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Serum paraoxonase-3 concentration is associated with insulin sensitivity in peripheral artery disease and with inflammation in coronary artery disease

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ABSTRACT

Objective: There are no data on the relationship between serum paraoxonase-3 (PON3) concentration and atherosclerosis in humans. Our aim was to investigate possible associations, using recently developed methods, in patients with peripheral artery disease (PAD) or coronary artery disease (CAD).

Methods: We studied 118 PAD and 72 CAD patients and 175 healthy volunteers. Serum PON3 was determined by in-house ELISA using polyclonal antibodies generated against a synthetic peptide with a sequence specific to PON3. Polymorphisms of the *PON3* promoter were analyzed by the Iplex Gold MassArray™ method.

Results: There was a significant increase in serum PON3 concentration in both groups of patients with respect to the control group. In PAD patients, we observed significant positive correlations between PON3, insulin levels and HOMA index. These associations were not observed in CAD. There were significant positive associations between serum PON3 and β-2-microglobulin, CCL2 and high-sensitivity C-reactive protein in CAD patients, but not in PAD. We did not find any significant differences in *PON3* gene promoter polymorphisms and their haplotypes between patients and controls, indicating that associations were not genetically determined.

Conclusion: In both atherosclerotic phenotypes, serum PON3 concentration was increased, but this was associated with decreased insulin sensitivity in PAD and with inflammation in CAD.

Abstract word count: 203

Key words: antioxidants; atherosclerosis; lipoproteins; myocardial infarction; paraoxonases; peripheral vascular disease.

1. Introduction

Atherosclerosis remains one of the leading causes of morbidity and mortality worldwide. The pathobiology of this disease is characterised by a sustained and silent progression, and the clinical consequences may vary depending on the anatomic localisation of the lesions. Patients with coronary artery disease (CAD) may develop sudden death or myocardial infarction, sometimes at relatively young age. On the other hand, patients with peripheral artery disease (PAD), have a higher burden of atherosclerosis than those patients with adverse events in other arterial territories, and this represents a differential aspect, since they can develop advanced and extensive arterial alterations without any immediate threat to survival. However, both forms of atherosclerosis share some underlying molecular mechanisms. To-date, it is generally accepted that one of the main metabolic causes of atherosclerosis is oxidative stress and the associated increased lipid peroxidation, leading to the activation of inflammatory cells. Thus, the enzymes involved in protection against oxidative stress have received a great deal of attention in recent years [1].

Paraoxonases (PON) are a family of enzymes comprising 3 members, PON1, PON2 and PON3, whose genes are located adjacent to each other on chromosome 7q21-22 [2]. In humans, PON1 and PON3 genes are produced in many cell types [3], and their protein products are found in the circulation bound to high-density lipoproteins (HDL) [4]. Conversely, PON2 is an intracellular enzyme which is not, however, found in plasma [5]. All these enzymes are able to retard low-density lipoprotein (LDL) oxidation and cellular oxidative stress [6]. In addition, studies using a variety of mouse models of atherosclerosis have

consistently shown that human PON1, 2 or 3 expression inhibits, or reverses, the development of atherosclerosis via mechanisms involving the reduction of oxidative stress, the promotion of cholesterol efflux from macrophages, and the normalisation of vascular endothelium function [6].

Although the knowledge on PON1 and PON2 structure and function is rapidly expanding, not much is known about the PON3 protein. Its gene was identified in 1996 when Primo-Parmo et al. [2] detected a large number of cDNA sequences in the Genome Data Base similar to human PON1. The percentage identity among human PON1, PON2, and PON3 genes is high (about 70%) and the genes are believed to derive from a common precursor [7]. Clinical research on PON3 has been hampered by the lack of methods for measurement [5], but we recently described a high-throughput, reliable enzyme-linked immunosorbent assay (ELISA) to analyse PON3 concentration in human serum [8]. The present study was aimed to investigate the possible association between serum PON3 concentration with PAD and CAD.

2. Methods

2.1. Study population

Patients with clinically diagnosed symptomatic PAD (n = 118, 85.6% men, 55 – 80 years old) were recruited from the outpatient clinics of *Hospital Universitari Joan XXIII*. At entrance, relevant data were collated by clinical records, interview and physical examination. Selected laboratory parameters were measured as well as the ankle to brachial index (ABI) and the degree of internal carotid artery (ICA) stenosis using widely accepted methods and recommendations [9,10]. The ABI testing was performed with the patient in the

supine position, after 5 min of rest, using a standard protocol. The brachial blood pressure measurement was obtained in the left arm, and in the right and left dorsalis pedis arteries, using a 5-7 mHz hand-held Doppler. The ABI was calculated by dividing the ankle blood pressure by the brachial blood pressure for each lower extremity. Chronic ischemia symptoms were detected using the Fontaine classification, a standardised physician-administered questionnaire that inquires about the presence of exertional calf discomfort related to walking uphill or walking rapidly. The degree of ICA was classified using the duplex criteria developed by the University of Washington based on the percentage of arterial diameter reduction (Supplementary Table 1). They had no signs of infection, renal impairment, liver disease, neoplasia, or autoimmune diseases.

Subjects with CAD (n = 72, all men, 47 – 51 years old) were patients that survived to an episode of myocardial infarction and were recruited in the Cardiology Unit of the *Hospital Universitari de Sant Joan*. They were matched with PAD patients for body mass index (BMI), incidence of diabetes, hypertension and corresponding treatments. The proportion of smokers and non-smokers was also similar in both groups. In these patients, there were no significant alterations in ICA, and ABI measurements were >1.0.

The control group consisted of 175 healthy volunteers (42.9% men, 50 – 80 years old) participating in an epidemiological study as described previously [11]. We recruited those volunteers that, after an interview, attended a clinical examination and provided a fasting blood sample. In the control group overweight was common (28.96 $\pm$ 0.30 Kg/m^2) and current smoking habit was less than 50% (23.6%). Incidence of diabetes, defined by history, use of diabetes medication or fasting serum glucose levels $\geq$ 6.99 mmol/L, was

present in an 8% of subjects. Dyslipidaemia, considered to be present by previous diagnosis, medication use or high levels of LDL-cholesterol ($\geq$ 4.13 mmol/L) and/or low levels of HDL-cholesterol ($\leq$ 1.03 mmol/L), and hypertension, defined by history and medication use, were not present in the control group. Also, there was no clinical analytical evidence of renal insufficiency, major liver disease, neoplasia or neurological disorders in these participants.

A fasting venous sample blood was obtained from all the participants and serum and EDTA-plasma were stored at –80ºC until measurements were performed. All the participants provided fully-informed consent to participation in the study on the understanding that anonymity of all data is guaranteed. The study was approved by the Ethics Committees of the participating hospitals (Institutional Review Boards).

2.2.- Measurement of serum PON1 and PON3 levels

Serum PON1 and PON3 concentrations were determined by in-house ELISA with rabbit polyclonal antibodies generated against synthetic peptides with sequences specific of mature PONs. The employed peptides were CRNHQSSYQTRLNALREVQ (specific for PON1) and CRVNASQEVEPVEPEN (specific for PON3). Details of these methods have been previously reported [8,12]. Serum PON1 lactonase activity was analysed by measuring the hydrolysis of 5-thiobutyl butyrolactone (TBBL) as described [13,14]. Lactonase activity was measured in an assay reagent containing 1mM $CaCl_2$, 0.25 mM TBBL and 0.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) in 0.05 mM Tris-HCl buffer, pH = 8.0. The change in absorbance was monitored at 412 nm.

Activities were expressed as U/L (1 U = 1 mmol of TBBL hydrolysed per minute). Serum PON1 esterase activity was determined by measuring the rate of hydrolysis of paraoxon at 410 nm and 37ºC in a 0.05 mM glycine buffer, pH 10.5 with 1 mM $CaCl_2$ [15]. Activities were expressed as U/L (1 U = 1 μmol of paraoxon hydrolysed per minute).

2.3. Other biochemical measurements

Serum β-2-microglobulin (β2M), high-sensitivity C reactive protein (hs-CRP), glucose, insulin, cholesterol, triglycerides, and HDL-cholesterol concentrations were measured in an automated analyser (UniCel^{TM} DxI 800, Beckman Coulter, Fullerton, CA, USA). LDL-cholesterol concentrations were estimated by the Friedewald formula [16]. Insulin resistance was estimated by using the homeostasis model assessment index (HOMA-IR) [17]. Plasma CCL2 concentration was measured by flow cytometry (FlowCytomix, Bender MedSystems).

2.4. PON3 genotyping

Genomic DNA was obtained from leukocytes (Puregene DNA Isolation reagent set, Gentra Systems Inc., Minneapolis, MN, USA). Selected single nucleotide polymorphisms (SNP's) of the *PON3* promoter were analysed by the Iplex Gold MassArray^{TM} method (Sequenom Inc., San Diego, CA, USA) at the Spanish National Genotyping Center (*Centro Nacional de Genotipado, Universitat Pompeu Fabra*, Barcelona, Spain).

2.5. Statistical analysis

Sample size was calculated with Open Epi (Open Epi: Open Source Epidemiologic Statistics for Public Health, Version 2.3.1. www.Openepi.com) using data from previously accepted biomarkers and own results [18].The normality of distributions was determined with the Kolmogorov-Smirnov test. Differences between two groups were assessed with the Student's t-test (parametric) or the Mann-Whitney U-test (non-parametric). Differences between multiple groups were analysed by the Kruskal-Wallis test. Pearson or Spearman correlation coefficients were used to evaluate the degree of association between variables. Each SNP was tested for Hardy-Weinberg equilibrium using Haploview 4.0 software [19]. Estimates of linkage disequilibrium between SNPs were calculated using Fisher's test. Results are shown as means and SEM in parenthesis. The SPSS 18.0 package was employed for all statistical calculations.

3.- Results

3.1. Serum PON3 and PON1 levels and other biochemical variables in CAD and PAD patients

Results of the biochemical measurements in patients with CAD or PAD compared to those obtained in the control group are shown in Fig. 1 and Table 1. There was a significant increase in serum PON3 concentration in the CAD and PAD patients with respect to the control group. Both groups of patients showed significantly lower levels of HDL-cholesterol, LDL-cholesterol and higher serum triglyceride concentrations. All PON1-related variables (serum

PON1 concentration, PON1 lactonase and paraoxonase activities) were significantly lower in PAD patients, but not in CAD patients who only presented a significant decrease in serum PON1 concentration compared to the control group. β2M and hs-CRP concentrations were higher in CAD and PAD patients than in normal subjects. CCL2 concentration was only significantly higher in PAD patients. It was also evident from Table 1 that the lowest values of PON1 activity were found in PAD with little overlapping with CAD and controls.

We observed significant positive associations between serum PON3 and PON1 concentrations in both CAD and PAD patients (Figure 1B), which were not observed in the control group (ρ = 004, P = 0.958). No significant associations between serum PON3 concentrations and PON1 lactonase and paraoxonase activities was observed in any of the groups (Supplementary Table 2).

When PAD patients were characterised by Fontaine classification, no significant differences were observed between groups in relation to biochemical parameters, inflammatory markers or PON-related measurements, although PON1 and PON3 concentrations showed a trend to be higher (Supplementary Figure 1 and Supplementary Table 3).

3.2. Relationships between serum PON3 concentrations and the clinical characteristics of the patients

No association was found between serum PON3 concentration and gender, smoking status or diabetes diagnosis in PAD and CAD patients. Only in PAD patients, we observed a significant positive correlation between PON3 and insulin levels and HOMA index (Figure 2). These associations were not

observed in CAD [Insulin: ρ = 0.017; *P* = 0.887; HOMA index: ρ = 0.026; *P* = 0.828]. Also in PAD patients we observed a significant positive association between serum PON3 concentrations and the presence of hypertension [2.07 (0.11) vs. 1.67 (0.08) mg/L; *P* = 0.038], not observed in CAD patients. When the influence of antihypertensive treatments in PAD patients was evaluated, a significant positive relationship was observed between PON3 and the combination of angiotensin converting enzyme inhibitor (ARE) and angiotensin receptor blocker (ARB) treatment [2.21 (0.13) vs. 1.82 (0.08) mg/L; *P* = 0.019). Multiple regression analysis confirmed the significant positive association between serum PON3 concentration and insulin in PAD patients (Supplementary Table 4).

3.3. Relationships between serum PON3 concentrations and inflammation-related markers

There were significant positive associations between serum PON3 levels and β2M, CCL2 and hs-CRP concentrations in CAD patients (Figure 3). However, these associations were not observed in subjects with PAD (Supplementary Figure 2). Most PAD patients had elevated serum CRP concentrations, indicating an acute inflammatory condition. Multiple regression analysis confirmed the significant positive association between serum PON3 concentration and β2M in CAD patients (Supplementary Table 5).

As described in the Methods section, all PAD patients were symptomatic, and most were included in grades III and IV of the Fontaine classification, meaning that most of them had ulceration or gangrene lesions. Less than 5% of PAD patients had serum CRP concentration ≤ 5 mg/L. These patients had

similar serum PON3 concentration than the control subjects (Supplementary Figure 3).

3.4. Influence of genotypic variants on serum PON3 concentrations in CAD and PAD patients

The distribution of *PON3* genotype variants among cases and controls and the Odds Ratios (95% CI) for CAD and PAD risk in relation to *PON3* genetic polymorphisms are shown in Supplementary Tables 6 and 7. More than 95% of the subjects were successfully typed in each *PON3* polymorphism. Genotype frequencies of each *PON3* polymorphism did not deviate from the Hardy-Weinberg equilibrium, neither in patients nor in controls. Linkage disequilibrium analyses revealed that all six *PON3* polymorphisms covered a unique central block region identical for patients and controls (Figure 4A). The expectation-maximization algorithm revealed three possible combinations with similar percentage frequency in all of the groups, but the frequency of ATGTGT showed a trend to be minor in control subjects than in patients.

Finally, the genotype-phenotype relationship was carefully assessed in control and patients. We observed that *PON3*$_{-746}$, *PON3*$_{-665}$ and *PON3*$_{-567}$ polymorphisms were those more significantly associated with changes in PON3 concentrations in the different groups (Figure 4B).

4. Discussion

Oxidative stress and inflammation play a key role in atherosclerosis [20-22]. Atherosclerotic plaque formation and progression is characterised by endothelial cell dysfunction, accumulation of lipoprotein aggregates in the

intima, and monocyte migration through the endothelium and their differentiation into macrophages [23]. Lipid peroxidation is linked to the progression of the disease from fatty streak to advanced atherosclerotic plaque [24]. Both PAD and CAD present with common biochemical characteristics linked to the onset and progression of atherosclerosis, but the extent of the diseased area in PAD is considerably higher to that in CAD. For this reason, it would be logical to expect qualitative and quantitative differences in the molecular factors involved in the progression of these diseases [25]. Manifestation of arteriosclerosis in lower extremity arteries may show characteristics which are not seen in other clinical events and to explore the similarities and differences between CAD and PAD may be clinically relevant. For instance, increase in hs-CRP and β2M concentrations is quantitatively much more important in patients with PAD than in patients with CAD, showing that the inflammatory response is extreme in these patients, which is probably related to the physical extension of this disease [26]. We recently reported that serum PON1 lactonase and paraoxonase activities, as well as concentration, are dramatically decreased in patients with PAD compared to controls, suggesting that this enzyme may be involved in the biochemical derangements leading to this disease [18].

In the present study we describe, for the first time, alterations in serum PON3 concentrations in patients with coronary and with peripheral atherosclerosis. Our patients with CAD or PAD had a significantly increased serum PON3 concentrations with respect to the control group which was accompanied by decreased PON1 concentrations (and activities in PAD) and decreased HDL-cholesterol levels. Although the increase in PON3 is quantitatively small, about 1 mg/L on average, compared to the 50 – 70 mg/L

decrease in PON1, it has to be taken into account that PON3 is about 100 times more potent per mg of protein than PON1 in protecting LDL against lipid peroxidation [27] and, thus, the increase in the enzyme's expression in these patients would be sufficient to balance the alterations in PON1 levels. This conclusion is in agreement with experimental studies showing that the overexpression of human PON3 in mice protected against the progression of atherosclerosis in mice [28]. It is to note, however, that PON1 activity is essentially normal in CAD patients and extremely low in PAD. This is important, due to the wide prescription of clopidogrel in these patients and, as recently suggested, the need for PON1 for its activation [29]. Whether this fact is relevant for clinical management of patients with PAD or CAD remains to be ascertained [30].

An interesting finding of the present article is the observed relationship between serum PON3 concentrations, hypertension and metabolic alterations in PAD, and particularly with insulin and the HOMA index, that was not found in subjects with CAD. Diabetes is the most common underlying disease causing PAD, and these results suggest a role for PON3 in the regulation of glucose metabolism, which is one of the most important derangements related to metabolic syndrome. Indeed, human PON3 transgenic mice have been reported to have lower insulin concentrations than their corresponding littermates and are protected against obesity development [31]. In PAD, however, serum PON3 concentrations were not associated to circulating inflammatory markers as β2M, CCL2 or hs-CRP, an association that was positively observed in CAD. We do not know the reason for these differences, but serum PON3 concentrations were significantly lower in PAD, while the levels of inflammatory markers were

much higher. The possibility exists that the magnitude of the physical extent of the diseased areas in PAD makes for PON3 impossible to thwart the massive arterial inflammation observed in these patients. We also analysed *PON3* gene promoter polymorphisms, to ascertain whether the observed differences in serum PON3 concentrations could be explained by differences in the gene frequency distribution between patients and controls. We did not find any significant differences in these polymorphisms and their haplotypes, and their influence on serum PON3 concentrations was similar in the three groups of subjects.

5. Conclusion

PON3 is still a relatively newly identified antioxidant enzyme and much work has yet to be done in order to elucidate its physiological functions and its implications in atherosclerosis. The present cross-sectional, pilot study found that serum PON3 concentration is increased in patients with CAD or PAD, two different manifestations of atherosclerosis with clearly differentiated phenotypes. We observed that PON3 seems to be more narrowly related to insulin sensitivity in PAD and to inflammation in CAD. However, further detailed studies would be necessary to fully understand the role of PON3 in human atherosclerosis.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:

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FIGURE LEGENDS

Fig. 1. (A) Serum PON3 concentrations in control subjects and patients with coronary artery disease (CAD) and peripheral artery disease (PAD). (B) Relationships between serum PON1 and PON3 concentrations in patients with CAD (yellow dots) and PAD (red dots).

Fig. 2. Relationships between serum PON3 concentrations and serum glucose, insulin and the HOMA index in patients with peripheral artery disease (PAD).

Fig. 3. Relationships between serum PON3 concentrations and inflammatory markers in patients with coronary artery disease (CAD).

Fig. 4. (A) Linkage disequilibrium structures across the *PON3* promoter gene, in control subjects, patients with coronary artery disease (CAD) and patients with peripheral artery disease (PAD), calculated by the Haploview software and labelled by their gene position. Pairwise linkage disequilibrium values are given in each square intersecting for each pair of SNPs and haplotype blocks outlined. There were not any significant differences in the haplotype frequencies between patients and controls. (B) Influence of the *PON3* gene promoter polymorphisms on serum PON3 concentrations in the three groups of subjects studied.

Figure 1

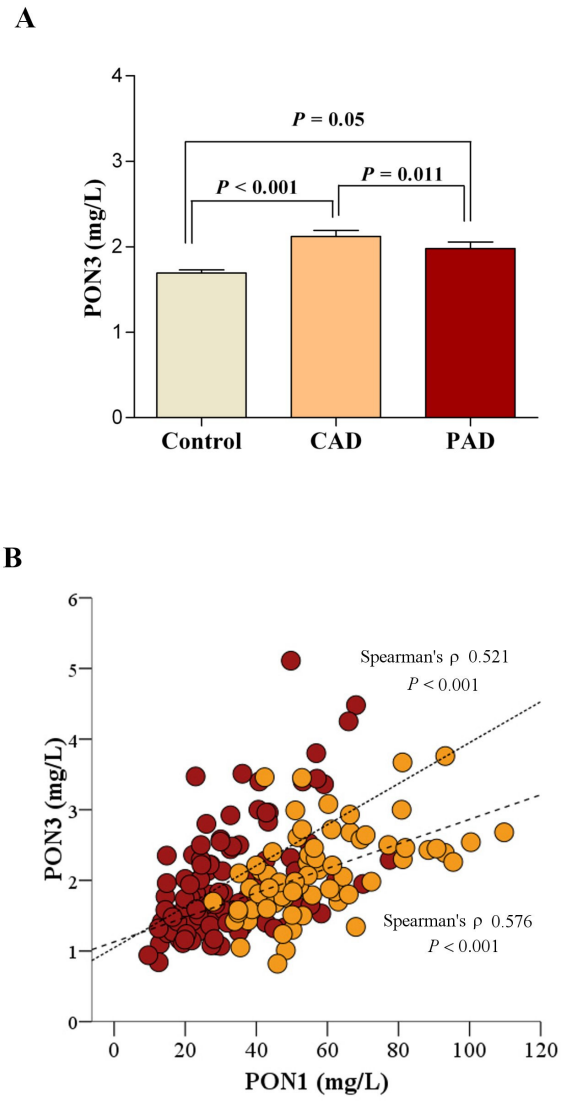

A
P = 0.05
P < 0.001
P = 0.011
PON3 (mg/L)
0
1
2
3
4
Control
CAD
PAD
B
Spearman's ρ 0.521
P < 0.001
Spearman's ρ 0.576
P < 0.001
PON3 (mg/L)
PON1 (mg/L)
0
20
40
60
80
100
120
1
2
3
4
5
6

Figure 2

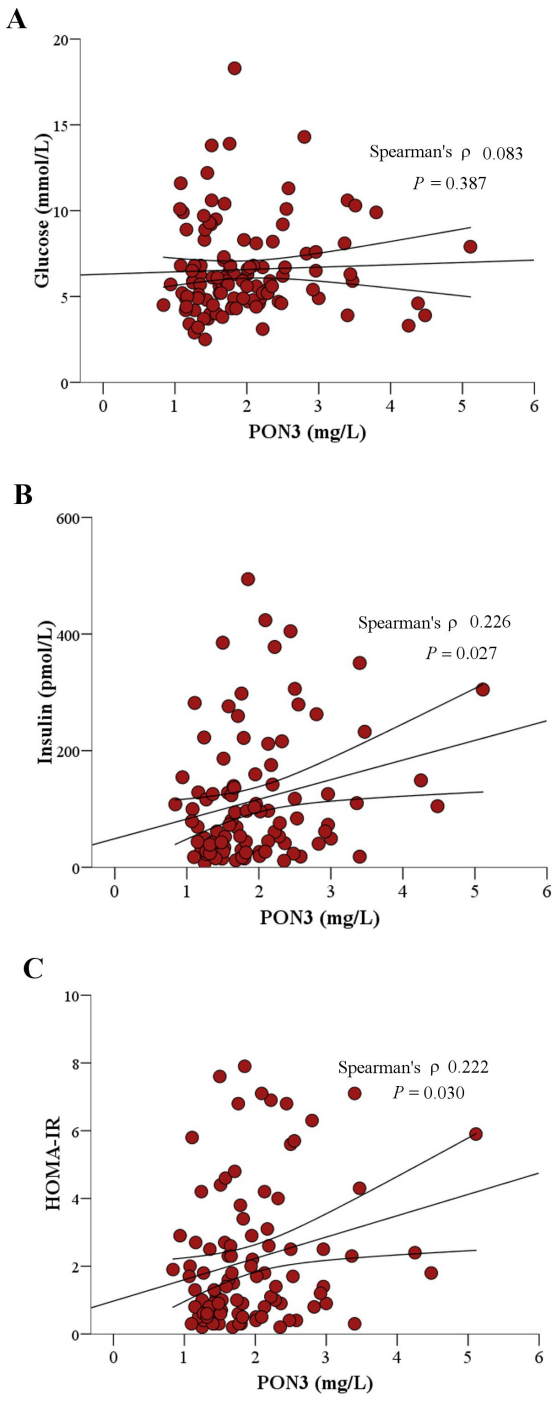

A
Glucose (mmol/L)
Spearman's ρ 0.083
P = 0.387
PON3 (mg/L)
B
Insulin (pmol/L)
Spearman's ρ 0.226
P = 0.027
PON3 (mg/L)
C
HOMA-IR
Spearman's ρ 0.222
P = 0.030
PON3 (mg/L)

Figure 3

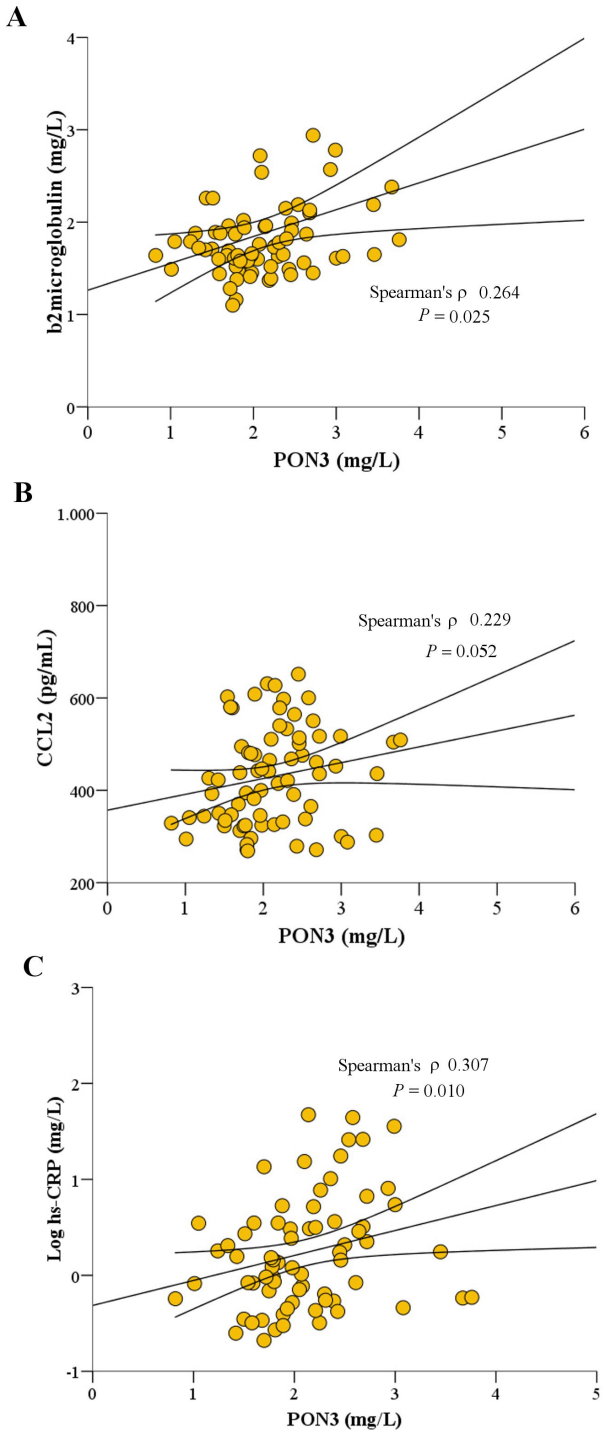
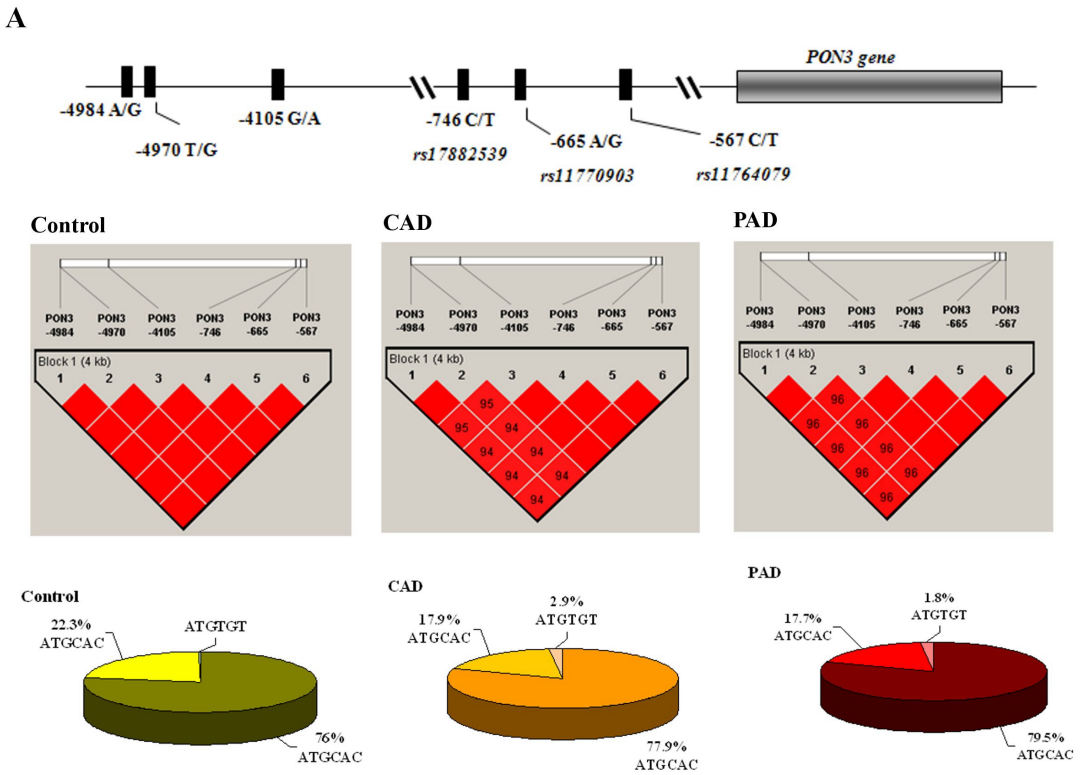
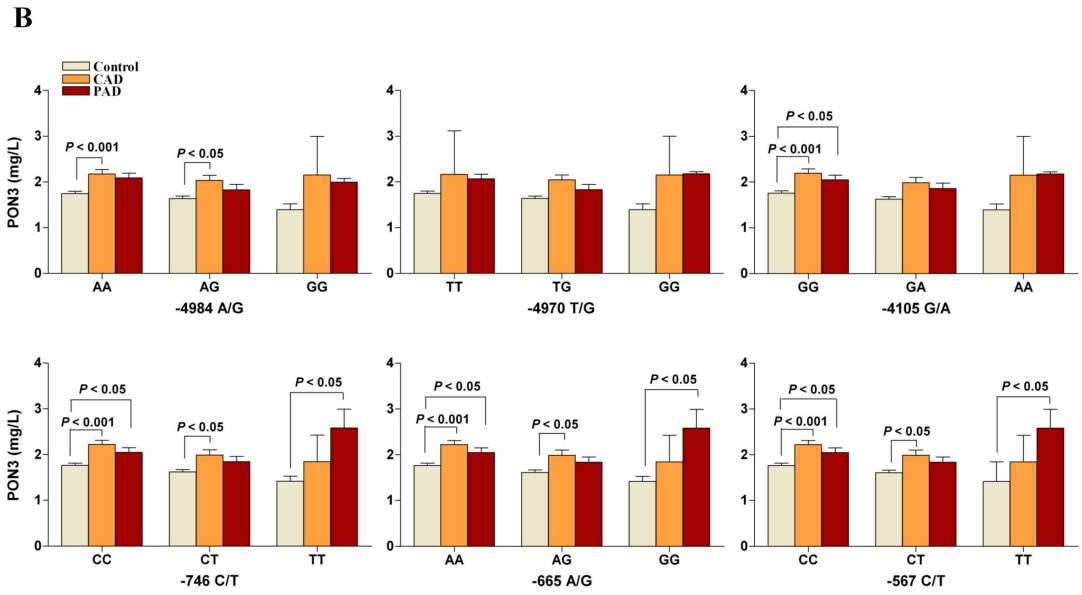

A
b2microglobulin (mg/L)
PON3 (mg/L)
Spearman's ρ 0.264
P = 0.025
B
CCL2 (pg/mL)
PON3 (mg/L)
Spearman's ρ 0.229
P = 0.052
C
Log hs-CRP (mg/L)
PON3 (mg/L)
Spearman's ρ 0.307
P = 0.010

Figure 4


A
PON3 gene
-4984 A/G
-4970 T/G
-4105 G/A
-746 C/T
rs17882539
-665 A/G
rs11770903
-567 C/T
rs11764079
Control
CAD
PAD
PON3 -4984
PON3 -4970
PON3 -4105
PON3 -746
PON3 -665
PON3 -567
Block 1 (4 kb)
1 2 3 4 5 6
95 95 94 94 94 94 94 94 94 94
96 96 96 96 96 96 96 96 96 96
Control
22.3% ATGCAC
ATGTGT
76% ATGCAC
CAD
17.9% ATGCAC
2.9% ATGTGT
77.9% ATGCAC
PAD
17.7% ATGCAC
1.8% ATGTGT
79.5% ATGCAC


B
Control
CAD
PAD
PON3 (mg/L)
P < 0.001
P < 0.05
AA AG GG
-4984 A/G
TT TG GG
-4970 T/G
GG GA AA
-4105 G/A
CC CT TT
-746 C/T
AA AG GG
-665 A/G
CC CT TT
-567 C/T