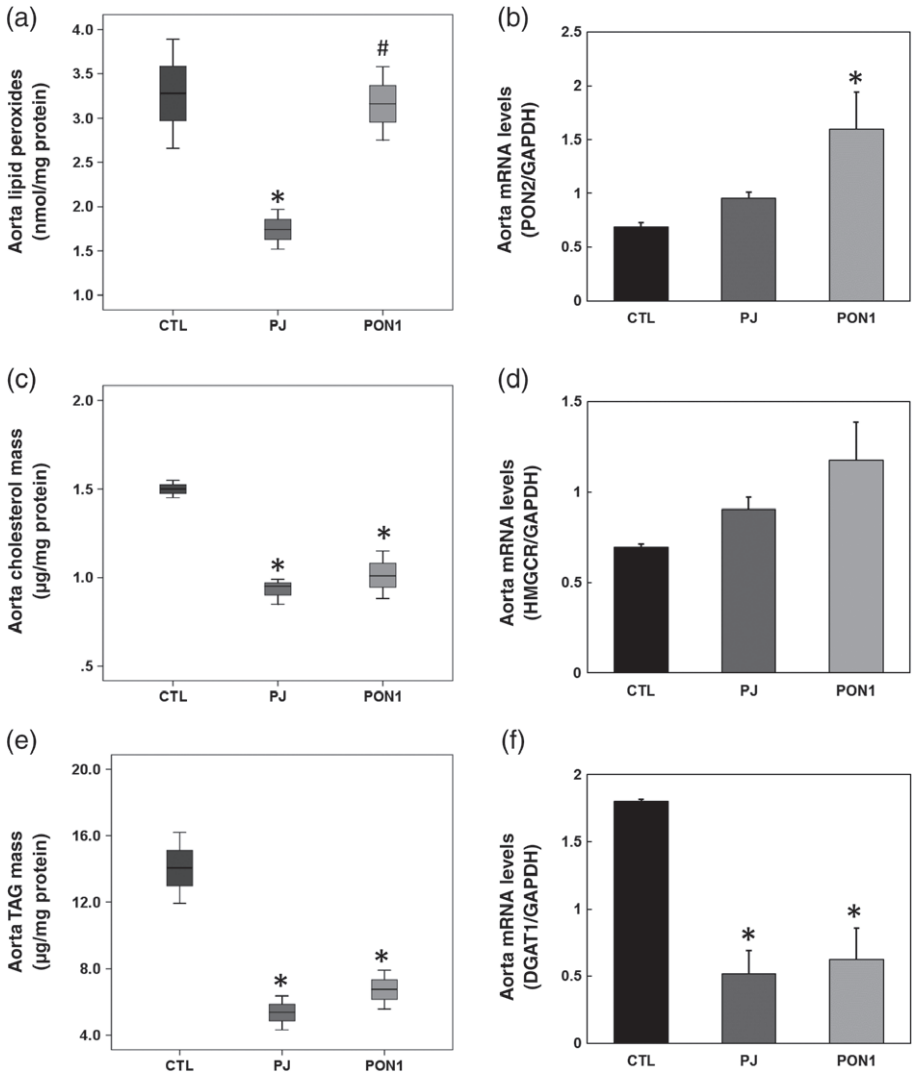

Fig. 2 Effects of pomegranate juice (PJ) or recombinant PON1 (rePON1) on aortic oxidation and lipid status. Aortic levels of (a) lipid peroxides, (b) paraoxonase2 (*Pon2*) mRNA, (c) cholesterol (d) 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*) mRNA (e) triacylglycerols (TAG), and (f) diacylglycerol O-acyltransferase 1 (*Dgat1*) mRNA, following 3 weeks of PJ consumption or rePON1 injection to C57BL/6 mice. $^{*}p < 0.05$ versus control (CTL) mice, $^{\#}p < 0.05$ versus mice treated with PJ

downregulation of the expression of *Dgat1*, a key enzyme TAG biosynthetic enzyme ex DGAT1 (by 71%, $p = 0.002$ or 65% $p = 0.006$, respectively, $p < 0.05$, Fig. 2f). These findings are in line with previous reports demonstrating the ability of PJ or PON1 to decrease aortic lipid accumulation and indicate their atheroprotective role related to inhibition of TAG biosynthetic pathways in the aorta as demonstrated by reduced *Dgat1* expression.

PJ Consumption or rePON1 Injection Decrease Heart TAG in Mice

The effects of PJ consumption or rePON1 injection on heart lipids were determined next. Although both PJ and rePON1 had no significant effects on the levels of lipid peroxides (Fig. 3a) and cholesterol (Fig. 3b), both antioxidants significantly decreased the content of TAG in the heart (by 31%, $p = 0.031$ and 42%, $p = 0.008$, respectively, Fig. 3c). A significant downregulation of DGAT1 (by 34%, $p = 0.011$) was observed in hearts from mice that consumed PJ, but not in mice that were injected with rePON1 (Fig. 3d).

PJ Consumption or rePON1 Injection Decrease Hepatic and Renal TAG in Mice

The effects of PJ consumption or rePON1 injection on hepatic and renal lipids were determined next. Similar to their effects on the heart, both antioxidants had no significant effects on lipid peroxides and cholesterol levels in the liver (Fig. 4a, b) or kidney (Fig. 4d, e). However, both PJ

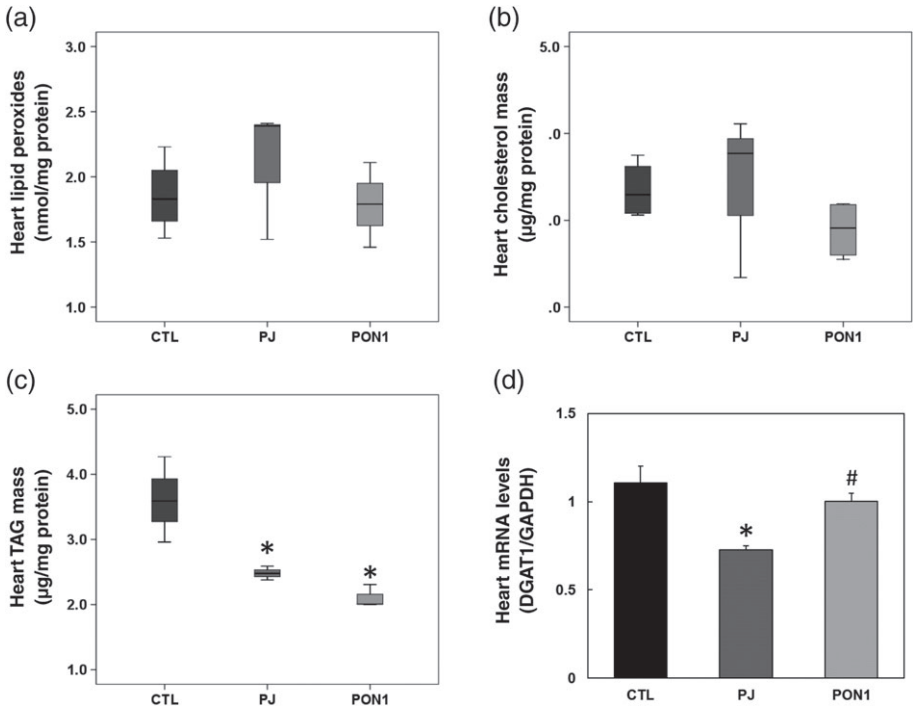

Fig. 3 Effects of pomegranate juice (PJ) or recombinant PON1 (rePON1) on cardiac oxidation and lipid status. Cardiac levels of (a) lipid peroxides, (b) cholesterol, (c) triacylglycerols (TAG), and (d) *Dgat1* mRNA, following 3 weeks of PJ consumption or rePON1 injection to C57BL/6 mice. $^{*}p < 0.05$ versus control (CTL) mice, $^{\#}p < 0.05$ versus mice treated with PJ

and rePON1 markedly decreased hepatic TAG mass (by 34%, $p = 0.03$ and 42%, $p = 0.01$ respectively, Fig. 4c) and renal TAG mass (by 42% $p = 0.045$ and 57% $p = 0.004$, respectively, $p < 0.05$, Fig. 4f). The marked TAG-lowering effects in the kidney were associated with downregulation of *Dgat1* expression by PJ administration (34%, $p = 0.02$) with a similar trend observed following rePON1 injection (Fig. 4g).

In Vivo and *Ex Vivo* Effects of PJ or rePON1 on Lipid Peroxides and TAG Content in VAT and SAT

Both in VAT and in SAT, rePON1, but not PJ, significantly decreased the levels of lipid peroxides (by 28%, $p = 0.046$ and 25%, $p = 0.02$, respectively Fig. 5a, c). Although PJ had more prominent effects to decrease the TAG content in VAT (by 28%, $p = 0.021$, Fig. 5b), both PJ and rePON1 significantly decreased the TAG content in SAT (by 22%, $p = 0.01$ and 18%, $p = 0.015$, respectively, Fig. 5d). Accordingly, in *ex vivo* experiments, incubation of SAT with serum derived from mice that consumed PJ or injected with rePON1 decreased SAT lipid peroxides (by 35%, $p = 0.02$ or 28%, $p = 0.043$, respectively, Fig. 5e) and TAG content (by 12%, $p = 0.001$ or 10%, $p = 0.006$ respectively, Fig. 5f).

Discussion

In the present investigation, we determined the effects of the exogenous antioxidant PJ or that of the endogenous antioxidant PON1 on oxidation and lipid status in CVD-related tissues from mice. In line with previous reports on the atheroprotective properties of PJ and PON1 (Aviram et al., 2000a; Mackness et al., 2006; Tward et al., 2002), we found marked lipid-lowering effects in aortas of mice that either consumed PJ or were injected with rePON1. Moreover, we now show, for the first time, that administration of PJ or rePON1 results in significant TAG-lowering effects in other CVD-related tissues, including serum, heart, liver, kidneys, VAT, and SAT.

Previous studies reported that PJ consumption or overexpression of PON1 in dietary or genetic hyperlipidemic mouse models decreases atherosclerotic lesion formation (Aviram et al., 2000a; Mackness et al., 2006; Tward et al., 2002). In the current study, PJ administration (but not rePON1) did not lower serum cholesterol levels, but significantly reduced the aortic cholesterol content. This could be related to the selective effects of PJ polyphenolic antioxidants and hypocholesterolemic compounds on cholesterol levels in various tissues. In the serum, and much less so in the aorta, the hypocholesterolemic effects of PJ are less pronounced because the serum (vs the arteries) is

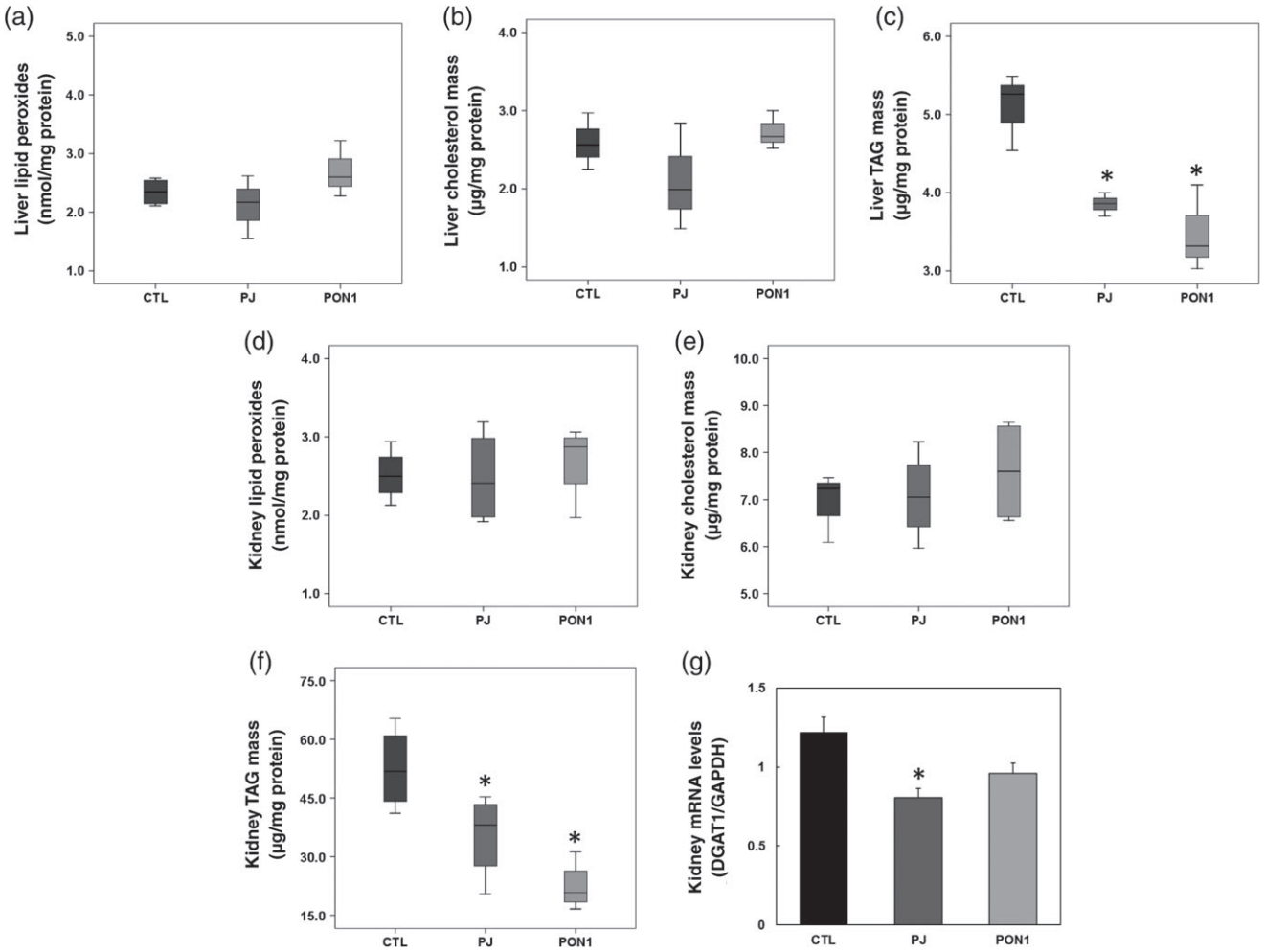

Fig. 4 Effects of pomegranate juice (PJ) or recombinant PON1 (rePON1) on hepatic and renal oxidation and lipid status. Hepatic levels of (a) lipid peroxides, (b) cholesterol, (c) triacylglycerols (TAG), and renal levels of (d) lipid peroxides, (e) cholesterol (f) TAG, and (g) *Dgat1* mRNA, following 3 weeks of PJ consumption or rePON1 injection to C57BL/6 mice. *$p < 0.05$ versus control (CTL) mice

selectively rich in potent hypocholesterolemic and antioxidant agents (Aviram et al., 2004). As for PON1, we show for the first time that injection of rePON1 to mice decreases aortic cholesterol and TAG contents. Although PON1 is associated with HDL in the circulation, PON2 is expressed in various major organs as an intracellular enzyme, and is known for its atheroprotective and cardioprotective roles (Li et al., 2018; Ng et al., 2006; Rom and Aviram, 2017). Here, we found that rePON1 injection upregulated PON2 in the aorta, whereas both PJ consumption and rePON1 injection reduced *Dgat1* expression, the key enzyme that catalyzes the final step in TAG biosynthesis by attaching a long-chain fatty acyl-CoA to diacylglycerol (Yen et al., 2008). In support, studies on *Pon2*-deficient mice have shown that PON2 inhibits DGAT1 and attenuates TAG accumulation in macrophages (Meilin et al., 2010; Rosenblat et al., 2009). These results suggest that the lipid-lowering effects of PJ and rePON1 in the aorta may be mediated by inhibition of DGAT1-induced TAG biosynthesis.

Recent reports indicate a role for PON1 in chronic heart failure. Lower PON1 arylesterase activity in subjects with chronic heart failure is a strong predictor of long-term major adverse cardiac events, indicating that PON1 activity may serve as a prognostic biomarker in chronic heart failure (Hammadah et al., 2017; Tang et al., 2011). Nevertheless, whether targeting PON1 can be used as a cardioprotective strategy and by which mechanisms it acts remain unknown (Rom and Aviram, 2017). Under pathological conditions such as obesity and type 2 diabetes, fatty-acid uptake and oxidation in the heart are imbalanced, leading to TAG accumulation and cardiac lipotoxicity (Goldberg et al., 2012). Indeed, intramyocardial TAG accumulation is evident in failing hearts of patients with obesity or diabetes (Sharma et al., 2004). We found that PJ consumption, which increased PON1 serum activity, as well as

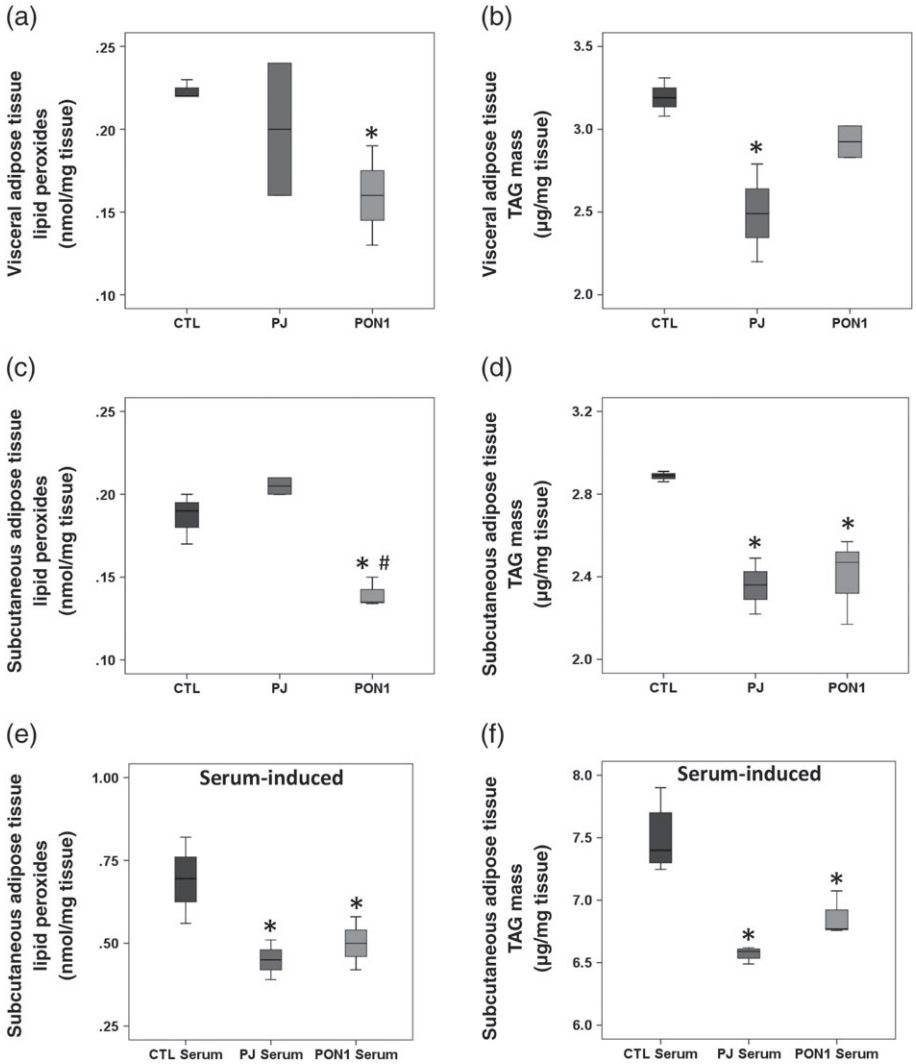

Fig. 5 *In vivo* and *ex vivo* effects of pomegranate juice (PJ) or recombinant PON1 (rePON1) on lipid peroxidation and triacylglycerols (TAG) content in visceral and subcutaneous adipose tissues (VAT and SAT). VAT levels of (a) lipid peroxides, and (b) TAG, SAT levels of (c) lipid peroxides, and (d) TAG, following 3 weeks of PJ consumption or rePON1 injection to C57BL/6 mice. SAT levels of (e) lipid peroxides, and (f) TAG, following incubation with 15% serum derived from mice that consumed PJ or injected with rePON1. $^{*}p < 0.05$ versus control (CTL) mice, $^{\#}p < 0.05$ versus mice treated with PJ

rePON1 injection to mice significantly decreased the TAG content in the heart. These results indicate potential cardioprotective effects of PJ and rePON1 that warrant further investigation using suitable animal models of heart failure (e.g. transverse aortic constriction, TAC, model) (Houser et al., 2012). In addition, whereas PJ treatment significantly decreased *Dgat1* expression in the heart, rePON1 treatment failed to bring about such an effect in the heart, suggesting that rePON1 may affect other enzymes that regulate TAG metabolism in the heart. Further studies are warranted to elucidate the mechanisms by which PJ or rePON1 regulate TAG metabolism in the heart or in other CVD-related tissues.

As for liver pathologies, NAFLD is known as the leading cause of chronic liver disease worldwide and is associated with increased mortality due to CVD (Younossi et al., 2018). The earliest stage of NAFLD is hepatic steatosis, characterized by accumulation of TAG in hepatocytes without signs of inflammation and hepatocellular damage (Haas et al., 2016). Recent reports demonstrated that consumption of PJ or pomegranate extract decreases high-fat diet-induced hepatic steatosis by promoting mitochondrial function, as well as by eliminating oxidative stress and inflammation (Noori et al., 2017; Zou et al., 2014). In line with the above reports, we found that the PJ consumption significantly decreased the hepatic TAG content in mice. Moreover, we show for the first time that rePON1 injection can also decrease hepatic TAG. Considering that no therapies are currently available for NAFLD (Brunt et al., 2015), further research targeting

PON1 (and other PON enzymes) as potential strategies for NAFLD treatment is still needed.

NAFLD patients are at increased risk of CKD that is also associated with higher CVD risk (Bonora and Targher, 2012). Lipid accumulation in the kidneys has been suggested to promote CKD and CVD in experimental models and humans (Herman-Edelstein et al., 2014; Montani et al., 2004). Obesity-induced accumulation of fat within the renal sinus was suggested to alter intrarenal physical forces favoring sodium reabsorption and arterial hypertension (Montani et al., 2004). In kidney biopsies from patients diagnosed with diabetic nephropathy, marked lipid deposition and increased intracellular lipid droplets are evident in association with downregulation of genes regulating β-oxidation and cholesterol efflux as well as upregulation of genes involved in lipoprotein uptake (Herman-Edelstein et al., 2014). To our knowledge, the current study is the first to report that PJ consumption or rePON1 injection can decrease the TAG content in the kidney. These findings should be further investigated using suitable models of renal disease accompanied by high-fat feeding, to elucidate the therapeutic potential of PJ or PON1 in renal disease.

Obesity, characterized by increased fat deposition in adipose tissues, is a worldwide epidemic and a major risk factor for CVD (Benjamin et al., 2017). Both PJ and PON1 have been suggested to play a role in obesity. In a randomized, double-blind, placebo-controlled clinical trial in 20 obese patients, PJ administration for 1 month halted the increase in the body weight and fat mass percentages that were observed in the placebo group (González-Ortiz et al., 2011). In CD-1 mice, pomegranate extract attenuated high-fat diet-induced dyslipidemia, obesity, and increased adipose pad weight (Lei et al., 2007). Similarly, administration of pomegranate seed oil to CD-1 mice decreased high-fat diet-induced weight gain and plasma leptin and increased plasma adiponectin (McFarlin et al., 2009). In line with the above studies, we found that PJ significantly decreased the TAG content in VAT and SAT *in vivo* and *ex vivo.*

Lower PON1 activity has been reported in obesity. In a case–control study of obese and lean children and adolescents, PON1 serum activity was found to be lower in obese subjects and to be associated with BMI, body fat percentage, and metabolic syndrome (Ferré et al., 2013). Polymorphisms in the PON1 gene affect the blood concentrations of the enzyme and its catalytic activity. The two most common polymorphisms of PON1 consist of a glutamine-to-arginine substitution at position 192 (Q192R) and a leucine-to-methionine substitution at position 55 (L55 M) (Rom and Aviram, 2017). Interestingly, PON1 polymorphism has been suggested to play a role in obesity. In a study of 127 Mexican adults, the frequency of the homozygous L genotype for the PON1-L55 M polymorphism was higher in the obese group than in the normal-weight group (Martínez-Salazar et al., 2011). In 373 Mexican-American children, higher odds of obesity were found in PON1 192QQ children compared to 192RR children (Huen et al., 2013). In the current study, we show for the first time that injection of rePON1 to mice results in decreased lipid peroxidation and TAG content in adipose tissue. The findings of the current study and that of previous reports suggest a protective role for PJ and PON1 against adiposity.

In addition to its high polyphenol content, PJ also contains sugars, mainly glucose and fructose (Rom and Aviram, 2016b). Sugar-containing juices may increase hyperglycemia in diabetic patients who are at increased risk for CVD (Benjamin et al., 2017). Interestingly, previous studies have shown that administration of PJ or pomegranate extracts to diabetic or obese patients as well as to streptozotocin-nicotinamide-induced diabetic rats or high-fat diet-induced obese mice did not exacerbate hyperglycemia and did not modify insulin secretion or sensitivity (González-Ortiz et al., 2011; Neyrinck et al., 2013; Rock et al., 2008; Rosenblat et al., 2006; Taheri et al., 2017). While PJ treatment did not worsen the diabetic parameters, it resulted in antioxidative effects in diabetic patients related to enhanced PON1 stability and catalytic activity (Rock et al., 2008; Rosenblat et al., 2006).

In summary, thus, our systemic analysis of the lipid and oxidation status in CVD-related tissues following PJ consumption or rePON1 injection to mice contributes to a better understanding of lipid metabolism in cardiovascular-related tissues. In line with previous reports on the atheroprotective role of the two antioxidants, marked lipid-lowering effects (mostly TAG) were found in association with downregulation of the key TAG biosynthetic gene—*Dgat1*.

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Conflict of Interest The authors declare that they have no conflict of interest.

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