Consumption of Pomegranate Decreases Serum Oxidative Stress and Reduces Disease Activity in Patients with Active Rheumatoid Arthritis: A Pilot Study

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ABSTRACT: **Background:** Pomegranate extract (POMx) consumption has been shown to reduce the incidence and severity of collagen-induced arthritis in mice.

Objectives: To investigate whether pomegranate consumption affects disease activity in patients with rheumatoid arthritis (RA), in relation to their serum oxidative status.

Methods: In this pilot 12 week open-labeled study eight patients with active RA consumed POMx (10 ml/day) for 12 weeks. Patients' joint status and serum oxidative status (lipid peroxidation, total thiols group, paraoxonase 1 activity) were evaluated at baseline and at week 12.

Results: Six patients completed the study. POMx consumption significantly ($P < 0.02$) reduced the composite Disease Activity Index (DAS28) by 17%, which could be related mostly to a significant ($P < 0.005$) reduction in the tender joint count (by 62%). These results were associated with a significant ($P < 0.02$) reduction in serum oxidative status and a moderate but significant ($P < 0.02$) increase in serum high density lipoprotein-associated paraoxonase 1 (PON1) activity. The addition of POMx to serum from RA patients reduced free radical-induced lipid peroxidation by up to 25%.

Conclusions: The pomegranate consumption reduced DAS28 in RA patients, and this effect could be related to the antioxidative property of pomegranates. Dietary supplementation with pomegranates may be a useful complementary strategy to attenuate clinical symptoms in RA patients.

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KEY WORDS: pomegranate, rheumatoid arthritis, oxidative stress, high density lipoprotein-paraoxonase 1 (PON1)

Rheumatoid arthritis is an autoimmune disease characterized by chronic joint inflammation and damage. A variety of mechanisms are involved in the pathogenesis of RA, many of which lead to increased generation of oxidants (reactive oxygen/nitrogen species). The initiation of RA is believed to result in an accumulation of macrophages and neutrophils in the synovial fluid, accompanied by production of free radical-producing enzymes. High levels of ROS/RNS in the synovial cavity increase inflammation and joint destruction [1]. While antioxidant treatment has been shown to reduce the damage caused by ROS/RNS, such as that formed during inflammation [2], most control studies investigating the therapeutic use of individual antioxidant supplementation have not shown any beneficial effects on RA symptoms [3]. However, supplementation with a combination of several antioxidants [4], or with a diet rich in certain antioxidants such as the Cretan Mediterranean diet, along with standard drug treatment improved the clinical condition of RA patients in comparison to standard treatment alone [5,6].

Pomegranate juice is rich in certain flavonoids (unique tannins such as punicalagin and several anthocyanins), which are potent antioxidants [7]. Previous studies demonstrated that consumption of pomegranate juice reduced serum oxidative stress in healthy subjects [8], in patients with diabetes mellitus [9], and in patients with carotid artery stenosis [10]. Consumption of pomegranate juice resulted in inhibition in the development of atherosclerotic plaques in the carotid arteries [10].

In addition to pomegranate juice, which is mainly prepared from the pomegranate arils (the fleshy seed coverings), other parts of the pomegranate fruit were also found to have antioxidative and antiatherogenic capacities [11]. Standardized extracts of pomegranate prepared from the red peel have been shown to possess antioxidative, anti-inflammatory and cardiovascular disease-preventing properties [12]. Moreover, consumption of POMx was shown to reduce the incidence and severity of collagen-induced arthritis in a mouse model [13]. We conducted a pilot study to investigate whether

RA = rheumatoid arthritis
ROS/RNS = reactive oxygen/nitrogen species
POMx = extracts of pomegranate

consumption of POMx would affect disease activity in RA patients, in relation to their serum oxidative status.

PATIENTS AND METHODS

We enrolled eight RA patients (according to American Rheumatism Association criteria) with inadequate response to disease-modifying anti-rheumatic drugs. The patients were postmenopausal women who signed an informed consent and wished to participate in the study. RA was defined as active disease by Disease Activity Score 28 > 3.2, based on erythrocyte sedimentation rate (Westergren) at the baseline visit [14]. The patients received stable doses of previous DMARDs and corticosteroids for at least 1 month before screening. The prednisone dose was restricted to 10 mg/daily. Changes in prednisone dose were not permitted during the study. Patients could continue taking their non-steroidal anti-inflammatory drugs if they had been taking them regularly for at least 1 month before the baseline visit. Reduction in NSAIDs was allowed during the study, according to the patient's condition. Exclusion criteria were: comorbidities such as diabetes mellitus, morbid obesity (body mass index > 35), uncontrolled hypertension, congestive heart failure, renal or hepatic insufficiency, acute infective illness, malignant conditions, hyperlipidemia (total cholesterol level > 220 mg/dl, triglyceride level > 200 mg/dl), RA functional class IV, treatment with parenteral corticosteroids and current or previous use of biological agents, current smoking, and supplementation with other antioxidants.

Patients were assessed at baseline (visit 1) and after 12 weeks (visit 2). During these visits, patients underwent a general examination, joint assessment (swollen and tender joint count), Health Assessment Questionnaire Disability Index, patient global assessment of overall RA activity measured by a visual analogue scale (100 mm), and blood tests. At both visits (visits 1 & 2) 25 ml of blood were collected from the patient's peripheral vein. At the baseline visit, the ESR test was performed immediately and the DAS28 (ESR) was calculated. Patients with DAS28 > 3.2 were included in the study.

The patients consumed 5 ml of POMx twice a day before meals for 12 weeks, in addition to their regular treatment. At week 6 the study coordinator checked patient compliance and safety issues by phone. Visit 3 was conducted at week 16 for safety follow-up and planning of further treatment. Blood samples collected at weeks 1 and 12 were centrifuged and stored at -80°C. All laboratory tests were performed together after completion of the study. Additional blood samples for in vitro studies were obtained from three healthy age-matched non-smoking women.

DMARDs = disease-modifying anti-rheumatic drugs
NSAIDs = non-steroidal anti-inflammatory drugs
ESR = erythrocyte sedimentation rate
DAS28 = Disease Activity Score 28

The study was approved by the local Ethics Committee (Helsinki approval No. 3067) and performed in accordance with the guidelines for good clinical practice in the European Community and the Declaration of Helsinki.

POMX PREPARATION

We used California-grown and processed Wonderful™ variety pomegranate (*Punica granatum* L) extract supplied by POM Wonderful (Los Angeles, CA, USA). The POMx was processed to provide polyphenol components similar to that of one serving of pomegranate juice (200 ml daily), produced in a two-step process. The fruit was crushed and squeezed for juice; after most of the juice was expelled from the pomegranate, the remaining fruit, including aril residues, was collected and processed to remove the seeds before going through a screw press to produce a pureed water extract with a high concentration of polyphenols. The polyphenols of the extract were concentrated via a membrane system and the resulting cloudy POMx 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. POMx contained 130,000 ppm gallic acid equivalents or 1300 mg GAE per each 10 ml daily dose consumed, which consisted of 95% polymolecular mixture ellagitannins, mainly punicalagin, and 5% ellagic acid.

SERUM BIOCHEMICAL PARAMETERS

C-reactive protein, cholesterol, high density lipoprotein-cholesterol, and triglyceride concentrations in serum were measured using an automated analyzer (Olympus™, Medtechnica, Israel).

SERUM OXIDATIVE STRESS PARAMETERS

Serum samples were diluted ×4 with phosphate-buffered saline and incubated with 100 mmol/L of 2,2'-azobis,2-amidinopropane hydrochloride (AAPH, Wako, Japan) for 2 hours at 37°C. The extent of lipid peroxidation was determined by the lipid peroxide test, which analyzes lipid peroxide formation by its capacity to convert iodide to iodine, as measured spectrophotometrically at 365 nm [16].

SERUM TOTAL THIOLS (SULFHYDRYL GROUPS)

Serum (10 µl) was mixed with 200 µl of Tris-EDTA buffer and the absorbance at 412 nm was then measured. To these samples, 8 µl from a stock solution of 10 mmol/L DTNB were added and the absorbance was measured again (together with a DTNB blank solution) after 15 minutes of incubation at room temperature. Total sulfhydryl groups were then calculated [17].

SERUM PON1 ARYLESTERASE ACTIVITY

Serum samples were diluted 1:10 (v:v) with a buffer containing 1 mmol/L CaCl2 in 50 mmol/L Tris HCl, pH 8.0. Then, 5

GAE = gallic acid equivalents

µl were incubated with 1.0 mmol/L phenyl acetate. The initial rate of hydrolysis was determined spectrophotometrically at 270 nm followed by monitoring for 3 minutes every 15 seconds. One unit of arylesterase activity is equivalent to 1 µmol of phenyl acetate hydrolyzed/min/ml [18].

STATISTICAL ANALYSIS

Student's paired *t*-test was used to compare differences between results obtained at the baseline visit, before POMx consumption, and after 12 weeks of POMx consumption for each individual.

RESULTS

EFFECT OF POMx ON RA DISEASE ACTIVITY

Eight patients were recruited but two were withdrawn before the final evaluation: one patient developed knee effusion at week 2 and needed corticosteroid joint injection (an exclusion criterion) and one patient was lost to follow-up. The baseline demographic and clinical features of the six patients who completed the study are presented in Table 1. The group consisted of non-smoking women over the age of 55 with a similar disease profile: seropositive and erosive RA with high DAS28 score (5.73 ± 0.71) and high HAQ-DI (1.83 ± 0.85) despite treatment with more than two DMARDs. Four patients needed NSAIDs. During the study, two patients experienced transient adverse events: one had dyspepsia and heartburn that responded to omeprazole, and one had an urticarial rash that responded to an antihistamine drug; both patients completed the study. There were no serious adverse events or hospitalizations during the study.

At the end of the study, the patients demonstrated a non-significant trend to reduction of DAS28 (CRP) and a significant decrease in DAS28 (ESR) [Figure 1A] in five of the six patients. In three of them, DAS28 (ESR) decreased by more than 1.2. No patient met DAS28 remission criteria. There was a trend for lower ESR at week 12 by 21%, which did not achieve statistical significance [Figure 1B]. The number of swollen joints by the end of the study decreased in four patients but increased in two [Figure 1C]. Similar changes were observed in patients' global disease assessment (VAS) [Figure 1D]. However, the most dramatic effect was observed in the number of tender joints, which significantly ($P < 0.005$) decreased by 62% after consumption of POMx [Figure 1E]. The HAQ-DI did not change during the study.

EFFECT OF POMX ON SERUM LIPID PROFILE AND OXIDATIVE STRESS

No significant changes were monitored in serum low density lipoprotein-cholesterol, HDL-cholesterol or triglyceride levels in RA patients after consumption of POMx for a period of 12 weeks (data not shown). POMx consumption induced a moderate (6%) but significant ($P < 0.02$) reduction in AAPH-induced serum lipid peroxidation, as measured by lipid peroxide formation [Figure 2A]. In parallel, serum paraoxonase 1 (PON1) activity exhibited a small increase that reached statistical significance ($P < 0.02$) in all examined patients at the end of 12 weeks of POMx consumption compared to baseline levels [Figure 2B]. A non-significant increase (17%) in the serum level of thiol sulfhydryl groups was reported after POMx consumption [Figure 2].

To determine whether the effect of POMx consumption on serum oxidative stress in RA patients can be attributed to a direct effect of POMx constituents, we incubated serum from RA patients and from healthy subjects for 2 hours at 37°C with increasing concentrations of POMx. Before the POMx addition, AAPH-induced oxidative stress was10% higher in

Table 1. Baseline characteristics of patients

No. of patients	6
Age, mean (SD) (yrs)	62.8 (9.3)
Duration of disease, mean (SD) (yrs)	9.4 (8.3)
Body mass index (kg/m2)	28.6 (4.4)
Fasting blood glucose (mg/dl)	90.1 (14.2)
Fasting total cholesterol (mg/dl)	212.4 (18.9)
Fasting triglycerides (mg/dl)	131.0 (58.3)
Rheumatoid factor positive (no. of patients)	6
Anti-CCP positive (no. of patients)	4
No. of patients with joint erosions	5
No. of previous DMARDs, mean (SD)	2.1 (0.6)
Current drugs	
Prednisone (mg) No. of patients Daily dose, mean (SD)	 5 7.0 (2.7)
Methotrexate (mg) No. of patients Weekly dose, mean (SD)	 6 13.6 (3.2)
Hydroxychloroquine (mg) No. of patients Daily dose, mean (SD)	 6 371.4 (75.6)
Sulfasalazine (Gr) No. of patients Daily dose, mean (SD)	 2 2 (0)
No. of patients taking NSAIDs	4
DAS28 (ESR)	5.73 (0.71)
HAQ-DI	1.83(0.85)

Data are presented as mean ± SD
DMARDs = disease-modifying anti-rheumatic drugs, HAQ-DI = Health Assessment Questionnaire Disability Index, DAS28 = Disease Activity Score 28-joint assessment, ESR = erythrocyte sedimentation rate, anti-CCP = anti-cyclic citrullinated peptide antibodies, NSAIDs = non-steroidal anti-rheumatic drugs

HAQ-DI = Health Assessment Questionnaire Disability Index
CRP = C-reactive protein
VAS = visual analogue scale
HDL = high density lipoprotein

AAPH = 2,2'-azobis,2-amidinopropane hydrochloride

Figure 1. Changes in DAS28 (ESR) components before and after 12 weeks of POMx consumption. Results are presented for each individual patient, and also as the mean ± SD (standard deviation). $P < 0.05$ statistically significant.

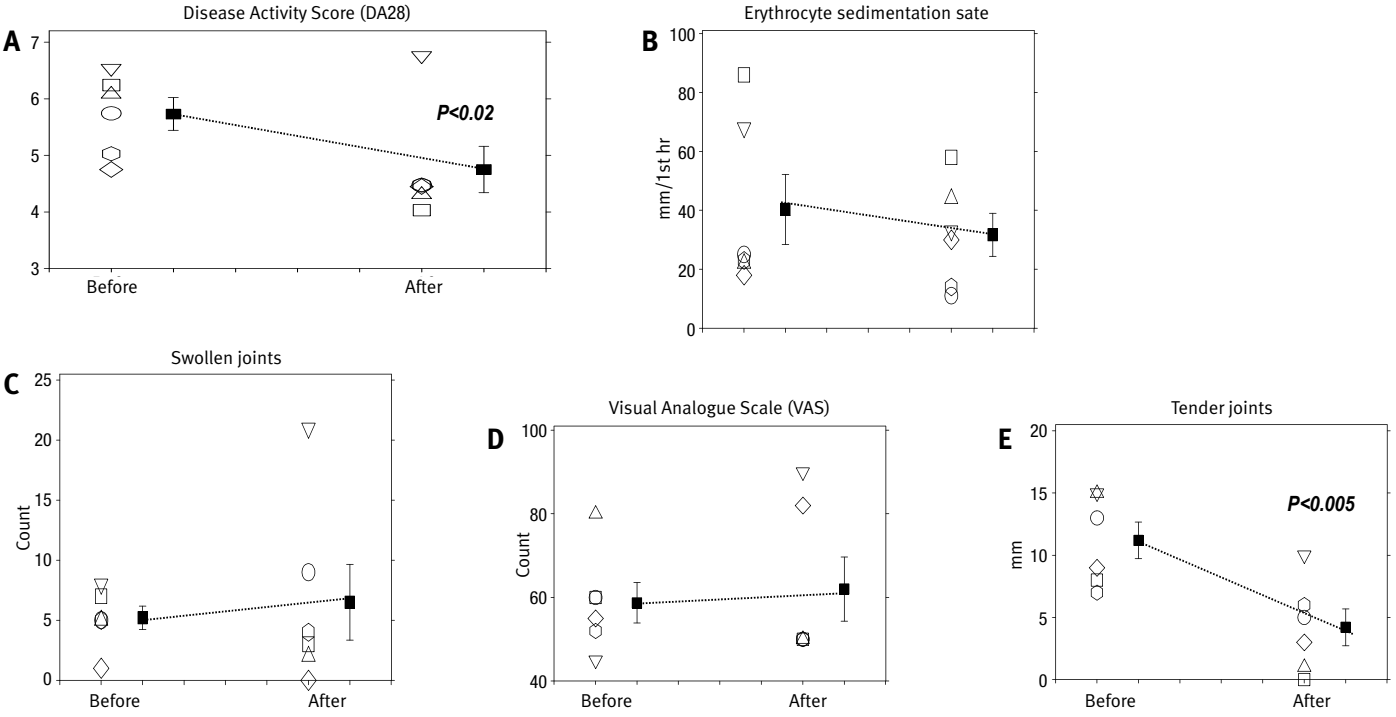


Figure 2. Effect of POMx consumption on serum oxidative stress in RA patients. **[A]** Serum from RA patients taken before and after POMx consumption was exposed to AAPH-induced oxidation. Lipid peroxidation was determined as the formation of lipid peroxides. **[B]** Paraoxonase 1 (PON1) activity was measured as arylesterase activity. **[C]** Serum antioxidant capacity was also evaluated as thiol sulfhydril (SH) groups. Results are presented as mean ± SD. $P < 0.05$ statistically significant.

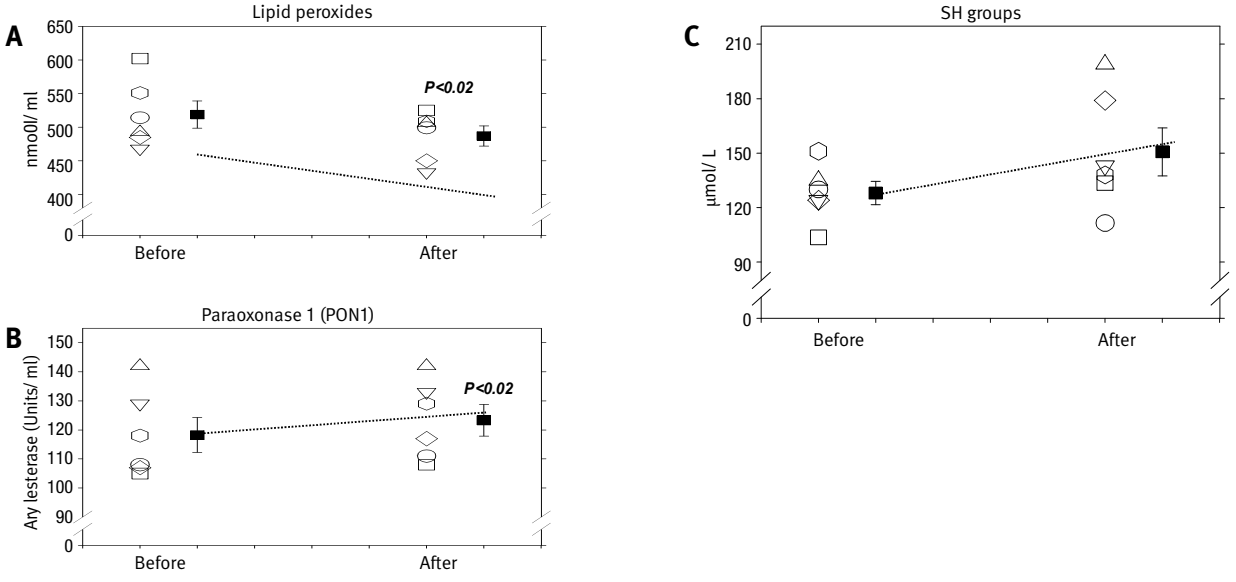
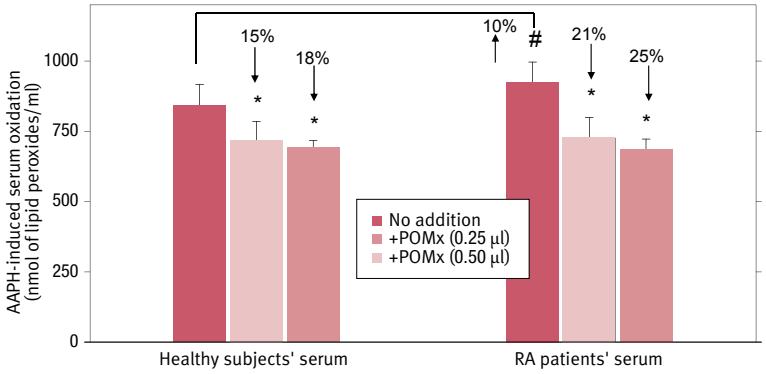


Figure 3. Direct in vitro effect of POMx on serum oxidative stress. Serum from RA patients withdrawn before POMx consumption, or from healthy subjects, was incubated with POMx (0.25 and 0.5 µl/ml) for 2 hours at 37°C. Oxidation was then induced by AAPH and determined by measuring the formation of lipid peroxides. Results are presented as mean of three separate experiments ± SD. *$P < 0.01$ with vs. without POMx, #$P < 0.02$ RA vs. healthy subjects (without POMx).



serum from RA patients compared to healthy subjects. After incubation of RA-derived serum with POMx, AAPH-induced serum oxidative stress decreased significantly ($P < 0.01$) by up to 25%, in comparison to an 18% decrease in serum derived from healthy subjects incubated with POMx under similar conditions [Figure 3].

DISCUSSION

This pilot study was performed to determine possible favorable effects of POMx consumption on clinical parameters in active RA, as related to serum oxidative status. We observed a significant reduction in DAS28 (ESR) after 12 weeks of POMx supplementation, since all our patients had a high initial mean DAS28 (~5.73) despite treatment with at least two DMARDs. In half the patients, a considerable (> 1.2) reduction in DAS28 was achieved. Our results demonstrate that the significant reduction in DAS28 (ESR) after 12 weeks of POMx supplementation could be attributed to a marked decrease in the tender joint count and ESR, whereas the patients' global assessment by VAS or swollen joint count was not significantly affected. This is in accordance with previous studies showing that DAS28 correlates better with tender joint count than with other measures of RA disease assessment.

The discrepancy between the more obvious reduction in the number of tender joints and only a trend in ESR reduction, unchanged CRP and the number of swollen joints could be explained by taking into consideration factors other than the DAS core components. There is increasing evidence that ROS/RNS contributes to the phenomenon of pain, such as chronic arthritic pain in RA patients. Supplementation of antioxidants, such as vitamin E, for patients with active RA resulted in a reduction in pain parameters (morning, evening, and after-activity pain) despite no influence on joint inflammation assessed by the duration of morning stiffness and the number of swollen joints [19]. Supplementing of RA patients with essential fatty acids, which exhibit anti-inflammatory characteristics, had no significant effect on clinical parameters measured by DAS28 but significantly reduced joint pain measured by patients' VAS. [20]. The reduction in the number of tender joints in RA patients following POMx consumption, as shown in our pilot study, is in accordance with previous studies by van Vugt et al. [21], who demonstrated a significant reduction in the number of tender joints and in DAS28 (ESR) in RA patients after consumption for 10 weeks of an antioxidant-enriched spread containing tocopherol, lycopene, carotenoids, lutein and vitamin C. Interestingly, 4 weeks after the antioxidant "wash-out" period, DAS28 increased again. Decreased tender joint count, inflammatory markers (ESR, CRP), and DAS28 were also demonstrated in RA patients who were treated with statins [22].

Inflammation in RA is mainly caused by activated neutrophils, monocytes and macrophages that, in addition to their immune activity, also produce a large amount of ROS/RNS, contributing to tissue injury. Indeed, we could show that the serum oxidative state in RA patients is significantly higher than in healthy subjects. This could be the result of lower antioxidant concentrations in patients' serum, which correlated with disease activity and higher CRP levels. A deficit in antioxidants in RA may have resulted from oxidative stress-induced antioxidant inactivation, from a lower intake of antioxidants, or from increased metabolism of antioxidants. The therapeutic activity of antioxidants in RA was previously explored in several studies. Canter et al. [23] summarized randomized trials that evaluated supplementation of several antioxidants (vitamin A, C, E and selenium) and showed little convincing evidence of a beneficial effect of a single antioxidant supplementation in arthritis. It has been suggested that an individual antioxidant may not reflect the complex vitamins and nutrients found in natural foods, which may explain the discrepancies between intervention trials on single antioxidant molecules and studies on the consumption of fruits and vegetables, which include a combination of several antioxidant molecules that can scavenge several different types of ROS/RNS [24]. In the present study we administered POMx containing a variety of polyphenols with impressive antioxidant capacity, including unique polymolecular ellagitannins, mainly the hydrolysable tannin punicalagin, as well as gallic and ellagic acid and several anthocyanins [7]. The improvement in RA clinical parameters after POMx consumption was accompanied by a significant improvement in indices of serum oxidative stress measured as serum lipid peroxide level, serum sulfhydryl-groups concentration and serum PON1 arylesterase activity. Incubation of serum from

RA patients with POMx resulted in a significantly greater reduction in AAPH-induced serum lipid peroxidation as compared to serum obtained from healthy subjects. This phenomenon could be related to the higher basal oxidative stress, which is present in RA patients, as it has been shown that patients with high oxidative stress may benefit more from antioxidant therapy [25].

It should be emphasized that patients with RA have a nearly threefold higher risk for new cardiovascular events (mean 1 year follow-up). Pomegranate in general and POMx in particular possess important antiatherogenic properties, which make POMx an attractive supplementation that may have a dual influence: decreasing oxidative stress and pro-atherogenic tendency, and ameliorating clinical symptoms in RA patients.

A limitation of the current study was the relatively small number of patients. However, our RA patient group was unique in that it was highly homogenous with a very narrow range of defined RA-related factors, which enabled us to evaluate the beneficial outcomes.

In this pilot open study, we demonstrated clinically beneficial effects of POMx supplementation for patients with active RA, supported by an improvement in serum parameters of oxidative stress. The combination of POMx antioxidative properties with its anti-inflammatory and antiatherogenic profile makes POMx an attractive over-the-counter supplementation to treat patients with active joint inflammation. Our results suggest the possibility of therapeutic co-administration of POMx with conventional drugs for the treatment of active RA. For further evidence of the beneficial effects of POMx, especially in terms of its safety and anti-inflammatory, antioxidative and antiatherogenic properties in patients with active RA, studies on a larger cohort may be required.

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"If a feller says, "It's not the money, it's the principle of the thing," it's the money"

Elbert Hubbard (1856-1915), American writer, publisher, artist and philosopher