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Paraoxonase 1 polymorphism Q192R affects the pro-inflammatory cytokine TNF-alpha in healthy males

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Abstract

Background: Human paraoxonase 1 (PON1) is an HDL-associated enzyme with anti-oxidant/anti-inflammatory properties that has been suggested to play an important protective role against coronary heart diseases and underlying atherogenesis. The common *PON1* Q192R polymorphism (*rs662*, A>G), a glutamine to arginine substitution at amino acid residue 192, has been analyzed in numerous association studies as a genetic marker for coronary heart diseases, however, with controversial results.

Findings: To get a better understanding about the pathophysiological function of PON1, we analyzed the relationships between the Q192R polymorphism, serum paraoxonase activity and serum biomarkers important for atherogenesis. Genotyping a cohort of 49 healthy German males for the Q192R polymorphism revealed an allele distribution of 0.74 and 0.26 for the Q and R allele, respectively, typical for Caucasian populations. Presence of the R192 allele was found to be associated with a significantly increased paraoxonase enzyme activity of 187.8 ± 11.4 U/l in comparison to the QQ192 genotype with 60.5 ± 4.9 U/l. No significant differences among the genotypes were found for blood pressure, asymmetric dimethylarginine, LDL, HDL, triglycerides, and cholesterol. As expected, MIP-2 alpha a cytokine rather not related to atherosclerosis is not affected by the *PON1* polymorphism. In contrast to that, the pro-inflammatory cytokine TNF-alpha is enhanced in R192 carriers (163.8 ± 24.7 pg/ml vs 94.7 ± 3.2 pg/ml in QQ192 carriers).

Conclusions: Our findings support the hypothesis that the common *PON1* R192 allele may be a genetic risk factor for atherogenesis by inducing chronic low-grade inflammation.

Introduction

Paraoxonase 1 (PON1) is a calcium-dependent enzyme exhibiting esterase, lactonase and peroxidase activity. It accepts a broad range of substrates including organophosphates, diverse lactones and lipid peroxides and has been studied for its ability to breakdown pesticides and nerve gases. PON1 is a glycoprotein of about 45 kDa that is predominantly synthesized by the liver, from where it is distributed to other tissues, mainly to serum [1,2]. In serum, PON1 is associated with high-density-lipoprotein (HDL) particles [3]. HDL-associated PON1 has been frequently shown to have anti-oxidant and anti-inflammatory potential mainly by protecting lipids of HDLs and low-density lipoproteins (LDL) from oxidative modifications [1,2]. Most likely, these protective effects depend on the peroxidase and esterase activity of PON1 allowing the detoxification of oxidized molecules such as phospholipids and lipid hydroperoxides [4,5]. Cardiovascular diseases and underlying atherosclerosis are associated with oxidative stress and inflammation. Hence, serum PON1 is suggested to contribute to the established anti-atherogenic function of HDLs which is, at least partly, attributable to their anti-oxidative properties [1,2,6].

This notion is further supported by animal model studies using *PON1* knock out and transgenic PON1 over-expressing mice. HDLs of *PON1*^{-/-} knock-out mice were

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found to prevent LDL oxidation less efficient than LDLs from control mice [7]. On the other hand, increased PON1 content in transgenic mice overexpressing murine or human PON1 resulted in HDLs that were more protected from lipid peroxidation [8,9]. Moreover, *PON1* deficiency in mice resulted in elevated levels of oxidative stress and endothelial adhesion molecules [10]. Accordingly, $PON1^{-/-}$ animals exhibited increased susceptibility to the development of large atherosclerotic lesions on a high-fat diet [7], whereas mice overexpressing human PON1 exhibited decreased atherosclerotic lesion sizes when fed an atherogenic diet [8,9].

The human *PON1* gene is located on the long arm of chromosome 7 between q21 and q22. Two common coding region polymorphisms occur: a glutamine to arginine substitution at position 192 (Q192R) which affects PON1 enzyme activity and is analyzed in this study, and a leucine to methionine substitution at position 55 (L55M) [11]. *PON1* gene polymorphisms have been examined with respect to their association to various human diseases including coronary heart disease (CHD), Parkinson's disease, type 2 diabetes and inflammatory bowel disease [12,13]. Most studies focused on the anti-oxidant/anti-inflammatory properties of PON1 in association with the development of atherosclerosis and the role of the Q192R polymorphism as a genetic marker for CHD. However, the results reported so far are controversial, some indicating an association between the Q192R polymorphism and atherosclerosis and CHD risk, while others do not as reviewed in [12,13].

In the present study, we have analyzed the influence of the *PON1* Q192R polymorphism on serum lipids and inflammatory biomarkers in a cohort of 49 healthy male individuals to get a better understanding of the role of the *PON1* Q192R polymorphism in the development of atherosclerosis and CHD.

Materials and methods
Participants and study design
The cohort of 53 healthy males investigated in the present study has been recently described [14]. In short, based on clinical laboratory tests, the participants aged between 21 and 48 had an average Body Mass Index (BMI) of 24.1 ± 2.5 and fulfilled four criteria: (i) no history of gastrointestinal, hepatic, cardiovascular or renal disease, (ii) no supplemental vitamin use for ≥ 2 weeks before the start of the study, (iii) non- or occasional smoking (≤ 3 cigarettes/day), and (iv) perpetuation of usual nutrition habits. Fasting blood samples were taken from each participant for genotyping, PON1 enzyme activity determination and inflammatory biomarker analyses. The study was approved by the ethics committee of the Medical Faculty of Kiel University, Germany,

(permission number A121/07) and was conformed to Helsinki Declaration. All volunteers gave written informed consent prior to participation.

Genotyping
Genomic DNA was isolated from whole blood samples. Genotyping of the *PON1* Single Nucleotide Polymorphism (SNP) A/A, A/G, G/G (rs662) responsible for the Q192R substitutions was performed by using the TaqMan system. Fluorescence was measured with ABI Prism 7900 HT sequence detection system (ABI, Foster City, USA).

PON enzyme activity assay
PON enzyme activity was determined spectrophotometrically in plasma samples following the protocol described in [15]. Briefly, the rate of hydrolysis of paraoxon (diethyl-p-nitrophenyl phosphatate; Supelco) was measured by monitoring the increase of absorbance at 405 nm using 100 mM Tris-HCl (pH 8.0), 1 mM paraoxon and 2 mM $CaCl_2$. One unit of PON activity is defined as 1 nmol of 4-nitrophenol formed per minute at 20 °C under standard assay conditions ($\varepsilon = 17600$ M^{-1} cm^{-1}).

Serum biomarkers
Supernatants of whole blood samples were measured with commercially available ELISA kits for TNF-alpha, MCP-1 (R&D Systems, Minneapolis, MN), oxLDL (KAMIYA Biomedical Company, Seattle, USA), asymmetric dimethylarginine (ADMA) (DLD Diagnostika, Hamburg, Germany) and MIP-2-alpha (Promocell, Heidelberg, Germany). Optical density was read on a microplate reader (Spectramax® 190, Molecular Devices). Laboratory measurements including serum lipid concentrations have been described previously [16].

Statistical analysis
Results are displayed as means ± SEM. Data were analyzed by an unpaired two-sided Student's t-test (Microsoft Excel Version 2003 or GraphPad Prism 4.0 software). P-values < 0.05 were considered statistically significant.

Results
Genotype distribution and basic characteristics
Genotype analysis of the *PON1* Q192R polymorphism (*rs662*, A>G) of 53 male volunteers revealed 25 homozygous for Q/Q (51%), 23 heterozygous for Q/R (47%) and 1 homozygous for R/R (2%), while 4 probes failed genotyping. Because of the small incidence, in further studies the G/G genotype (phenotype R/R) was combined with the A/G genotype (phenotype Q/R) group. Basic characteristics of the cohort have been recently
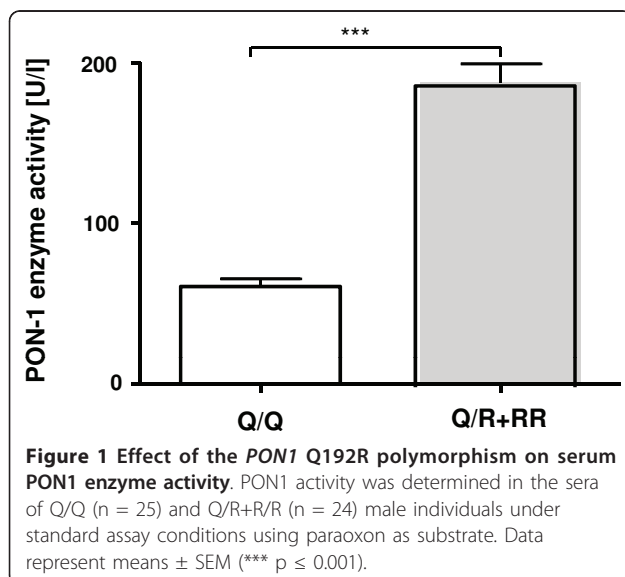
reported by our group [14]. Briefly, the values for age (30.13 ± 6.71 years), weight (79.11 ± 10.17 kg), height (1.81 ± 0.06 m), BMI (24.12 ± 2.50 kg/m^2) and fasting glucose level (86.47 ± 10.68 mg/dl) as well as for the kidney and liver parameters creatinine (1.05 ± 0.10 m/dl), aspartate aminotransferase (30.09 ± 8.67 U/l), glutamate pyruvate transaminase (37.79 ± 14.73 U/l) and γ-glutamyl transpeptidase (20.49 ± 10.36 U/l) were in accordance with the inclusion criteria of the study and show values within the physiological range for healthy men.

Effect of the Q192R polymorphism on PON1 activity

PON1 enzyme activity was found to be significantly elevated in the serum of Q/R and R/R individuals with a mean value of 187.8 ± 11.4 U/l, when compared to the Q/Q group having a mean value of 60.5 ± 4.9 U/l (p < 0.001; unpaired two-sided Student's t-test) (Figure 1). These data confirm previous studies that have demonstrated a similar effect of the Q192R polymorphism on serum PON1 activity [4,15].

Effect of the Q192R polymorphism on blood pressure and ADMA

Subsequently we analyzed a possible association between the Q192R polymorphism and blood pressure. However, no significant differences for blood pressure and ADMA levels, a blood pressure biomarker, were found between the Q/Q and the combined Q/R + R/R group with mean values of 82.5 ± 2.0 versus 82.9 ± 1.9 for diastolic and 125.0 ± 2.4 versus 128.0 ± 2.6 for systolic pressure as well as 0.96 ± 0.05 versus 0.98 ± 0.07 µmol/l for ADMA.

Figure 1 Effect of the *PON1* Q192R polymorphism on serum PON1 enzyme activity. PON1 activity was determined in the sera of Q/Q (n = 25) and Q/R+R/R (n = 24) male individuals under standard assay conditions using paraoxon as substrate. Data represent means ± SEM (*** p ≤ 0.001).

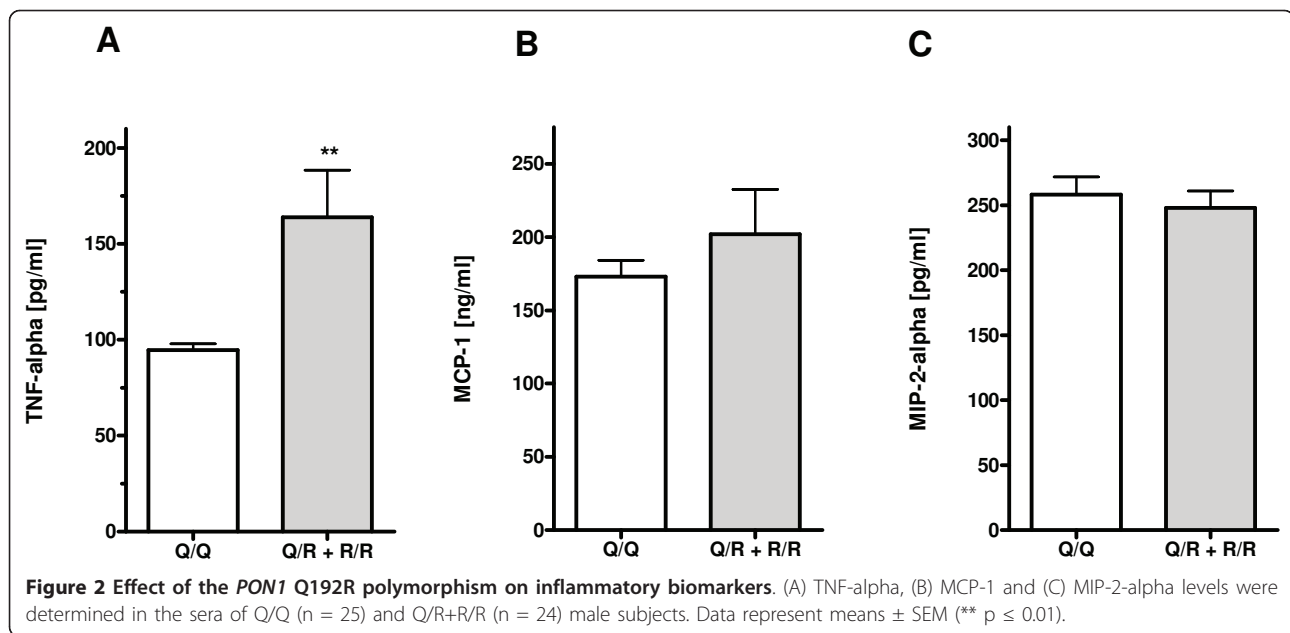### Effect of the Q192R polymorphism on serum lipoproteins and lipids

The Q192R polymorphism had no significant effect on HDL (51.7 ± 2.8 versus 50.8 ± 2.4 mg/dl), LDL (95.8 ± 5.7 versus 92.8 ± 6.1 mg/dl), triglycerides (TG) (91.0 ± 8.1 versus 102.9 ± 11.6 mg/dl) and cholesterol (165.7 ± 5.1 versus 164.1 ± 6.7 mmol/l). In addition, we found a slight but not significant increase in the oxLDL levels for the Q/R + R/R group with a mean of 21.7 ± 2.8 U/ml compared to Q/Q individuals with a mean value of 17.8 ± 3.0 U/ml.

Effect of the Q192R polymorphism on inflammatory biomarkers

Finally, we analyzed the effect of the Q192R polymorphism on the inflammatory biomarkers TNF-alpha, MCP-1 and MIP-2-alpha. As shown in Figure 2A, the mean level of TNF-alpha for the Q/Q group was found to be 94.7 ± 3.2 pg/ml. In the sera of Q/R and R/R individuals the respective level was significantly enhanced with a mean value of 163.8 ± 24.7 pg/ml (p = 0.007; unpaired two-sided Student's t-test). Furthermore, the inflammatory biomarker MCP-1 was slightly but not significantly enhanced in Q/R and R/R individuals when compared with the Q/Q group (202.0 ± 30.7 versus 173.0 ± 11.2 ng/ml) (Figure 2B). In contrast to that, MIP-2-alpha levels were not affected by the Q192R polymorphism (258.2 ± 13.7 versus 248.1 ± 12.9 pg/ml; Figure 2C).

Discussion

The role of the *PON1* Q192R polymorphism in cardio-vascular diseases is still under debate [1,2,12,13]. In the present study, genotyping of a cohort of 49 German males revealed a frequency of 0.74 for the Q192 allele confirming previous reports on Western populations with Caucasian origin [17-20]. Moreover, we found that the occurrence of the R192 allele led to an elevated serum paraoxonase activity which is also in good accordance with previously published data on the Q and R allozymes [21,22]. In this regard it is noteworthy that paraoxon represents a non-natural substrate and that the natural substrate(s) of PON1 has/have not been identified so far [2]. Consequently, it is not a contradiction that elevated specific paraoxonase activities of PON1 allozymes have been demonstrated to be negatively correlated with their antioxidant capacity in HDLs, i.e. protecting LDLs against oxidation, reversing the biological effects of oxidised LDLs and preserving the function of HDLs [13,21,22]. Since the oxidation of LDL and the accompanied formation of foam cell layers are thought to represent crucial steps in the initiation process of atherosclerosis [23,24], an enhanced antioxidant activity of HDLs has been suggested to prevent atherosclerosis and CHD [1,2]. Accordingly, a low-active paraoxonase allele such as *PON1* Q192 should protect

Figure 2 Effect of the *PON1* Q192R polymorphism on inflammatory biomarkers. (A) TNF-alpha, (B) MCP-1 and (C) MIP-2-alpha levels were determined in the sera of Q/Q (n = 25) and Q/R+R/R (n = 24) male subjects. Data represent means ± SEM (** p ≤ 0.01).

against atherosclerosis when compared with the corresponding high-active R192 allele. Although such an association has been found in some studies [17-19,25-31], no relationship has been revealed by others [20,32-38]. Recent meta-analyses suggested a weak association between the *PON1* Q192R polymorphism and CHD risk [39], however, no or only a population-specific effect of the R192 allele on human longevity [40]. Here we addressed the question whether the Q192R polymorphism and the related differences in PON1 activity are linked to changes in biomarkers indicative for a pro-atherogenic status.

Our analyses revealed that *PON1* genotypes are not associated with alterations in blood pressure and ADMA levels, elevation of both are linked with atherosclerosis [41]. Moreover, there were no differences in the serum lipid profiles including TG, HDL, LDL and cholesterol, except for slightly but not significantly enhanced oxLDL levels. Similar results for TG, HDL, LDL and cholesterol have been reported previously [19,20,25,29,31-34,36-38], whereas a few studies found a more pro-atherogenic serum lipid and/or lipoprotein pattern in association with the R192 allele [35,42]. The principal finding of this study is that the frequency of the low-antioxidant R192 allele is associated with significantly increased levels of the pro-inflammatory cytokine TNF-alpha. Since chronic low grade alterations of inflammatory markers are known to be associated with increased atherogenic risk [43], elevated R192 allele-dependent TNF-alpha levels may thus represent a putative risk marker.

Only recently, it has been demonstrated that adenovirus-based overexpression of human PON1 in apolipoprotein E knock-out mice caused enhanced serum anti-oxidative and anti-inflammatory capabilities reflected among other factors by decreased TNF-alpha levels [44]. Hence, enhanced PON1 antioxidant capacity was found to be associated with reduced TNF-alpha levels, probably protective against atherosclerosis. Considering the anti-oxidant property of HDL-associated PON1, it is intriguing to speculate that low amounts of serum PON1 or low-anti-oxidant *PON1* alleles such as R192 lead to elevated levels of reactive oxygen species in this way triggering the redox-sensitive NfkappaB signaling pathway that is known to stimulate TNF-alpha expression. However, further mechanistic investigations are necessary to decipher this proposed PON1 TNF-alpha relationship. It is remarkable that, in turn, enhanced TNF-alpha levels have been shown to down-regulate PON1 expression in murine and human hepatoma cell lines as well as *in vivo* in mice, most probably via an NF-kappaB- and nuclear receptor peroxisome proliferator-activated receptor-alpha (PPAR-alpha)-dependent mechanism thereby diminishing the antioxidant and anti-atherogenic activity of HDLs [45,46]. Moreover, a TNF-alpha antagonist therapy in rheumatoid arthritis patients led to enhanced PON1 levels concurrent with elevated anti-oxidative capacities of HDLs and lowered inflammatory status [47]. Interestingly, anti-TNF-alpha therapy response was found to be associated with SNPs in the *PON1* locus [48] and a recent case-control study on atherosclerosis in rheumatoid arthritis found a

correlation between PON-1 activity and serum TNF-alpha and IL-6 levels [49], emphasizing the close regulatory interrelation between PON1 activity and TNF-alpha levels.

In addition to TNF alpha, we found that levels of a second pro-inflammatory cytokine MCP-1 were slightly although not significantly enhanced in R192 carriers that usually express PON1 allozymes with less antioxidant capacity. MCP-1 expression and secretion of endothelial cells are known to be induced by oxidized LDL and accordingly, are thought to represent a crucial step in the initial phase of the inflammatory processes in atherosclerosis [43]. Consistent with its anti-oxidant function that leads to reduced lipid peroxidation, PON1 has been demonstrated to attenuate MCP-1 expression of cultured endothelial cells [50]. In good accordance with that are data on HDLs isolated from wild type and *PON1* knock-out mice. In a human endothelial cell culture model $PON1^{-/-}$ HDLs exhibited significantly less antioxidant capacity accompanied with elevated MCP-1 levels, hence, linking *PON1* deficiency to lipid hydroperoxide-triggered expression of pro-inflammatory MCP-1 [7]. Since the amount of MIP-2 alpha, a cytokine rather not related to atherosclerosis, is not affected by *PON1* polymorphism, the observed changes of TNF-alpha and MCP-1 levels observed in the current study are most likely specific.

Conclusion

Our data indicate that the low-antioxidant *PON1* R192 allele is associated with increased pro-inflammatory cytokines known to be involved in the initiation process of atherosclerosis. However, our finding needs to be confirmed in further studies and especially in larger study population.

Abbreviations used

ADMA: asymmetric dimethylarginine; CHD: coronary heart disease; PON1: paraoxonase 1; SNP: single nucleotide polymorphism; TG: triglycerides.

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Authors' contributions
KL analysed the data and wrote the manuscript. CS participated in the design of the study, acquired and analysed data. CBS performed enzyme assays. CK carried out genotyping studies. GR participates in the design of the study and critically revised the manuscript. FD conceived and designed the study, analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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