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Research article

Distribution of Paraoxonase 1 polymorphisms and activities in obese patients

Distribuția polimorfismelor Paraoxonazei 1 şi activităților ei la pacienții obezi

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Abstract

Study objective. The objective of this study was to investigate PON1 phenotype and genotype in Romanian patients with abdominal obesity. Materials and methods. The study groups consisted of 88 patients with abdominal obesity and 46 subjects with normal waist circumference, matched for age and gender. For each patient, we determined the clinical parameters that may influence PON1 activities. Q192R and L55M polymorphisms analysis in the PON1 gene were performed by PCR-RFLP using specific primers and restriction enzymes. PON1 lactonase, paraoxonase and arylesterase activities were assayed by spectrophotometric methods. Analysis of PON1 genotypes and activities distribution in the obese and non-obese individuals was performed with MedCalc Software (Version 12.4.0.0). Results. There was no statistically significant difference between obese and controls in regards to age and gender. The study revealed that PON1 activities were not influenced by gender. Of all PON1 activities, only the paraoxonase activity was inversely correlated with age, being significantly reduced in patients with abdominal obesity compared to non-obesity (p=0.009). Abdominal circumference independently influenced only the variation of arylesterase activity (R^2=6.5%, p=0.003). Distribution of PON1 genotypes in the study groups was significantly different (p=0.007) only for the Q192R but not for the L55M genotypes. The QR

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genotype had the highest influence on paraoxonase activity (R^2=40.6; p<0.001). The MM genotype had the greatest influence on arylesterase (R^2=11.3%, p<0.001) and lactonase activities (R^2=7.4%, p<0.001). Conclusions. Q192R genotypes distribution was significantly different in obese patients and the QR genotype influenced greatly the paraoxonase activity. The MM genotype had the most important independent influence on the lactonase and arylesterase activities.

Keywords: paraoxonase, arylesterase, lactonase, activity, genotype, obesity

Rezumat

Obiectivul studiului a fost de investigarea fenotipului şi genotipului PON1 la pacienţii români cu obezitate abdominală. Material şi metodă. Loturile din studiu au constat din 88 de pacienţi cu obezitate abdominală şi 46 subiecţi cu circumferinţă abdominală normală. Pentru fiecare pacient am determinat parametri clinici care ar putea influenţa activităţile PON1. Polimorfismele Q192R şi L55M ale genei PON1 au fost identificate prin PCR-RFLP (polimorfismul lungimii fragmentelor de restricţie) prin utilizarea unor primeri şi enzime de restricţie specifice. Activităţile PON1 lactonază, paraoxonază şi arilesteraza au fost măsurate prin metode spectofotometrice. Analiza statistică a genotipurilor PON1 şi activităţilor ei la pacienţii obezi şi nonobezi a fost realizată cu programul MedCalc (versiunea 12.5.0.0). Rezultate: Nu am găsit o diferenţă semnificativă între pacienţii obezi şi nonobezi în ceea ce priveşte vârsta şi sexul. Studiul a revelat faptul că activităţile PON1 nu sunt influenţate de sexul pacienţilor. Dintre activităţile PON1, doar activitatea paraoxonazei a fost invers corelată cu vârsta (p=0,05). Circumferinţa abdominală a influenţat independent doar variaţia activităţii arilesterazei (R^2=6,5%); p=0,003). Distribuţia genotipurilor PON1 între loturi a fost semnificativ diferită doar pentru Q192R (p=0,007), dar nu şi pentru genotipurile L55M. Genotipul QR a avut cea mai mare influenţă asupra activităţii paraoxonazei (R^2=40,6; p<0,001). Genotipul MM a avut cea mai mare influenţă asupra variaţiei arilesterazei (R^2=11.3%; p<0,001) şi lactonazei (R^2=7,4%, P<0.001). Concluzie. Distribuţia genotipurilor Q192R a fost semnificativ diferită la pacienţii obezi, comparativ cu martorii, iar genotipul QR a influenţat mult activitatea paraoxonazei. Genotipul MM a avut influenţa independentă cea mai mare asupra activităţilor arilesterazei şi lactonazei.

Cuvinte cheie: *paraoxonază, arilesterază, lactonază, activitate, genotip, obezitate*

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Introduction

Although it has been demonstrated that there is a direct correlation between obesity and oxidative stress markers, the mechanisms by which obesity *per se* could induce oxidative stress have not yet been elucidated.

Human plasma paraoxonase 1 (PON1), encoded by a gene located on chromosome 7q21.3 (1, 2), is a high-density lipoprotein (HDL)-associated esterase/lactonase, HDL containing apolipoprotein J (clusterin) (2-4). HDL-associated PON1 hydrolyzes paraoxon, organophosphorus compounds, unsaturated aliphatic esters and aromatic carboxylic esters (2, 5).

PON1 enzymatic activity is characterized by large interindividual variation and its low values predict a high risk of developing diseases involving oxidative damage and lipid peroxidation (5).

Recent studies have reported low HDL-associated PON1 activity in obese individuals (6) due to changes in HDL content. Changes in HDL shape and size adjusted the binding affinity and stability of PON1 (1).

Relatively recent comprehensive studies have shown that PON1 native activity is lactonase (7), its arylesterase and paraoxonase activities being fortuitous. Thus, correct characterization of PON1 requires the assessment of

all 3 activities: arylesterase (A-ase), paraoxonase (P-ase) and lactonase (L-ase).

Two better known single nucleotide polymorphisms (SNPs) have been identified in the PON1 coding region, Q192R and L55M (8), partly explaining the interindividual variation in PON1 activity (9). Gln (Q)→Arg (R) substitution at position 192 leads to the likelihood of two holoenzymes which differ in their ability to hydrolyze paraoxon, Arg holoenzyme having a greater activity (8).

SNPs at position 55 affect the stability (9) and influence the concentration of the enzyme (*via* an interaction with the -108C>T polymorphism in the promoter region), Leu (L) holoenzyme being associated with higher serum concentrations (8).

Protection against lipid peroxidation is higher in R192R and L55L homozygous individuals, for each one alone and cumulated (10). The LL homozygous genotype was found to be positively correlated with obesity in Mexicans (6).

To assess the correlation between PON1 and obesity, recent studies have emphasized the importance of simultaneously assaying PON1 activity and functional phenotypes at the expense of any number of point mutations (11).

Various studies have reported significant correlations between obesity and PON1 gene variants (5, 6). Controversial results have been obtained when assessing the correlation between obesity and enzymatic activity: some report lower levels of PON1 in obese individuals than in healthy subjects (6). An increased PON1 activity has been reported in obese subjects treated with tetrahydrolipstatin (Orlistat) (6). Obese women (5) or obese patients with non-diabetic metabolic syndrome (SM) showed no changes in PON1 activity, although there have been changes in inflammatory processes and oxidative stress (6).

The objective of this study was to assess whether there is an association between Q192R and L55M polymorphisms and PON1 activity in patients with abdominal obesity. To our knowledge, until now, there have been no studies of PON1 genetic polymorphisms in patients with abdominal obesity in Romania, nor has PON1 activity been assessed by determining all 3 types of activities in subjects with abdominal obesity in this geographical area.

Material and method

Study participants

The study included 88 subjects with abdominal obesity hospitalized for multidisciplinary assessment. The control group consisted of 46 subjects who had normal waist circumference. Initially the obese group included 91 subjects, but we could not isolate the DNA in three of them, therefore we excluded them from the final analysis.

The groups were balanced in terms of age (patients 55.4±12.6 vs. controls 55.1±12.6, P=0.90) and gender (35.5% male patients vs. 38.2% male control subjects, P=0.8).

The diagnosis of abdominal obesity was obtained based on the National Cholesterol Education Program (Adult Treatment Panel III) (NCEP-ATP III) guidelines. Elevated waist circumferences (>94 cm for male subjects and >80 cm for female subjects) led to the diagnosis of abdominal obesity (12).

Before patient recruitment, all participants signed an informed consent, the study being approved by the Ethics Committee of "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca. The informed consent and the research protocol were in accordance with the World Medical Association, Declaration of Helsinki.

Subjects were excluded from the study in case of relevant medical evidence for liver disease, such as acute or chronic viral hepatitis, declared consumption of alcohol in the last 6 months (>20g/day in men and >10g/day in women), autoimmune hepatitis, primary biliary cirrhosis, primitive sclerosing cholangitis, alpha-1 antitrypsin deficiency, hemochromatosis, porphyria, Budd-Chiari syndrome, liver cirrhosis and various malignancies. Subjects with

renal diseases, infections and psychiatric disorders that can cause changes in PON1 activity were also excluded.

Anthropometric measurements and clinical examination

General (age, gender, area of origin), clinical and laboratory data were recorded from each subject. Case history and physical examination provided data on the presence of co-morbidities: hypertension, heart failure, type 2 diabetes, stroke, coronary heart disease or angina. In order to obtain the same demographics, participants were chosen from the same geographical area. Standing height was measured in orthostatism, without shoes, using a measuring rod (stadiometer) and three determinations have been made. The arithmetic mean of these measurements, expressed in meters (m), was calculated.

Abdominal circumference was measured with a flexible inch tape, also in orthostatism. Three measurements have been performed, midway between the umbilicus and xiphoid process, patients performing maximal expiratory flow. The average of these determinations, expressed in centimeters (cm), was recorded (13).

Body mass index (BMI, kg/m^2) was calculated by the equation weight in kilograms (kg) divided by height in squared meters. Patients wore light clothing, no shoes, and the margin of error was +/-50 grams (g). Body mass index (BMI) was determined as normal <25 kg/m^2, overweight between 25-30 kg/m^2, class 1 obesity between 30.1-34.9 kg/m^2, class 2 between 35-40 kg/m^2, and class 3 >40 kg/m^2 (14).

Blood and serum samples

Venous blood samples were drawn in the morning, after 12 hours of fasting, using clot-activator and EDTA vacutainers, which were immediately placed at 4°C. For enzymatic determinations, serum samples were obtained by centrifuging the blood (3 minutes at 3000 rpm). They were frozen at -80°C and stored until analysis. For genetic determinations, the whole blood genomic DNA was purified and stored at -20°C until genotyping.

Paraoxonase activities

Serum paraoxonase, arylesterase and lactonase activities were measured spectrophotometrically according to the protocol described by Eckerson et al. (15, 16), with minor changes. Paraoxonase (P-ase) activity was determined using paraoxon (O, O-diethyl-O-p-nitrophenyl phosphate, Sigma) as a substrate. Basic sample mixture included 1mM paraoxon, 2M NaCl and 1mM $CaCl_2$ in 50mM glycine sodium hydroxide buffer (pH 10.5). The reaction was initiated by addition of serum sample and absorption was monitored at 405 nm for 90 seconds. Arylesterase (A-ase) activity was measured using phenyl acetate as a substrate: 1mM in 20mM Tris-HCl (pH 8.0) containing 1mM $CaCl_2$. Hydrolysis of phenyl acetate at 25°C was determined by monitoring the increase in absorption at 270 nm over a period of 90 seconds. A blank sample containing incubation mixture without serum was run simultaneously to correct spontaneous decomposition of substrate, in both determinations. All samples were run in duplicate and the mean value was used to calculate the activity by means of molar extinction coefficient of 18290 $M^{-1}cm^{-1}$ at 412 nm for p-nitrophenol and 1310 $M^{-1}cm^{-1}$ at 270nm for phenol. Lactonase activity (L-ase) was determined by a pH-sensitive colorimetric assay, with slight variations. Briefly, 3 µl of serum was incubated with 1mM δ-valerolactone in 1.2 ml Bicine buffer 2.5mM (pH 8.3), containing 0.15M NaCl, 1mM $CaCl_2$ and 0.2mM *m*-Cresol-Purple. The bleaching rate, resulting from the carboxylic acid formation, was monitored spectrophotometrically at 577 nm, for 1 minute. A standard calibration curve was performed with 10mM acetic acid, for the rate factor. Results are expressed in U/l for P-ase and in kU/l for A-ase and L-ase activities.

Genotype analysis

Genomic DNA was isolated from whole peripheral blood using the Wizard® Genomic DNA Purification Kit and protocol. Genotyping of DNA samples was performed by PCR-RFLP technique.

PCR reaction was adapted from a previously published protocol (17) and used the follow-

ing primers which were designed and verified with PRIMER 3 free software: FW 5'-TATTGTTGCT-GTGGGACCTGAG-3' and RV 5'-CCTGA-GAATCTGAGTAAA-TCCACT-3' for rs662 (Q192R) and FW 5'-GAAGAGTGATGTATAGC-CCCA-3' and RV 5'-TGAAAGCCAGTC-CATTAGGC-3' for rs 854560 (L55M).

PCR products were digested with *AlwI* and *Hin1II* (Thermo Scientific) for Q192R and for L55M polymorphisms, respectively, and migrated on 2% agarose gel (17). The resulting fragments were of 66 and 172 bp for the RR genotype and a non-digested 238 bp fragment for the QQ genotype; 66 and 106 bp fragments for the MM and a non-digested 172 bp fragment for the LL genotype.

Statistical analysis was performed using the MedCalc Software, Version 12.5.0.0.

Data were considered as nominal or quantitative variables. Nominal variables were characterized using frequencies. Quantitative variables were tested for normality of distribution using Kolmogorov-Smirnov test and were characterized by median and percentiles (25-75%) or by mean and standard deviation (SD), when appropriate. A chi-square test was used in order to compare the frequencies of nominal variables. Quantitative variables were compared using t test, Mann-Whitney test, ANOVA test or Kruskal–Wallis test, when appropriate. Some variables needed to be log-transformed before applying parametric tests. The correlation between quantitative variables was assessed using Pearson correlation or Spearman's rho, when appropriate. Deviations of allelic frequencies from Hardy–Weinberg equilibrium were calculated using a Chi-square test.

Multivariate analysis was carried out using linear regressions. We used as dependent variable the PON1 activities: paraoxonase (log-transformed), arylesterase and lactonase. We included as independent variables the data that achieved the criterion of significance at $p<0.2$ in univariate analysis. We used the Bonferroni correction in order to account for multiple comparisons.

The level of statistical significance was set at $p<0.05$.

Results

Clinical and demographic characteristics and laboratory findings in patients in the two groups are presented in *Table 1*.

We observed the existence of an inverse correlation between age and P-ase ($r =-0.170$, $P=0.05$). We did not find a correlation between age and the A-ase and L-ase ($r=-0.058$, $P=0.5$, respectively $r=-0.045$, $P=0.6$). Patient gender had a significant influence on P-ase, A-ase and L-ase activities ($P=0.9$, $P=0.1$, respectively $P=0.2$). We have established a correlation between HDL-Cholesterol (HDL-C) and P-ase, A-ase and L-ase values ($r=0.213$, $P=0.01$, $r=0.303$, $P<0.001$, respectively $r=0.271$, $P=0.002$).

Patients with RR genotype had statistically significantly higher values of P-ase than those with QQ genotype ($P<0.001$). Also, QR heterozygotes had statistically significantly higher values of P-ase than QQ homozygotes ($P<0.001$). Q192R polymorphism did not influence the values of A-ase and L-ase activities ($P=0.7$, respectively $P=0.4$).

L55M polymorphism influenced the values of all three PON1 activities. Thus, LL individuals had greater values of P-ase than LM ($P=0.001$) or MM ($P<0.001$) individuals. LL individuals had higher values of P-ase than MM homozygotes ($P<0.001$), and also higher values of A-ase than LM ($P<0.001$) or MM ($P<0.001$) individuals. Subjects with LL genotype had higher values of L-ase than those with LM ($P=0.01$) or MM ($P<0.001$) genotypes.

Multiple linear regression (forward method) was used to assess to what extent is P-ase independently explained by the following six variables: age, overweight status, L55M polymorphism, Q192R polymorphism and HDL-C levels. The model chosen in our study reported a 69.1% variation in P-ase, QR genotype having the greatest influence on its variation ($R^2=40.6\%$, $P<0.001$). It was followed, in order of importance, by the RR genotype ($R^2=12.6\%$, $P<0.001$), the MM genotype

Table 1. Clinical and demographic characteristics and laboratory findings in the two study groups

Variable	Non-obese patients (n=46)	Obese patients (n=88)	P
Age (years)*	55.11±12.64	55.38±12.593	0.9
Men**	17 (36.9%)	31 (35.2%)	0.8
Women**	29 (63%)	57 (64.7%)	
Waist circumference (cm)*	79±7.7	108.1±11	<0.001
Height (cm)*	167.1±6.35	166.2±8.13	0.4
Weight (kg)*	61.9±8.16	86.5±14	<0.001
BMI (kg/m^2)*	22.1±2.57	31.1±3.89	<0.001
A-ase (kU/l)*	69.48±21.45	63.53±15.75	0.1
L-ase (kU/l)*	55.29±16.94	57.52±14.95	0.4
P-ase (U/l) ***	347 (225; 531)	222 (151; 390)	0.009
HDL-C (mg/dl) *	52.62±12.83	47.61±10.31	0.01
RR **	0	7 (7.9%)	0.007
QR **	26 (56.5%)	28 (31.8%)	
QQ **	20 (43.5%)	53 (60.2%)	
MM **	7 (15.2%)	15 (17%)	0.4
LM **	16 (34.7%)	39 (44.3%)	
LL **	23 (50%)	34 (38.6%)	
Q/R	0.71/0.29	0.76/0.24	0.2
L/M	0.67/0.33	0.60/0.40	0.1

* mean±SD; ** number (%); *** median (percentile 25%; 75%)

(R^2=8.5%, P<0.001), HDL-C levels (R^2=4.6%, P<0.001) and LM genotype (R^2=1.7%, P=0.01). Age and obesity had no significant influence on P-ase activity. When measurement error correction technique was applied, LM genotype did no longer independently influence this activity.

We built a model to explain the variation in A-ase using multiple linear regression (forward method). The following variables were introduced in the final model: gender, obesity, L55M polymorphism, Q192R polymorphism, HDL-C levels. The model reported a 40.3% variation in A-ase activity. MM genotype had the greatest influence on its variation (R2=11.3%, P<0.001), being followed, in order of importance, by the LM genotype (R^2=13.4%, P<0.001), obesity (R^2=6.5%, P=0.003), HDL-C levels (R^2=4.1%, P=0.009), QR genotype (R^2=2.2%, P=0.01) and RR genotype (R^2=2.8%, P=0.01). Gender did not significantly influence A-ase. When we applied a measurement error correction technique, QR and RR genotypes did no longer independently influence this activity.

We built a model to explain the variation in L-ase using multiple linear regression (forward method). The following variables were introduced in the final model: obesity, L55M polymorphism, Q192R polymorphism, HDL-C levels. The model reported a 28.6% variation in L-ase activity. MM genotype had the greatest influence on its variation (R^2=7.4%, P<0.001), followed, in order of importance, by HDL-C (R^2=6.9%, P=0.001), QR genotype (R^2=6%, P=0.001), LM genotype (R^2=4.1%, P<0.001) and RR genotype (R^2=2.7%, P=0.01). Obesity did not significantly influence L-ase activity. When we applied a measurement error correction technique, QR genotype did no longer independently influence this activity.

Discussions

Recent studies indicate that HDL-associated PON1 may be involved in the pathogenesis of metabolic syndrome and obesity by demonstrating low A-ase, P-ase (6) and L-ase (18) activities and by presenting some associations with less active *PON1* gene variants in obese individuals (5, 6).

This study aimed to investigate all three PON1 activities (A-se, P-ase, L-ase) in a representative cohort of subjects with abdominal obesity, as well as the two most important polymorphisms in the coding region of the *PON1* gene (i.e. Q192R and L55M).

For the first time in Romania, this report provides evidence regarding the association of the L55M and Q192R polymorphisms with abdominal obesity, confirming other studies (6, 10). Even though there is no other data about the distribution of *PON1* gene variants in this geographical area, the frequencies observed are similar to those described for the European Caucasian race (9, 11).

The distribution of the studied polymorphisms in the 2 groups only showed statistically significant differences in the case of the Q192R polymorphism, similar to other reports showing influences of this polymorphism on the bioavailability of the enzyme (19-21). The L55M polymorphism was comparably distributed in both groups, so in view of these circumstances it would be expected not to find any difference in PON1 concentration. Differences in P-ase may be explained by the increased frequency of the QQ genotype in the obese group but other non-genetic factors, such as structural alterations of HDL, PON1 instability, increased levels of inflammatory mediators, changes in adipokine secretion profile may be involved as well.

L-ase and A-se activities, although showing lower values in the obese group, did not reach the significance threshold, most likely due to the small percentage of the population studied. Similar results with no changes in PON1 activities have been reported (22) for P-ase and A-ase activities in a young Turkish population with non-diabetic metabolic syndrome and obesity. In contrast to our results, a recent study showed a direct association of L-ase activity with obesity in obese women (18).

Our observations indicate that the group of participants (obese vs. control) influenced only P-ase and, in accordance with other studies (1, 21), we found a negative influence of age on this activity. Age did not have a significant influence when analyzing P-ase depending on genetic factors, HDL levels or gender. Thus, changes in P-ase are probably related to the development of oxidative stress, as a result of the alteration of sulfhydryl groups with aging. Similar to other studies, A-ase and L-ase showed no significant changes with age (1).

Similar to other reports, there was no significant influence of gender on any of the PON1 activities, most likely due to genetic heterogeneity (21).

HDL-C levels showed a weak direct association with each of the three activities. The data are consistent with other studies demonstrating a weak association of PON1 with HDL in obese individuals (22) and showing that PON1 is not distributed across the entire spectrum of HDL, but it is only associated with a subset of HDL particles (HDL3), with a poorer lipid content (11) and which contain apoJ (2-4). Another explanation could be the decrease in the apoprotein content of HDL, which leads to an altered binding affinity and stability of PON1 (1). Due to the obese condition (in fact, due to the large quantities of leptin, proportional to the body fat mass), *apoA1* gene, essential for PON1 transfer from hepatocytes to HDL and especially for PON1 activity (2-4, 11), acquires mutations. On the other hand, adipocytes secretion profile, consisting of numerous biologically active peptides, might change (3, 23). Adipokine imbalance leads to an increase of inflammatory mediators, which, in turn, will trigger the proatherogenic and proinflammatory activities of the HDL-PON1 complex (24).

Similar to other cross-sectional studies (25), multiple regression analysis showed that the Q192R polymorphism is the major determinant of P-ase, while L55M polymorphism influenced all three activities.

Even if RR individuals displayed the highest values of P-ase, it was the QR genotype that mostly influenced P-ase, due to higher frequency. Our results confirm another recent study (19) where the Q(Arg) allele carriers or QR heterozygotes display an increased risk of metabolic syndrome.

Most subjects in the obese group were LM heterozygotes and in the normal weight group were LL homozygotes, the LL variant strongly influencing all three activities. Without statistically significant difference, probably due to the small number of study participants, the group with abdominal obesity showed a prevalent homozygous MM genotype, which negatively influenced A-ase. While this activity is reduced from the early stages of oxidative stress and given that this decrease is dependent on obesity, we conclude that homozygous MM genotype is a risk factor for oxidative stress and indirectly for metabolic syndrome in obese individuals. In this context, genotyping could be a screening test for oxidative stress and risk of metabolic syndrome in individuals with abdominal obesity.

However, even if large interindividual variability in serum PON1 activities (11) was reported within a genetic class and PON1 phenotype is a better predictor of disease (9), it would be wrong to consider only the SNPs in an epidemiological study.

We also noticed that within the two Q192R and L55M polymorphisms, RR and LL genotypes show the highest P-ase, as previously reported (19).

We failed to reveal any statistically significant direct association of obesity with L-ase, though, given the physiological function of PON1 and obesity representing one of the preceding stages of insulin resistance and indirectly of metabolic syndrome, we would have expected the obese condition to influence PON1 activity providing antioxidant protection. Our results are in contradiction with other reports that have found a direct association of obesity with L-ase (18), probably due to small number of study participants and presence of subjects with metabolic syndrome in the obese group. Under these conditions, some of the subjects could have shown significant changes in A-ase, as this activity is frequently associated with changes in oxidative stress, and therefore, the antioxidant protection (given by the lactonase function) has been reduced.

There are several limitations of this study: it is a cross-sectional study, with all the deriving consequences, since more information may be obtained by following the dynamics of PON1 status. We did not analyze the linkage disequilibrium and the cumulative effect between of the two polymorphisms. We did not exclude subjects with metabolic syndrome from the obese group and part of our observations may even be due to this condition. Smokers were not excluded from the study and smoking is known to decrease PON1 activity (21, 24). We did not analyze the influence of environmental factors on PON1 activity and such an analysis should be carried out in future studies. It also seems essential to further consider other *PON1* gene polymorphisms and to conduct a functional genomic analysis in order to define with greater accuracy the molecular mechanisms of regulation, the role and importance of this enzyme.

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The authors declare that they have no conflict of interest.

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