

Paraoxonase 1 activities and polymorphisms in autism spectrum disorders

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Abstract

Autism spectrum disorders (ASD) comprise a complex and heterogeneous group of conditions of unknown aetiology, characterized by significant disturbances in social, communicative and behavioural functioning. Recent studies suggested a possible implication of the high-density lipoprotein associated esterase/lactonase paraoxonase 1 (PON1) in ASD. In the present study, we aimed at investigating the PON1 status in a group of 50 children with ASD as compared to healthy age and sex matched control participants. We evaluated PON1 bioavailability (*i.e.* arylesterase activity) and catalytic activity (*i.e.* paraoxonase activity) in plasma using spectrophotometric methods and the two common polymorphisms in the *PON1* coding region (Q192R, L55M) by employing Light Cycler real-time PCR. We found that both PON1 arylesterase and PON1 paraoxonase activities were decreased in autistic patients (respectively, $P < 0.001$, $P < 0.05$), but no association with less active variants of the *PON1* gene was found. The PON1 phenotype, inferred from the two-dimensional enzyme analysis, had a similar distribution in the ASD group and the control group. In conclusion, both the bioavailability and the catalytic activity of PON1 are impaired in ASD, despite no association with the Q192R and L55M polymorphisms in the *PON1* gene and a normal distribution of the PON1 phenotype.

Keywords: autism • arylesterase • paraoxonase • polymorphisms

Introduction

Autism spectrum disorders (ASD), which include the prototypic autistic disorder, Asperger's syndrome and pervasive developmental disorders not otherwise specified (PDD-NOS), are a category of neurobehavioural conditions falling under the umbrella term of PDD. This severe and sustained group of disorders is characterized by qualitative impairments in reciprocal social interaction, impairments in verbal and nonverbal communication and a

pattern of repetitive, stereotypical behaviours and interests [1]. Although the neurobiological underpinnings of ASD are currently undeciphered, it is becoming increasingly clear that it is a systemic genetically influenced disorder with significant epigenetic and environmental contributors [2–4]. There is growing evidence suggesting that low-level chronic exposure to organophosphate (OP) compounds (*e.g.* insecticides and nerve agents) may affect neurodevelopment [5–7], and a recent study demonstrated that both prenatal and postnatal dialkylphosphate metabolites were associated with an increased risk for PDD at 24 months of age [8].

Human serum paraoxonase 1 (PON1), encoded by the *PON1* gene on chromosome 7q21.3, is a high-density lipoprotein (HDL)-associated esterase/lactonase that catalyses the hydrolysis of arylesters,

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toxic OP compounds, carbamates and lactones [9]. Although the physiological role is still uncertain, PON1 plays a role in protection against oxidative modification of low-density lipoproteins (LDL) [10, 11], homocysteine-thiolactone [12, 13] and bacterial endotoxins [14]. There is an impressive interindividual variation in PON1 activity and concentration [15]. Two common polymorphisms in the PON1 coding region, Gln→Arg (Q192R) and Leu→Met (L55M), have been described to contribute to this variability [16–18]. While phenylacetate hydrolytic activity (ARE.ase) is a reliable surrogate for serum PON1 bioavailability, PON1 catalytic efficiency can be evaluated by measuring the paraoxon rate of hydrolysis (PO.ase). The PO.ase activity is determined in part by the Q192R polymorphism: the Q form of PON1 is more efficient at hydrolyzing sarin and soman, whereas the R form more efficiently hydrolyzes paraoxon [19]. The second coding region polymorphism, PON1 L55M, does not affect catalytic activity, but may affect PON1 protein stability [20], and has been associated with plasma PON1 protein levels *via* an interaction with the C-108T promoter polymorphism [21–23]. Several recent studies have underlined the importance of determining both PON1 activities and functional alloform phenotypes as opposed to analysing any number of PON1 single nucleotide polymorphisms (SNPs), when inferring associations between PON1 and disease [15, 24, 25].

Recent evidence pointed to a possible implication of PON1 in ASD. D'Amelio and collaborators demonstrated that Caucasian-American, but not Italian families, display a significant association between autism and less active *PON1* gene variants [26], and Paşca *et al.* reported low ARE.ase activity in a cohort of children with autism [27]. The aim of the present study was to evaluate whether the measurement of arylesterase (ARE.ase) and NaCl stimulated paraoxonase (ssPO.ase) enzymatic activities of PON1, together with the assessment of PON1 Q192R and L55M polymorphisms might yield more information than either genotype or activity alone in a cohort of patients with ASD.

Material and methods

Participants

The participants enrolled in this study were 50 children with a diagnosis of ASD and 30 healthy children, balanced both with regard to age (respectively, 6.54 ± 0.48 years, 6.74 ± 0.50 years, $P = 0.76$) and sex (respectively, 78.0% males, 73.3% males, $P = 0.78$) (Table 1). For the genetic analysis, additional participants were recruited for the control group, with a total of 85 apparently healthy participants being genotyped. All participants in this study were Romanians. Each patient was examined by an experienced child neuropsychiatrist and assigned a diagnosis of autism (29 patients, 58%), PDD-NOS (17 patients, 34%) or Asperger syndrome (4 patients, 8%) based on the criteria defined in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Revised* (DSM-IVR) [1]. None of the patients followed any special diet (*i.e.* gluten free, casein free, high-dose vitamin supplementation). Comparison participants were drawn from the same geographical area as our patients, aiming to recover the same demographics for the control and patient groups. All the controls were somatically and behaviourally healthy, had no past or present history of neuropsychiatric disorders

and none of them had ever taken medications for psychiatric conditions. Informed consent was obtained and the research protocol was in agreement with the Declaration of Helsinki of the World Medical Association.

Blood samples

Blood specimens were obtained after overnight fast. Samples were withdrawn from a cubital vein into blood tubes and immediately stored on ice at 4°C. For the enzymatic determinations, plasma was separated by centrifugation at 3000 rpm for 10 min. and stored at –20°C until analysis.

Paraoxonase 1 activities

PON1 ssPO.ase and ARE.ase activities in heparinized plasma were measured spectrophotometrically according to Eckerson *et al.* [28], with minor modifications. Determination of ssPO.ase activity was performed by using paraoxon (O,O-diethyl-O-p-nitrophenyl phosphate, Sigma Chemical Co., Seelze, Germany). The basal assay mixture included 1.0 mM paraoxon, 2M NaCl and 1 mM CaCl₂ in 50 mM glycine-NaOH buffer (pH 10.5). The reaction was initiated by the addition of the plasma sample, and the absorbance was monitored at 405 nm for 90 sec. ARE.ase activity was measured using phenyl acetate (Sigma Chemical Co., Steinheim, Germany) 1 mM in 20 mM Tris-HCl (pH 8) containing 1 mM CaCl₂. The rate of phenyl acetate hydrolysis at 25°C was determined by monitoring the increase of absorbance at 270 nm over a 90 sec. period. A blank sample, containing incubation mixture without plasma, was run simultaneously to correct for spontaneous substrate breakdown, for both activity determinations. All samples were run in duplicate; the average value was used for activity calculation using a molar extinction coefficient of $18,290 \text{ M}^{-1} \text{ cm}^{-1}$ at 412 nm for p-nitrophenol, and $1310 \text{ M}^{-1} \text{ cm}^{-1}$ at 270 nm for phenol. Results are expressed as U/I for PON1 ssPO.ase activity (nanomole paraoxon hydrolyzed per minute), and kU/I for PON1 ARE.ase activity (micromole phenyl acetate hydrolyzed per minute).

Paraoxonase 1 genotyping

Genomic DNA was isolated from whole peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and was kept at –20°C until further determinations. Gene polymorphisms were determined using Light Cycler real-time PCR technology based on fluorescence resonance energy transfer. PON1 L55M and Q192R polymorphism analyses were performed according to the method proposed by Pocsai *et al.* [29], using Light Cycler™ high-speed thermal cycler (Hoffmann-LaRoche, Basel, Switzerland) and Light Cycler™ DNA Master HybProbe kit (Roche Molecular Biochemicals, Mannheim, Germany). Melting curve analysis allowed the identification of the PON1 L55M and Q192R polymorphisms according to the melting point temperatures: 61.0°C for M allele, 57°C for L allele, 57.5°C for Q allele and 51.5°C for R allele.

Statistical analysis

The data are presented as means \pm S.E.M. (standard error of the mean). The normal distribution of continuous data was checked using the Kolmogorov–Smirnov test. In parameters with normal distribution comparisons between the ASD group and their age and sex matched controls were

Table 1 Group composition by gender and age, PON1 enzymatic activities and PON1 polymorphisms distribution in ASD patients *versus* control participants

	ASD (<i>n</i> = 50)	Controls (<i>n</i> = 30)	<i>P</i>	
Age (years)	6.54 ± 0.48	6.74 ± 0.50	0.769	
Sex: male, <i>n</i> %	39 (78.0%)	22 (73.3%)	0.787	
Paraoxonase 1				
Arylesterase activity (kU/l)	69.87 ± 2.04	85.05 ± 3.32	<0.001	
Paraoxonase activity (U/l)	422.72 ± 36.18	610.79 ± 69.19	0.021	
	ASD (<i>n</i> = 50)	Controls (<i>n</i> = 85)	χ^2	<i>P</i>
PON1 Q192R				
QQ	26 (52.0%)	43 (50.6%)	0.02	0.98
QR	21 (42.0%)	37 (43.5%)		
RR	3 (6%)	5 (5.9%)		
Q/R	0.73/0.27	0.72/0.28	0.01	0.90
PON1 L55M				
LL	15 (30.0%)	31 (36.5%)	1.13	0.56
LM	30 (60.0%)	43 (50.6%)		
MM	5 (10.0%)	11 (12.9%)		
L/M	0.60/0.40	0.62/0.38	0.08	0.77

All values are the mean ± S.E.M.

made using Student's *t*-test. When normality was not present and equal variance could not be assumed, the Mann-Whitney rank-sum test was applied. The effect size (Cohen's *d* based on sample size) for variables with a significant group mean difference was computed. For the polymorphisms under study, genotypic and allelic frequencies were calculated. The distribution of genotypes in all groups was tested for deviation from Hardy-Weinberg equilibrium. The Pearson's chi-square (χ^2) test and Fisher's exact test were applied to assess differences in the genotype and allelic (respectively) distributions between groups of patients and controls. Linkage disequilibrium (LD) was assessed using the THESIAS software, version 3.1 [30]. The multiple regression analysis for the ssPO.ase activity and ARE.ase was conducted using five independent variables (age, sex, PON1 L55M, PON1 Q192R and group). The linear regression analysis (performed on the square-root transformed data) was performed in order to check for the influence of the two polymorphisms in the *PON1* gene on ARE.ase and ssPO.ase. A *P*-value of less than 0.05 was considered statistically significant. SPSS software (version 15.0) was used for data analysis.

Results

We evaluated the activity of PON1 using as substrates both phenylacetate (ARE.ase activity) and paraoxon (ssPO.ase activity) in

50 children with ASD and 30 age and sex matched healthy individuals. As shown in Table 1, both ARE.ase (69.87 ± 2.04 kU/l *versus* 85.05 ± 3.32 kU/l, *P* < 0.001) and ssPO.ase (422.72 ± 36.18 U/L *versus* 610.79 ± 69.19 U/L, *P* < 0.05) activities were significantly lower in the patient group than in the control group; the effect size (Cohen's *d* based on sample size) was 0.92 for ARE.ase and 0.57 for ssPO.ase.

We investigated the PON1 genotypes distributions as a possible explanation for our observation of decreased PON1 function. We found that the distribution of SNPs is unaffected. Allele and genotype frequencies for the ASD group and extended control group (*n* = 85) are summarized in Table 1. Genotype distributions were at or near Hardy-Weinberg equilibrium for ASD patients and controls (Fisher exact test, *P* = 0.20 and 0.80 for the L55M and 0.41 and 0.40 for the Q192R polymorphisms in patients and controls, respectively). We found that PON1 L55M and PON1 Q192R genotype and allele distribution was similar in patients and controls (Table 1). Neither were there any differences in the frequencies of combined haplotypes (QM: QL: RL: RM was 0.38: 0.35: 0.25: 0.01 in patients and 0.37: 0.35: 0.26: 0.01 in controls; χ^2 = 0.31, *P* = 0.95). LD between markers was estimated by both $|D'|$ calculation and determination of the associated *P*-value. A strong LD between

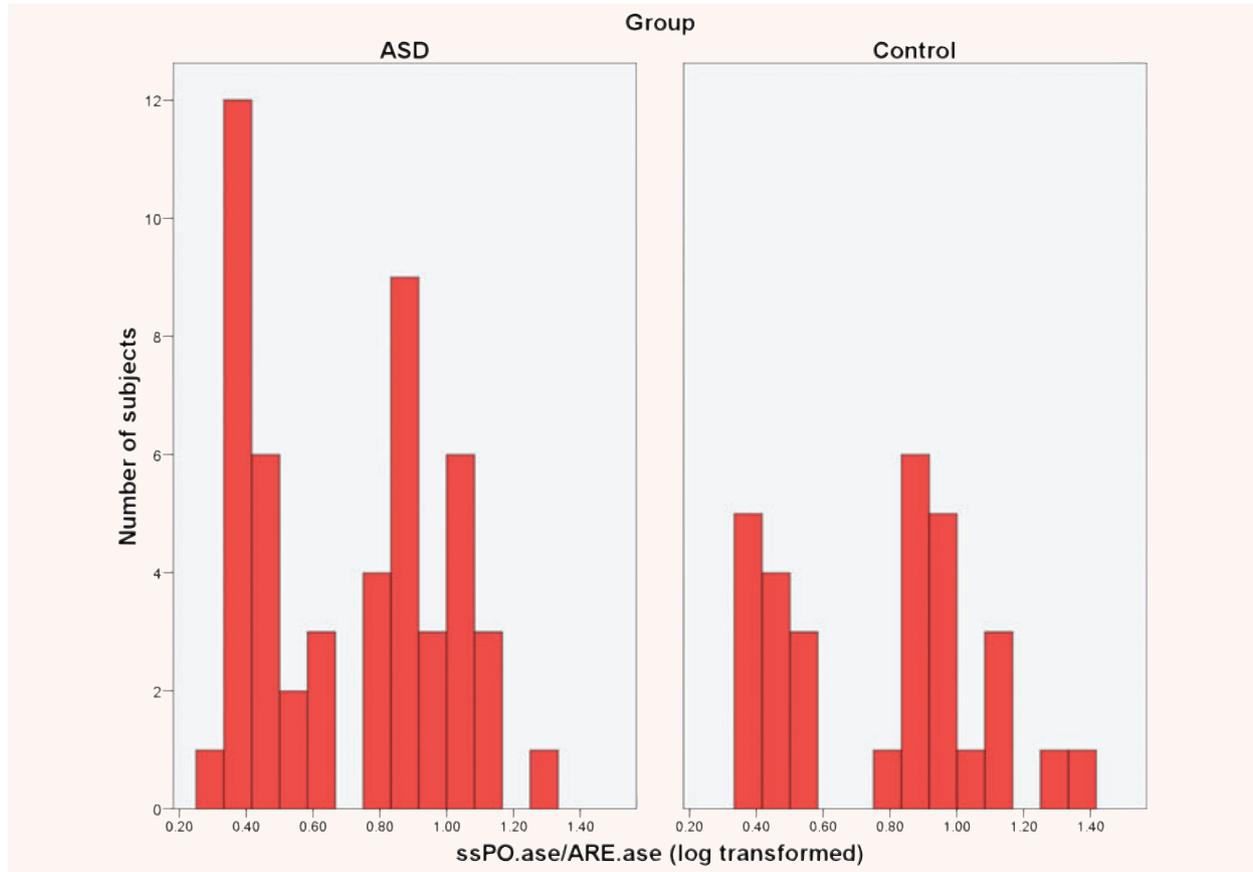


Fig. 1 Histogram of the PON1 phenotype distribution in the ASD and control groups. The PON1 phenotype was determined by computing the ratio of ssPO.ase to ARE.ase activities (double-substrate method applied on the logarithm-transformed data). The discriminative values, which separate the low-activity phenotype AA and the high-activity phenotype BB from the intermediate AB phenotype, were found as 0.7 and 1.2, respectively. The phenotype frequencies were similar in patients *versus* control participants ($P > 0.05$).

the R allele of the PON1 192 polymorphism and the L allele of the PON1 55 polymorphism was observed in both ASD participants ($D' = 0.84$, $P < 0.001$) and controls ($D' = 0.90$, $P < 0.001$).

Multiple regression analysis showed that ssPO.ase activity was independently affected (adjusted $R^2 = 0.42$, $F_{5, 74} = 12.69$, $P < 0.001$) by the factors of PON1 Q192R ($\beta = 0.57$, $P < 0.001$) and group ($\beta = -0.23$, $P = 0.008$), whereas the ARE.ase activity was affected (adjusted $R^2 = 0.20$, $F_{5, 74} = 5.04$, $P < 0.001$) by the factors of PON1 L55M ($\beta = -0.30$, $P = 0.008$) and group ($\beta = -0.39$, $P < 0.001$).

The linear regression analysis (performed on the square-root transformed data) showed that the PON1 Q192R polymorphism explained 49.9% of ssPO.ase activity variance in ASD patients (adjusted $R^2 = 0.499$, $P < 0.001$), and 25.9% of the variance of the ssPO.ase activity in the control group (adjusted $R^2 = 0.259$, $P < 0.001$). On the other hand, the PON1 L55M polymorphism did not influence the ARE.ase activity in the ASD group (adjusted $R^2 = 0.035$, $P = 0.102$), but it showed a borderline influence in the control group (adjusted $R^2 = 0.085$, $P = 0.065$).

The ratio of ssPO.ase to ARE.ase activities (double-substrate method applied on the logarithm-transformed data) was used to identify individual phenotypes according to Eckerson *et al.* [31]. PON1 ratio was trimodally distributed on the histograms in both groups, and the discriminative values, which separate the low-activity phenotype AA and the high-activity phenotype BB from the intermediate AB phenotype, were found as 0.7 and 1.2, respectively (Fig. 1). The AA : AB : BB phenotype frequencies obtained were similar in the ASD group and the control group (respectively, 0.480 : 0.480 : 0.040, 0.400 : 0.533 : 0.067; $\chi^2 = 0.64$, $P = 0.72$).

Discussions

Recent investigations indicated a putative involvement of the HDL-associated esterase/lactonase PON1 in the pathogenesis of autism by demonstrating low ARE.ase activity in ASD [27], and by showing

an association with less active *PON1* gene variants in autistics from North America [26]. In the current study, we aimed at investigating, in a representative cohort of Romanian children with ASD, both *PON1* catalytic activity and bioavailability (*i.e.* ssPO.ase and ARE.ase) and the two most common polymorphisms in the *PON1* gene (Q192R and L55M).

The present report does not lend support to either the L55M or the Q192R polymorphism or combined haplotypes of the *PON1* gene being associated with ASD in Romania, which is congruent with the lack of association reported in another European autistic group [26]. A previous study [32], investigating the transmission disequilibrium of the *PON1* L55M polymorphism in 196 trios, did not demonstrate any association with autism. In our sample, the Q and the L alleles were predominant, and the previously described strong LD between *PON1* codon 55 and *PON1* codon 192 was observed [33]. Although there are no available data on the *PON1* gene variants distribution in Romania, the observed frequencies are similar to those described for other European populations [34–37].

Because wide interindividual variability in plasma *PON1* levels within a genetic class has been reported and the *PON1* phenotype is a better predictor of disease [24], one should not be misled by SNPs analysis alone in an epidemiological study. Indeed, we provide evidence that both *PON1* enzyme activities are significantly decreased in ASD patients compared with healthy individuals, irrespective of the polymorphisms distribution. In a previous report, we observed a decreased ARE.ase activity in a cohort of autistic children [27], but ssPO.ase activity failed to reach significance; most probably because of the high variance seen in this activity and the small population sample.

Our current findings showed that the participants' group (ASD *versus* control) influenced both activities, but no influence of age or sex was detected. The multiple regression analysis performed in our participants also indicated that, as previously demonstrated in a large cross-sectional investigation [38], the major determinant of the *PON1* catalytic activity (PO.ase) was the Q192R genotype, whereas *PON1* bioavailability (ARE.ase) was affected only by the L55M genotype. Interestingly, in the ASD group, the ssPO.ase/Q192R dependency was more pronounced than in controls. On the other hand, the dependency of the bioavailability of *PON1* (ARE.ase) on the L55M polymorphism was absent in the ASD patients, whereas it reached a borderline small level in healthy participants.

The phenotype distribution, computed by graphically examining the ssPO.ase/ARE.ase ratio, was analogous in ASD and controls. The similar phenotypic distribution and the fact that overall there was no difference in the ssPO.ase/ARE.ase ratio between groups suggest that the relationship between the two activities is generally maintained in autistics, regardless of a reduction in the hydrolytic protection.

In recent years, great concern has been raised about the negative impact of chronic low OP exposure on the nervous system development. Prospective studies recorded significant decreases in birth weight, head circumference and slower reflexes in prenatally exposed infants [5, 39, 40]. Developmental disorders in

children, including atypical autism, have been described among farming families who generally use OP [7]. In the same line of evidence, it has recently been reported an adverse association of OP pesticide exposure, as measured by six non-specific dialkylphosphate metabolites, with mental development and pervasive developmental problems at 24 months of age [8]. Alternatively, postnatal exposure to chlorpyrifos, a well-known neurotoxic OP, reduced the expression of nerve growth factor and reelin (RELN) in the forebrain of rats [41].

Studies of the *PON1* enzyme in newborns showed that its levels are on average three to fourfold lower than those of adults [42], suggesting that newborn children and infants will be more susceptible to OP compounds. Taking maternal *PON1* status into consideration greatly increases the risk for this adverse behaviour [6]. Because the dose–response relationship for OP toxicity are steep [43], a small percentage difference in *PON1* hydrolytic rates should account for large differences in toxicity [19].

The low levels of *PON1* activity in our ASD cohort are congruent with the recently proposed model of gene–environment interactions in ASD [3], which involves *PON1*, prenatal exposure to OP and RELN, a large extracellular serine protease that orchestrates neuronal positioning during corticogenesis. Within this framework, an autistic phenotype could arise in genetically or epigenetically vulnerable individuals producing lower amounts of RELN that are exposed to OP during critical neurodevelopmental periods and in which neuronal migration would be affected to a different extent depending on the enzymatic efficacy of *PON1* [44].

The implications of a low level of *PON1* activity for the pathogenesis of ASD can also be translated to other factors than just the OP toxicity. *PON1* displays a peroxidase-like activity [45], being involved in the protection against oxidative stress [46]. Several studies suggest increased oxidative stress in autism by showing alterations in antioxidant enzymes (*e.g.* superoxide dismutase, glutathione peroxidase, catalase), increased lipid peroxidation, disturbances in transsulfuration and altered glutathione levels [47–49]. In this context, a reduction in the *PON1* activity level could be one of the contributing factors to the redox alterations in autism. *PON1* is also able to protect cells against the toxicity of bacterial endotoxins, lipopolysaccharides (LPS), and prevent or greatly reduce the release of cytokines [14]. Recent evidence suggests that ASD may be accompanied by aberrant immune responses, and peripheral blood mononuclear cells from ASD children produce excessive proinflammatory cytokines with a suboptimal dose of endotoxin (LPS) at high frequency [50, 51]. Therefore, low levels of *PON1* activity in children with ASD might contribute to this exuberant cytokine response and to the subsequent disturbances in the cellular immunity. Previous studies have showed a link between *PON1* alleles that debilitate its activity with Alzheimer's dementia [52] and Parkinson's disease [53], indicating an impact on the cholinergic system. Therefore, an altered *PON1* status could also be translated at the level of the previously described alterations in the cholinergic system in autism [54].

Our study does not bring any direct explanation for the low *PON1* activity in ASD. The mechanisms behind the observed decrease in the level of *PON1* activity are yet to be investigated and

here we can only mention a few hypotheses. Some inhibitors of the enzymatic activity of PON1 in children with autism or factors influencing liver *PON1* gene expression could be involved. PON1 could also be inactivated under oxidative stress [55] or following pesticide exposure [56]. PON1 can also undergo S-glutathionylation with a consequent reversible inactivation [57]. The enzymatic activity of PON1 is also modulated by subtle dietary factors, as well as other polymorphisms in the *PON1* gene [9]. A lessening in PON1 activities in ASD, especially in the PON1 bioavailability, could also be a consequence of a dysfunction of liver conjugation capacity or an altered synthesis and/or secretion of HDL [58]. The fact that neonatal PON1 activity is decreased in infants from mothers having a chronic viral hepatic disease [59] raises also the possibility of an influence of mother's liver status and/or infection. PON1 has a potentially imprinted expression in mouse placenta [60], which, if confirmed in human beings, could have implications for the imprinting hypothesis for the development of autism [61].

There are a few limitations in our study. First, it is a retrospective study and more information could be gained by following the PON1 status dynamics in children who develop ASD. Second, we did not obtain more accurate clinical phenotypic descriptions in order to balance our patient group for the heterogeneity of ASD [62]. Third, we did not collect data on OP exposure in our participants.

On the other hand, because many of the developmental brain abnormalities associated with ASD arise during the first two semester of the pregnancy, environmental factors are more likely to play a role in the pathophysiology of ASD *via* maternal factors, and consequently, it would seem essential to look, in future studies, for an effect of mother's PON1 activity in children with ASD.

Conclusions

Both PON1 bioavailability and catalytic activity are impaired in children with ASD. There is a normal distribution of PON1 phenotype and no association with the two most common polymorphisms in the coding region of the gene.

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