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No influence of increased intake of orange and blackcurrant juices and dietary amounts of vitamin E on paraoxonase-1 activity in patients with peripheral arterial disease

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Abstract *Background* Paraoxonase-1 (PON1) is an antioxidative enzyme associated with HDL and its serum activity is associated with risk of cardiovascular disease. The interindividual variation in PON1 activity is partly determined by genetic factors, such as polymorphisms in the *PON1* gene, but also by dietary factors like the antioxidants. *Aim of the study* We examined the effect of antioxidant-rich orange and blackcurrant juices and vitamin E supplement on PON1 activity in patients with peripheral arterial disease. Furthermore, we studied whether genetic polymorphisms in the PON1 gene predicted the change in PON1 activity. *Methods* The study was designed as a cross-over trial with 48 participants who received two of the four possible treatments: (1) 250 ml orange juice and 250 ml blackcurrant juice; (2) 15 mg vitamin E; (3) 250 ml orange juice and 250 ml blackcurrant juice and 15 mg vitamin E; or (4) control/placebo (energy-equivalent sugar-containing beverage). The treatments were given for 28 days, separated by a 4-week wash-out period. *Results* The PON1 activity was not affected by juice or vitamin E supplement neither was there evidence of synergetic effects. However, a statistically significant interaction was observed between treatment and PON1 genotype, such that PON1 activity increased after juice alone in patients carrying the PON1 L55-allele. Results need to be interpreted with care since the study population was relatively small. *Conclusion* Consumption of orange and blackcurrant juice and vitamin E supplement does not affect the activity of PON1 in patients with peripheral arterial disease. However, a gene-diet interaction may be present.

Key words PON1–orange juice–blackcurrant juice–vitamin E–oxidative stress–F_2-isoprostanes

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Introduction

Serum paraoxonase-1 (PON1) is associated with high-density lipoprotein HDL in the plasma and is one of the component that contributes to the cardioprotective effects of HDL including the reverse cholesterol transport, as well as the antioxidative and anti-inflammatory properties [1]. PON1 is able to hydrolyze oxidized lipids in HDL and LDL as demonstrated both in in vitro and in vivo experiments (reviewed in [2]). Furthermore, PON1 appears to be able to hydrolyze hydrogen peroxide [3], a major reactive oxygen species that is implicated in the development of atherosclerosis [4]. Consequently, PON1 may protect against atherosclerosis. Some of the best evidence for a protective role of PON1 arises from studies on mice. In a mouse model of metabolic syndrome, overexpression of PON1 inhibited the atherosclerotic process [5]. Likewise, PON1 knock out mice express an increased atherosclerotic burden, as well as increased lipid peroxidation [6, 7] in contrast with human PON1 transgenic mice that have increased PON1 concentration with a concomitant decrease in atherosclerotic lesions [8]. These experimental animal studies are supported by epidemiological evidence demonstrating that low PON1 activity is associated with an increased risk for cardiovascular events [9], as well as the presence of vascular disease [10]. Thus, good evidence exists to indicate that PON1 plays an important role in the protection against atherosclerosis. It is, therefore, of interest to identify modulators of PON1 activity and concentration, and diet may be one of these factors [11]. It could be anticipated that intake of food rich in antioxidants, like fruit and vegetable juices [12, 13] may affect the activity and/or concentration of the antioxidative enzyme PON1 in humans. Thus, administration of pomegranate juice to healthy subjects or to patients increases PON1 activity [14, 15], as well as decreases carotid stenosis [15]. On the other hand, juices made of vegetables have a minute effect on PON1 activity [16, 17]. Therefore, the purpose of this study was to investigate the effect of orange and blackcurrant juices on PON1 activity. These juices were selected as they are common in the Nordic diet. Furthermore, we wished to examine the role of a dietary dose of vitamin E on PON1 activity as it has been suggested that antioxidants, and especially vitamin C interacts positively with vitamin E in vivo [18] and a supplement with both may, therefore, be more effective than with either one alone. Finally, it is well-known that PON1 activity and concentration are determined by polymorphisms in the *PON1* gene [19] and we, therefore, wished to examine whether potential effects of supplement were specific for the PON1 C-108T, M55L, and Q192R genotypes.

Methods

Subjects

Participants were patients diagnosed with peripheral arterial disease (PAD) who were identified through the registers of the Department of Vascular Surgery, Ribe County Hospital, Esbjerg and the Department of Vascular Surgery, Odense University Hospital, Denmark. A diagnosis of PAD is based on an examination programme including medical history, vascular physical examination, and presence of a low ankle-brachial index (< 0.9 [20]). Patients were eligible for the study if they were between the ages of 40 and 70 years, and were without diabetes or renal disease. Patients who had undergone any surgical procedure 3 months prior to the study start or were awaiting surgical procedures within the time frame of the study period were ineligible. Finally, also the patients unwilling to stop consuming self-prescribed nutritional supplements (like vitamin C) 1 month prior to study start and during the course of the study could not participate.

A total of 144 patients were identified from the hospital records from the period 1999–2001 and contacted by mail. Of 130 patients that could be reached by phone, 37 declined to participate, and 28 were excluded for various reasons. Two subjects did not come to the initial visit and two more subjects were excluded at the initial visit. Five subjects (one deceased) did not come to the second visit where randomization take place. Eight subjects dropped out during first period or before start of the second period. Thus, 48 subjects completed both treatment periods.

The study protocol was approved by the local Ethical Committee of Ringkjøbing, Ribe and South Jutland Counties (M-2242-01).

Study design

The study was designed to test the effects of vitamin E (15 mg RRR-α-tocopherol per day) (Winter Medico A/S, Odense, Denmark) and fruit juice (250 ml orange juice (Rynkeby Foods A/S, Denmark) and 250 ml blackcurrant juice (Cadiso Foods A/S, Denmark) per day) on PON1 activity and concentration. Therefore, a controlled crossover study was performed in which the patients were randomly assigned to a sequence of two of the four treatments:

(a) juice + vitamin E (JE)
(b) juice + placebo-vitamin E (JP)
(c) reference beverage + vitamin E (RE)
(d) reference beverage + placebo-vitamin E (RP).

The reference beverage was a carbohydrate-only containing beverage with an energy content equivalent to the juice.

The factorial design permitted assessment of the juice and vitamin E supplements independently (main effects) and their synergistic effect. The order in which subjects took their supplement was decided by randomization, so that each patient was randomized to one of the 12 possible sequences of two different interventions within blocks of 12 patients. The study investigator was blinded to sequence allocation until the end of study. With 48 participants, a statistical power of at least 0.75 at significance level 0.05 is obtained testing the global null hypothesis of no difference among all four treatments and the assumption that in any of the three groups JP, JE, and RE two thirds of the subjects will show a change in the same direction. This corresponds to an expected change of at least 0.43 times the standard deviation of the change. Furthermore, it is assumed that there is no change in the reference group (i.e. group RP) and that the correlation of repeated measurements within a patient is at least 0.25.

Patients were asked to drink one juice at breakfast and one at dinner, and to take the tablet during dinner. The treatment period was 4 weeks with an intervening 4-week wash-out period. After each period, the patients were asked to return their leftovers for estimation of the apparent adherence to treatment.

Patients were carefully instructed not to change their dietary habits during the study period. Dietary habits at baseline were assessed by a validated Danish food frequency questionnaire (FFQ) [21]. The patients were asked to keep logbooks on their intake of fruit and vegetables for 2 weeks within each intervention period. Additionally, patients were asked about smoking habits, which included simple questions on smoking status (never, ex- or current smoker) and categories of the daily number of cigarettes smoked, level of daily physical activity, use of medications and previous regular use of vitamins or antioxidants. Finally, after each period patients were asked whether they had changed their habitual dietary habits, physical habits, medications, and smoking habits to control for possible changes.

Weight in light clothing was measured to the nearest 0.1 kg at the initial visit, and again after each intervention period and at the beginning of the second period. Height without shoes was measured to the nearest 0.1 cm.

Blood sampling

Non-fasting blood samples were obtained at the start (average of two samples 3 days apart) of each treatment period (baseline) and at the end of each treatment period. All blood samples were collected between 8:30 and 10:00 am to avoid diurnal fluctuations. Venous blood samples were collected with minimal stasis in vacutainers without additives and kept at room temperature until centrifuged within 1 h at 2,000*g* for 20 min for PON1 activity and concentration; in EDTA tubes in melting ice and kept on ice and in the dark until centrifuged within 1 h at 2,000*g* at 4°C for 20 min for analysis of α-tocopherol (vitamin E), vitamin C, and F_2-isoprostane. To stabilize vitamin C, plasma samples were processed immediately after collection by centrifugation at 5,000*g* for 1 min followed by addition of 15% metaphosphoric acid to plasma (1:1). For F_2-isoprostane analyses, butylhydroxy-toluene (BHT) was added to EDTA plasma (final BHT concentration 0.005%) to reduce the risk of lipid auto-oxidation. Plasma and serum samples were snap frozen in small aliquots in liquid nitrogen and stored at −80°C until analysis, which was performed in one series of each variable in order to prevent between-day analytical variation.

Biochemical analysis

PON1 activity measurements were performed with paraoxon as substrate as previously described [9]. Briefly, serum was added to Tris buffer (100 mmol/l, pH 8.0) containing 2 mmol/l $CaCl_2$ and 5.5 mmol/l paraoxon (*O,O*-diethyl-*O*-*p*-nitrophenylphosphate, Sigma Chemical Co). The rate of *p*-nitrophenol generation was thus determined at 405 nm, 25°C, using a continuously recording spectrophotometer (Beckman DU-68) [22].

PON1 concentration was determined by an in-house enzyme-linked immuno assay as previously described [23] with minor modifications. Briefly, the reference curve and diluted samples were applied to a microtiter plate and incubated overnight at room temperature. The wells were washed once with 0.1% BSA in PBS and remaining absorption sites were blocked for 1 h with PBS/1% BSA. The wells were washed three times and incubated for 1 h with rabbit anti-human paraoxonase monospecific antibodies. After they were washed twice, wells were incubated for 1 h with anti-rabbit IgG peroxidase conjugate. After the final wash, hydrogen peroxide was added and the plate was incubated for 15 min while shaking. The reaction was stopped by adding H_2SO_4 and the absorbance was read at 405 nm with a Multiskan Accent ELISA reader (Thermo Labsystems).

Plasma concentrations of vitamins C and E were determined by colorimetric electrochemical detection [24] and fluorometric detection [25], respectively as described previously. Vitamin C is the sum of ascor-

bic acid plus dehydrascorbic acid. In a subgroup of 23 patients, free F_2-isoprostane level was measured as a biomarker of oxidative stress, i.e. lipid peroxidation [26]. It was expected that the JE and RP would give the largest difference in this measure of oxidative stress hence samples were selected from these two groups. The specific samples were selected to be from every second subject. Thus, 12 samples were analyzed from group juice + vitamin E (JE) and 11 from reference group (RP). For the analysis of free F_2-isoprostane plasma samples were purified on C18 and silica Sep-Pak solid phase extraction columns followed by trimethylsilyl ether derivatization. The analysis was performed on GC/MS with negative-ion chemical ionization, and has a sensitivity <5 pg/ml and a variability of 6% [27]. Non-fasting total-cholesterol and high-density lipoprotein (HDL) were determined by established methods in the hospital laboratory.

PON1 genotypings

DNA was extracted from the white blood cells by a salting-out method, and PON1 genotype for the C-108T (rs705379), M55L (rs854560) and Q192R (rs662) polymorphisms were determined by the Taqman technology based allele discrimination assay using the ABI Prism 7700 Sequence Detection System [28].

Statistical analyses

Data are presented as means (SD) or as median (25–75th percentiles) for skewed data. The main endpoints were changes in PON1 activity or concentration; the change was calculated as post-supplement value minus baseline values for each supplement period. The effect of juice and vitamin E on the main endpoints was examined by multiple regression analyses via modeling of changes in activity or concentration on its baseline value with treatment and period as indicator variables using robust estimates of variance. This was done by using Stata's regress command together with the cluster option, which takes into account that data are not independent within clusters (i.e. each subject) although independent across clusters. We used log-transformed PON1 activity and concentration in these regression models. To study whether PON1 genotype was an effect modifier, the multiple regression analyses were repeated with an interaction term between genotype and supplement. Treatment effects within group were analyzed by the two-sided paired *t*-test (F_2-isoprostanes) or the Wilcoxon matched pairs signed rank sum test (Vitamins C and E). The differences between groups in the changes for these variables were analyzed by unpaired *t*-test or Mann-Whitney *U* test, respectively.

A two-tailed value of $P < 0.05$ was considered statistically significant. Stata version 8.2 (Stata Corporation, Texas, USA) software was used for statistical analyses.

Table 1 Baseline characteristics of the population

	Patients (n = 48)
Age, years	61 (6)
Female, %	27
Body weight, kg	79.3 (16.3)
BMI, kg/m^2	26.9 (4.2)
Smokers, %	
Current	50
Former	40
Never	10
Medication, %	
Aspirin	77
Cholesterol lowering	33
Blood pressure lowering	38
Cholesterol, mmol/l	5.67 (0.91)[a]
HDL-cholesterol, mmol/l	1.48 (0.49)[a]
Glucose, mmol/l	5.8 (5.3–6.8)[a,b]

Values are mean (SD)
[a] Non-fasting values
[b] Median (25–75th percentiles)

Results

Table 1 summarizes the clinical and metabolic characteristics for the patients. Body weights before and after each intervention were comparable. There were no significant changes in blood glucose, plasma total cholesterol or HDL-cholesterol between groups at baseline or after interventions (data not shown). Moreover, patients reported no changes in smoking habits, physical activity or medication during the study period. Finally, no changes in intake of vegetables and fruits during the intervention periods were reported comparing with the intake assessed by a validated questionnaire at baseline (data not shown).

The effect of orange and blackcurrant juice and vitamin E on plasma vitamins

After the 4-week orange and blackcurrant supplement, which contained ~200 mg of vitamin C per day, median plasma levels of ascorbate increased to 62.4 (47.8–87.7) compared with the baseline levels of 43.0 (26.3–57.0) μmol/l ($P < 0.0001$)(Table 2). After supplementation with 15 mg RRR-α-tocopherol for 4 weeks, median plasma levels of vitamin E increased to 12.0 (10.3–13.9) from 10.6 (9.1–12.4) μg/ml ($P = 0.001$)(Table 2). These changes indicate that

Table 2 Effects of orange juice and blackcurrant juice and vitamin E supplementation for 4 weeks on PON1 activity and concentration

	Reference beverage + placebo (n = 23)	Reference beverage + vitamin E (n = 25)	Juice + placebo (n = 24)	Juice + vitamin E (n = 24)	P value[a]
PON1 activity, μmol/min/l					
Baseline	107.8 (61.2–234.8)	94.3 (78.8–154.4)	82.4 (70.1–153.0)	134.8 (94.5–221.5)	
Change[b]	−3.5 (−11.8–20.2)	1.0 (−11.8–12.5)	6.4 (−8.1–10.8)	−2.8 (−12.9–1.6)	$P > 0.05$
PON1 concentration, μmol/l					
Baseline	69.7 (48.2–85.7)	58.6 (47.9–74.7)	63.3 (50.9–75.1)	68.6 (49.9–80.1)	
Change	−3.2 (−10.4–7.1)	5.2 (−13.1–14.4)	2.8 (−11.4–10.2)	1.0 (−17.5–7.1)	$P > 0.05$
F_2-isoprostane, pg/ml[c]					
Baseline	21.3 (15.7–26.9)			25.6 (20.5–30.7)	
Change	3.9 (0.3–7.5)			−0.7 (−4.5–3.1)	$P = 0.066$
Vitamin C, μmol/l					
Baseline	31.50 (18.90–51.60)	47.30 (32.25–69.35)	46.38 (28.75–60.88)	40.25 (24.30–54.58)	
Change	−2.95 (−7.90–2.80)	−5.95 (−12.80–0.85)[d]	13.00 (0.08–24.18)[e]	20.2 (9.50–42.75)[f]	
Vitamin E, μg/ml					
Baseline	10.76 (9.11–12.85)	10.79 (9.48–12.25)	9.93 (8.33–11.65)	10.19 (8.75–13.07)	
Change	−0.27 (−0.99–0.71)	0.81 (−0.15–1.62)[d]	−0.38 (−0.78–0.69)	1.82 (1.12–2.44)[e]	

Values are median (25–75th percentiles)
[a] Regression analysis comparing the four groups as explained in section on statistics
[b] Change indicates difference between treatment and baseline absolute values, thus a negative change indicate a decrease in post-value compared with baseline value
[c] Values are mean and 95% CI. For RP, n = 11 and for JE, n = 12
[d] P = 0.05 within group, Wilcoxon matched pairs signed rank sum test
[e] P = 0.005 within group, Wilcoxon matched pairs signed rank sum test
[f] P = 0.0001 within group, Wilcoxon matched pairs signed rank sum test

participants took their supplement regularly and this was confirmed by the compliance, as measured by tablet count, which was 96%.

The effect of orange and blackcurrant juice and vitamin E on PON1 activity and concentration

Median baseline and changes in PON1 activity and concentration after supplementation are provided in Table 2. No significant changes in either enzyme activity or concentration were observed in patients who received either juice alone (JP, n = 24), vitamin E alone (RE, n = 25) or both combined (JE, n = 24) compared with the reference group (RP, n = 23).

The effect of orange and blackcurrant juice and vitamin E on F_2-isoprostanes

As described in the method section, F_2-isoprostanes were measured in a subgroup of patients from group JE (n = 12) and RP (n = 11). There was no statistically significant difference in the changes between the groups, but the F_2-isoprostanes significantly increased between baseline and 4 week in the RP group (Table 2).

Genotype – supplement interaction

At baseline, PON1 activity was highest in the subjects with the 192RR or 55LL genotype, lowest in the 192QQ or 55MM, and intermediate in the heterozygotes. The C-108T genotype had no influence on activity (Fig. 1a). PON1 concentration was not affected by the three SNPs (Fig. 1b).

In the complete multivariate model, a significant interaction (P = 0.046) between genotype and supplement on the change in PON1 activity was observed. Specifically the patients carrying the L allele (n = 19) had an increase while the 55MM homozygotes (n = 5) had a decrease in PON1 activity after orange and blackcurrant juice + placebo (Fig. 2). No such interactions were observed for the other genotypes and/or the other treatments (Table 3). No interaction was observed for changes in PON1 concentration, genotype and treatment (data not shown).

Discussion

A low PON1 activity predicts future cardiovascular events [9] and is associated with increased risk of cardiovascular disease [29]. Furthermore, it has been reported previously that individual PON1 activity varies between 10 and 40-fold and that this variation is partly determined only by genetic factors [30]. Thus, knowledge of nutritional and environmental factors that modulate PON1 activity is of potential relevance [11].

In our study, we did not observe an effect of the juices or vitamin E on PON1 activity or antigen levels. Our results are in line with a recent study showing

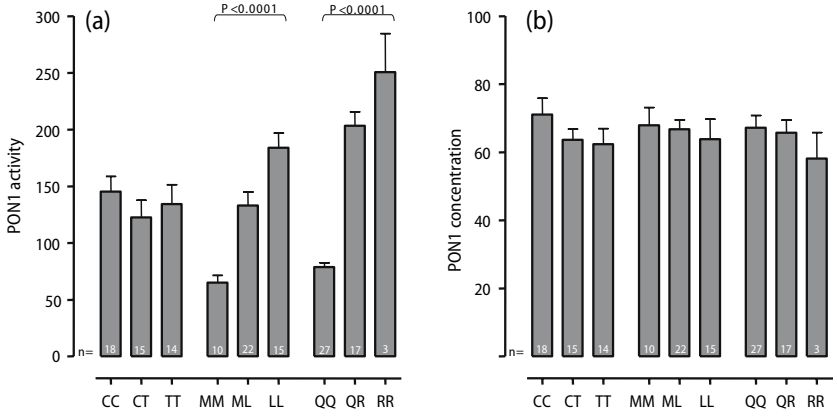

Fig. 1 Paraoxonase-1 activity (a) and concentration (b) in relation to three PON1 polymorphisms; C-180T, L55M and Q192R. Differences between genotypes are analysed by using the Kruskal-Wallis test. Numbers in columns reflect group size.

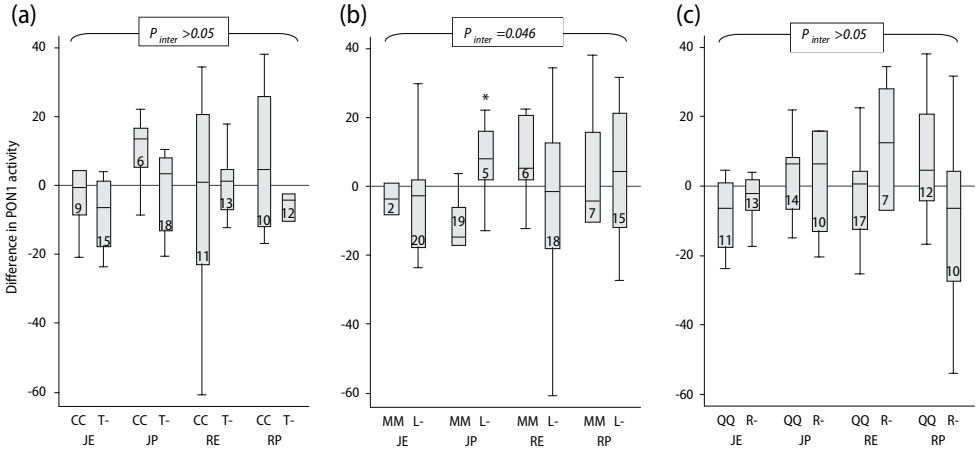

Fig. 2 Relationship between changes in PON1 activity after treatment and genotypes at PON1 C-108T (CC homozygous or T-allele carriers) (a), M55L (MM homozygous or L-allele carriers) (b) and Q192R (QQ homozygous or R-allele carriers) (c). JE: Juice + vitamin E; JP: Juice + placebo vitamin E; RE: reference beverage + vitamin E; RP: reference beverage + placebo vitamin E. P-values are from genotype-treatment interaction analyses. Boxes represent lower and upper quartiles and the centre line the median value. Numbers in boxes reflect group size.

that a tomato and carrot juice supplement for 2 weeks to healthy young men had no effect on either PON1 paraoxonase or phenylacetate activity [17]. In contrast, in a tomato juice supplement study using elderly subjects the intervention increased PON1 phenylacetate activity, but so did water in the control group [16]. Also, pomegranate juice supplement increased PON1 phenylacetate activity in healthy men, diabetic patients or patients with severe carotid artery stenosis [15, 31, 32]. Red wine rich in polyphenolic compounds increased PON1 paraoxonase activity in healthy men, but so did the beer and spirits, thus it may have been an alcohol-induced increase and not a result of the increased antioxidant intake [33]. The inconsistencies among the studies including the present one may be explained partly by differences in the beverages and therefore in active ingredients, choice of enzyme substrate, study populations and length of supplementation period. Clearly, it is difficult at the present time to depict with certainty the general effect of antioxidant rich beverages on PON1 activity or concentration, and it warrants further studies to clarify the role of dietary antioxidants as modulators of the enzyme activity or concentration.

There are several possible explanations for the absence of a general effect of the supplements. Firstly, it is possible that the amount of juice used in the present study was too low to influence the level of oxidative stress. This is supported by the absence of changes in the systemic marker of oxidative stress, F_2-isoprostanes. However, a recent study in healthy subjects has demonstrated that 500 ml orange juice per day for 2 week, providing approximately 250 mg vitamin C per day, decreases the isoprostane level [34, 35]. Also, in the present study the vitamin E dose and/or the supplementation period may have been too low, as mostly only very high vitamin E doses for a prolonged period of time have been shown to influence lipid peroxidation as indicated by the isoprostane concentration [36, 37]. However, pharmacologic vitamin E doses at 200 or 400 mg/day do not affect PON1 activity either [38]. F_2-isoprostanes are considered to be the most reliable and accurate markers of lipid peroxidation [39]. However, results are conflicting regarding intervention studies on the association between for instances diet (food) rich in antioxidants or specific antioxidants and isoprostanes [36].

Table 3 The effect of PON1 genotypes[a] on the response in enzyme activity after supplementations

	C-108T		
	CC	CT	TT
(A) C-108T			
Group JE	$n = 9$	$n = 7$	$n = 8$
Baseline	112.9 (97.8–135.8)	189.4 (87.8–236.4)	162.8 (102.3–254.8)
Change	0.5 (−8.3–4.4)	−6.7 (−17.4–−3.5)	0.0 (−12.7–2.7)
Group JP	$n = 6$	$n = 10$	$n = 8$
Baseline	107.0 (80.7–219.0)	78 (68.8–124.9)	99.4 (50.2–162.0)
Change	13.6 (5.6–16.7)	5.4 (2.3–8.1)	−7.0 (−13.8–8.3)
Group RE	$n = 11$	$n = 8$	$n = 5$
Baseline	110.3 (82.5–177.1)	76.7 (69.9–85.1)	125.2 (78.8–145.6)
Change	1.0 (−22.7–20.6)	−5.3 (−12.1–2.7)	12.5 (4.6–17.6)
Group RP	$n = 10$	$n = 5$	$n = 7$
Baseline	107.9 (75.1–234.8)	131.9 (56.8–191.9)	69.5 (49.1–211.4)
Change	4.6 (−11.8–25.7)	−3.5 (−27.4–20.2)	−4.2 (−8.6–−2.5)
	L55M		
	LL	ML	MM
(B) L55M			
Group JE	$n = 8$	$n = 14$	$n = 2$
Baseline	214.9 (124.4–275.4)	124.1 (96.4–189.4)	50.6 (30.4–70.9)
Change	−0.1 (−48.9–3.1)	−6.6 (−17.4–1.4)	−3.6 (−8.1–0.9)
Group JP	$n = 6$	$n = 13$	$n = 5$
Baseline	131.8 (88.3–219.0)	78.3 (71.4–168.0)	68.8 (55.6–77.9)
Change[b]	13.6 (9.5–16.7)	7.2 (2.3–8.1)	−14.8 (−16.9–−6.3)
Group RE	$n = 7$	$n = 11$	$n = 6$
Baseline	154.4 (110.3–287.4)	86.5 (79.0–145.6)	69.9 (25.3–76.3)
Change	17.2 (−22.7–28.0)	−6.5 (−17.8–1.0)	5.3 (2.3–20.6)
Group RP	$n = 9$	$n = 6$	$n = 7$
Baseline	191.9 (107.8–234.8)	154.2 (44.3–289.3)	61.2 (55.6–75.1)
Change	4.2 (−11.8–22.3)	0.2 (−8.6–20.2)	−4.2 (−10.2–15.8)
	Q192R		
	QQ	QR	RR
(C) Q192R			
Group JE	$n = 11$	$n = 10$	$n = 3$
Baseline	96.4 (71.9–112.9)	197.9 (155.3–243.4)	307.3 (193.3–320.3)
Change	−6.5 (−17.4–0.9)	−0.3 (−6.7–3.9)	−96.9 (−230.3–0.9)
Group JP	$n = 14$	$n = 10$	$n = 0$
Baseline	76.5 (55.6–80.7)	177.1 (124.9–219.0)	–
Change	6.4 (−6.3–8.11)	6.3 (−12.7–16.0)	–
Group RE	$n = 17$	$n = 6$	$n = 1$
Baseline	81.1 (74.4–94.3)	202.3 (145.6–290.0)	287.4
Change	0.7 (−12.3–4.2)	8.6 (−6.7–28.0)	17.6
Group RP	$n = 12$	$n = 8$	$n = 2$
Baseline	65.4 (52.4–86.0)	227.3 (201.7–272.8)	197.9 (107.8–288.1)
Change	4.6 (−4.2–20.7)	−6.4 (−19.6–13.2)	−82.5 (−162.5–2.5)

Results are median (25–75th percentiles) for subjects given juice plus vitamin E (JP, $n = 24$), juice plus placebo vitamin E (JP, $n = 24$), reference beverage plus vitamin E (RE, $n = 24$) or reference beverage plus placebo vitamin E (RP, $n = 22$)

[a] Due to lost sample material, genotypes were only determined in 47 subjects

[b] $P < 0.05$, for a significant interaction between treatment group and genotype

We consider it unlikely that patients did not drink their juice or did not take their tablets since we observed the expected increases in plasma vitamin C and E concentrations.

Secondly, the choice of orange and blackcurrant juices was based on their common use in the Danish diet, especially for the orange juice. However, the use of these juices may explain the absence of an effect since so far, the pomegranate juice is the antioxidant-rich beverage that has shown the strongest increase in PON1 activity (measured as arylesterase activity). It has been shown recently that this juice has higher

total polyphenolic content than most juices [40] and may, therefore, be more potent in the protection against oxidative stress. Furthermore, pomegranate juice may contain specific polyphenolic substances in contrast to the orange and blackcurrant juices used in the present study; compounds that may either have a higher bioavailability or may possess a more efficient antioxidant activity.

Thirdly, we used paraoxon as a substrate and did not observe an effect of the supplement. It is well-known that paraoxon is not a natural substrate for PON1 and the ability to hydrolyze several organophosphates are simply a part of the enzyme's promiscuous activities [41]. It has been demonstrated recently that lactones are among the likely physiological substrates [42]. Accordingly, it is possible that using a native substrate would have given a better indication of the physiological enzymatic activity, as well as changes herein. Unfortunately, native substrates are not commercially available yet.

Our results suggest that there may be a moderate interaction between supplement and the L55M genotypes, such that within the group supplemented with juice + placebo only in L allele carriers PON1 activity increased significantly while in the MM homozygotes a decrease was observed (Fig. 2). No other studies have examined the L55M diet-genotype interaction with respect to PON1 activity and antioxidant-rich beverage supplement. A biological explanation for this interaction is yet unknown, although it has been demonstrated that the L55M affect enzyme stability and therefore also enzyme activity [43]. As in the present study, Bub and co-workers found no influence of Q192R on the effect of tomato juice consumption on PON1 activity [17]. However, the sample size is small and therefore, these results should be interpreted with caution. Still, the results illustrate, that genetic variation may contribute to individual variation in response to nutrient intake. Further studies including larger sample sizes are needed to clarify this putatively important public health issue.

The study results may have been influenced by the study medication, such as statins, which are known to increase PON1 activity. However, patients did not change their medication and continued to take the medications prescribed by their medical doctors during the total study. Unfortunately, the study is too small to make subgroup analyses. Furthermore, the patients were non-fasting and PON1 activity may be affected by the post-prandial phase. However, since the patients were allowed only a light breakfast before blood sampling, we expect that this effect will be minimal.

In conclusion, the present study demonstrates that in patients with peripheral arterial disease orange and blackcurrant juices and vitamin E supplement have no effect on either the activity or the concentration of the antioxidative enzyme paraoxonase-1. There may be a modest gene-nutrient specific response on activity, but the sample size is small and a clear biological mechanism is lacking to explain the response only in that specific genotype group. Therefore, the results should be verified in a larger population sample.

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