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ORIGINAL RESEARCH ARTICLE

Paraoxonase gene variants are associated with autism in North America, but not in Italy: possible regional specificity in gene–environment interactions

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Organophosphates (OPs) are routinely used as pesticides in agriculture and as insecticides within the household. Our prior work on Reelin and APOE delineated a gene–environment interactive model of autism pathogenesis, whereby genetically vulnerable individuals prenatally exposed to OPs during critical periods in neurodevelopment could undergo altered neuronal migration, resulting in an autistic syndrome. Since household use of OPs is far greater in the USA than in Italy, this model was predicted to hold validity in North America, but not in Europe. Here, we indirectly test this hypothesis by assessing linkage/association between autism and variants of the paraoxonase gene (PON1) encoding paraoxonase, the enzyme responsible for OP detoxification. Three functional single nucleotide polymorphisms, PON1 C−108T, L55M, and Q192R, were assessed in 177 Italian and 107 Caucasian-American complete trios with primary autistic probands. As predicted, Caucasian-American and not Italian families display a significant association between autism and PON1 variants less active *in vitro* on the OP diazinon (R192), according to case–control contrasts (Q192R: $\chi^2 = 6.33$, 1 df, $P < 0.025$), transmission/disequilibrium tests (Q192R: TDT $\chi^2 = 5.26$, 1 df, $P < 0.025$), family-based association tests (Q192R and L55M: FBAT $Z = 2.291$ and 2.435 respectively, $P < 0.025$), and haplotype-based association tests (L55/R192: HBAT $Z = 2.430$, $P < 0.025$). These results are consistent with our model and provide further support for the hypothesis that concurrent genetic vulnerability and environmental OP exposure may possibly contribute to autism pathogenesis in a sizable subgroup of North American individuals.
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Introduction

Autistic disorder[1] (MIM 209850) is a severe neuropsychiatric disorder, with an incidence that has apparently risen during the last two decades from 2–5 to 15–20/10 000 children. Strong genetic contributions to autism pathogenesis have received support from family and twin studies.[2] Despite initial suggestions of a 'simple' genetic predisposition conferred by a relatively small number of major genes, an unexpected degree of complexity has later been acknowledged, with numerous contributing loci, broad interindividual genetic heterogeneity, epistasis, and possibly gene–environment interactions.[3–6]

A prenatal timing for neurodevelopmental alterations ultimately leading to autism, dating possibly to as early as the first trimester of pregnancy, is most compatible with the cytoarchitectonic abnormalities found in *post-mortem* studies of brains of individuals

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with autism.[7,8] Furthermore, neonates later diagnosed with autism display elevated plasma levels of brain-derived neurotrophic factor and NT4 already at birth,[9] as well as fine motor abnormalities.[10] Genetic and environmental factors, acting either alone or in combination, could thus contribute to autism pathogenesis by interfering with prenatal neurodevelopmental processes.

Searching for variants conferring vulnerability to autism in neurodevelopmentally relevant genes, we initially described an association with 'long' alleles of a polymorphic GGC repeat located in the 5′ untranslated region (UTR) of the RELN gene, which encodes Reelin, a pivotal protein for neuronal migration during neurodevelopment.[4,11] We also found that long GGC alleles are functional, resulting in significantly reduced gene expression both *in vivo*[12] and *in vitro* (Persico *et al*, submitted). In addition to binding to a variety of receptors, including the VLDL receptor, APOE-R2, and $\alpha 3\beta 1$ integrins, Reelin exerts proteolytic activity on extracellular matrix proteins, a process critical for neuronal migration.[11,13] As with all serine proteases, this enzymatic activity is specifically and potently inhibited by organophosphates (OPs),[13] compounds routinely used as pesticides in agriculture and insecticides within the household.[14] The agricultural use of OPs is equally intensive in the USA and in Italy, whereas household use by home owners and exterminators is largely more widespread and intensive in North America than in Europe (see Discussion).[14–18] We therefore hypothesized that a subgroup of genetically vulnerable individuals characterized by decreased Reelin gene expression, if exposed prenatally to OPs during critical periods in neurodevelopment, could undergo altered neuronal migration resulting in an autism spectrum disorder,[6] and that this pathogenetic pathway should be more likely to occur in North American than in European households.

Several lines of evidence have since provided further support to this model: (1) the association between long RELN alleles and autism, almost entirely carried by Caucasian-American and not by Italian families in our initial study,[4] was independently replicated in two out of three studies assessing also simplex families in North America,[19–21] whereas no replication has been reported in European samples recruited in France, the UK, and Germany,[22,23] or in one US-based study assessing multiplex families only;[24] (2) a recent post-mortem study confirms and extends previous findings of decreased Reelin gene expression in autism,[11] reporting coincident changes in Reelin, Dab-1, and VLDL receptor gene expression in frontal cerebral and cerebellar cortices of brains from individuals with autism compared to age-, sex-, and post-mortem interval-matched controls;[25] (3) several epidemiological studies have recently found evidence of prenatal exposure to OP compounds in the USA, particularly for diazinon and chlorphyrifos, which have been most widespread in household use (see Discussion).

This model based on gene–environment interactions limited to specific geographical regions is tested in the present study from yet another prospective, namely by assessing the distribution of three functional single nucleotide polymorphisms (SNPs) (C−108T, L55M, and Q192R) located in the promoter and coding sequence of the paraoxonase gene (PON1) in human chromosome 7q21.3.[26,27] Paraoxonase is the serum enzyme physiologically involved in protecting low-density lipoproteins (LDL) and high-density lipoproteins (HDL) from oxidation, and is responsible for OP inactivation in humans.[28,29] The three SNPs assessed here are the best-characterized functional polymorphisms affecting either the amount of serum paraoxonase or its affinity for specific substrates, ultimately leading to impressive interindividual differences in human serum paraoxonase activity.[26–29] In particular, T−108 is associated with approximately 50% reduction in paraoxonase serum levels compared to C−108;[27] L/M55 alleles only marginally affect PON1 gene expression, but are in tight linkage disequilibrium (LD) with C/T−108 and Q/R192 alleles, respectively;[26,28,29] Q192 displays reduced affinity for chlorpyrifos, while R192 is significantly less active on diazinon *in vitro*.[26–29] This hypothesis-driven study therefore tests whether (a) Caucasian-American, and not Italian, families show a significant association between PON1 alleles and autism; (b) PON1 alleles associated with autism are those conferring reduced protection against OP exposure, by yielding either lower amounts of serum paraoxonase (T−108/M55) or possibly decreased affinity for diazinon (R192) or chlorphyrifos (Q192); and (c) there is evidence for potential gene–gene interactions between PON1 and RELN gene variants in autism pathogenesis.

Subjects and methods

Subjects

This study involved 312 autistic patients and 676 first-degree relatives, belonging to 177 Italian families and 107 Caucasian-American families. The latter group also includes 15 simplex and 23 multiplex families recruited by the AGRE consortium.[30] Table 1 summarizes the composition of the sample by recruitment center, including the number of simplex and multiplex families, as well as the number of complete trios (ie including both parents and the affected child) and incomplete trios (ie mother and one affected child). Demographic and clinical characteristics, as well as inclusion criteria, and diagnostic screening methods have been previously reported in detail.[31] All parents gave written informed consent for themselves and for their children, using the consent form approved by the IRB of UCBM (Rome, Italy).

We also assessed 180 unaffected Italian controls, including 166 individuals whose blood was drawn at the Laboratory of the 'S Cuore' Clinic (Rome, Italy), as prescribed by family practitioners for a broad range of

Table 1 Composition of the Italian and Caucasian-American samples of families with a primary autistic proband, assessed in this study

Site	*Number of individuals with autism*	*Number of families*		*Number of trios*	
		Simplex	*Multiplex*	*Complete*	*Incomplete*
II University of Naples (Naples, Italy)	71	71	—	61	10
IRCCS 'Ospedale Bambino-Gesù' (Rome, Italy)	42	42	—	38	4
IRCCS 'Oasi Maria SS' (Troina, Italy)	42	42	—	41	1
UCBM (Rome, Italy)	22	22	—	22	—
Italian families	*177*	*177*	—	*162*	*15*
AGRE Consortium	60	15	23[a]	38	—
Southwest Autism Research Center (Phoenix, AZ)	44	34	4[b]	38	—
University of Iowa College of Medicine (Iowa City, IA)	31	31	—	31	—
Caucasian-American families	*135*	*80*	*27*	*107*	—
Total sample	*312*	*257*	*27*	*269*	*15*

[a]DNA was not available for one of the two affected children from one multiplex family from the AGRE Consortium. This family is still listed as 'multiplex' in the table.
[b]All multiplex families include two affected children, except for one quadruplet family recruited in Phoenix, AZ, USA.

physical complaints unrelated to psychiatric disorders, and 14 medical and nursing students recruited at UCBM (Rome, Italy).

An additional sample of 252 individuals with autism studied by the ASD-CARC and the Autism Research Program (Ongwanada, Kingston, ON, Canada), and previously genotyped at the RELN locus,[19] was genotyped only at the PON1 Q192R SNP, to complete the assessment of gene–gene interactions between the PON1 and RELN loci (see below).

Genotyping

The PON1 C−108T, L55M, and Q192R SNPs were each genotyped separately by PCR amplification and restriction digest, as described.[26,27] Briefly, (a) the C−108T SNP was amplified using primers GACC GCAAGCCACGCCTTCTGTGCACC and TGCAGCCG CAGCCCTGCTGGGGCAGCGCCGATTGGCCCGCCGC with 5% DMSO and an annealing temperature of 63°C for 35 cycles. The 109 bp fragment was digested with *Bst*UI, yielding 67 and 42 bp fragments in the presence of the C allele. (b) The L55M SNP was amplified with primers GAGTGATGTATAGCCCCAG TTTC and AGTCCATTAGGCAGTATCTCCG and an annealing temperature of 55°C for 40 cycles. The 144 bp fragment was digested using *Hin*fI, producing two 122 and 22 bp fragments in the presence of the L allele. (c) The Q192R SNP was amplified using primers TATTGTTGCTGTGGGACCTGAG and CACG CTAAACCCAAATACATCTC at an annealing temperature of 60°C for 35 cycles. The 99 bp fragment was digested with *Alw*I, yielding 66 and 33 bp fragments with the R allele.

Mutational analysis by DHPLC and DNA sequencing

The nine exons of the PON1 locus,[32] their flanking intronic sequences, and the 5′ and 3′ UTR were PCR amplified from the genomic DNA of 58 individuals with autism to yield 226–445 bp fragments, which were assessed by denaturing high-performance liquid chromatography (DHPLC)[33] using the Transgenomic Wave rapid DNA analysis system (Transgenomic Inc., Omaha, NE, USA). The acetonitrile gradient was formed mixing Buffer A (0.1 mM TEAA) and Buffer B (0.1 mM TEAA, 25% acetonitrile) at a flow rate of 1.5 ml/min, and increasing Buffer B by 5% per minute over 2 min. Primer sequences, PCR conditions, and DHPLC temperatures are available from the authors upon request. PCR products yielding chromatographic variations were sequenced using a 3100 Genetic Analyser (PE Applied Biosystems, Foster City, CA, USA).

Serotonin blood levels

Blood samples were centrifuged for 25 min at 4°C and 140 *g* within 20 min of venipuncture; 1 ml of supernatant (ie platelet-rich plasma) was stored at −80°C and assessed by HPLC, as described.[34]

Urinary peptide excretion rates

Urinary peptide excretion analysis was performed by HPLC on the first morning urine samples of all family

members, as described.[35] The total area of peaks under the 215 nm absorption curve (AUC) in the peptide region following the hippuric acid peak was calculated and expressed in μm^2.

Statistical analyses

Hardy–Weinberg analyses were performed using the HWE program (available at http://linkage.rockfeller.edu/ott/linkutil.htm). Case–control allelic and genotypic distributions were contrasted using the χ^2 statistics, following randomized selection of one patient per multiplex family. Family-based linkage/association analyses were performed applying the transmission/disequilibrium test (TDT), where preferential allelic transmission from heterozygous parents to affected offspring is tested by applying the $(b-c)^2/(b+c)$ statistics and the χ^2 ('McNemar test').[36] TDT analyses were performed only on complete trios and using one trio per multiplex family, in order to avoid introducing a bias by reconstructing missing parental genotypes or by assuming sampling independence in multiplex families.[37] To overcome this limitation, family-based association tests were also performed using the FBAT program.[38] By emphasizing contrasts between siblings, FBAT uses the full potential of intrafamilial genetic information particularly in multiplex families, and is therefore more powerful than the TDT.

Haplotype and LD were estimated from pedigree data with the HBAT program[39] and from unrelated individuals using the 3LOCUS program, determining statistical significance for the G test statistic by comparison with a simulated null distribution derived from 1000 replications.[40] Gene–gene interactions between the PON1 and RELN loci were assessed by logistic regression to test for the independence of allelic segregation, and using Fisher's exact test on genotype distributions, following genotype dicotomization by the presence/absence of PON1 R192 and of 'long' RELN alleles.

Data are expressed as mean ± SEM, except for head circumference and urinary peptide excretion rates, expressed as median percentile ± semi-interquartilic range (semi-IQR). Unless otherwise specified, this study employs conservative two-tail *P*-values with statistical significance set at $P<0.025$ to account for parallel analyses of two distinct ethnic groups (Italians and Caucasian-Americans), with no further correction for the number of SNPs, which are in tight LD (see Results).

Results

Case–control association study

Case–control association analyses for single markers are reported in Table 2. Caucasian-Americans, but not Italian patients, differed significantly from ethnically matched controls in allelic distributions at the Q192R SNP ($\chi^2=6.33$, 1 df, $P<0.025$), with genotypic distributions reaching a nonsignificant nominal $P=0.032$. The R allele was associated with autism

Table 2 Genotypic and allelic distributions of PON1 SNPs in unrelated Italian and Caucasian-American individuals with autism and ethnically matched controls

C−108T	*Italian patients (N=177)*	*Italian controls (N=180)*	*American patients (N=107)*	*American controls (N=376)*		*Italian patients (N=354)*	*Italian controls (N=360)*	*American patients (N=214)*	*American controls (N=752)*
C/C	38 (21.5%)	34 (18.9%)	20 (18.7%)	94 (25.0%)	C	175 (0.4944)	166 (0.4611)	99 (0.4626)	376 (0.5000)
C/T	99 (55.9%)	98 (54.4%)	59 (55.1%)	188 (50.0%)	T	179 (0.5056)	194 (0.5389)	115 (0.5374)	376 (0.5000)
T/T	40 (22.6%)	48 (26.7%)	28 (26.2%)	94 (25.0%)					
	$\chi^2=0.93$, 2 df, $P=0.63$, n.s.		$\chi^2=1.88$, 2 df, $P=0.39$, n.s.			$\chi^2=0.79$, 1 df, $P=0.37$, n.s.		$\chi^2=0.93$, 1 df, $P=0.33$, n.s.	
L55M	*Italian patients (N=170)*	*Italian controls (N=180)*	*American patients (N=106)*	*American controls (N=376)*		*Italian patients (N=340)*	*Italian controls (N=360)*	*American patients (N=212)*	*American controls (N=752)*
L/L	64 (37.6%)	62 (34.5%)	43 (40.6%)	147 (39.1%)	L	216 (0.6353)	209 (0.5806)	140 (0.6604)	479 (0.6370)
L/M	88 (51.8%)	85 (47.2%)	54 (50.9%)	185 (49.2%)	M	124 (0.3647)	151 (0.4194)	72 (0.3396)	273 (0.3630)
M/M	18 (10.6%)	33 (18.3%)	9 (8.5%)	44 (11.7%)					
	$\chi^2=4.21$, 2 df, $P=0.12$, n.s.		$\chi^2=0.87$, 2 df, $P=0.64$, n.s.			$\chi^2=2.20$, 1 df, $P=0.14$, n.s.		$\chi^2=0.39$, 1 df, $P=0.53$, n.s.	
Q192R	*Italian patients (N=174)*	*Italian controls (N=180)*	*American patients (N=107)*	*American controls (N=376)*		*Italian patients (N=348)*	*Italian controls (N=360)*	*American patients (N=214)*	*American controls (N=752)*
Q/Q	82 (47.1%)	97 (53.9%)	41 (38.3%)	195 (51.9%)	Q	248 (0.7126)	266 (0.7389)	137 (0.6402)	548 (0.7287)
Q/R	84 (48.3%)	72 (40.0%)	55 (51.4%)	158 (42.0%)	R	100 (0.2873)	94 (0.2611)	77 (0.3598)	204 (0.2713)
R/R	8 (4.6%)	11 (6.1%)	11 (10.3%)	23 (6.1%)					
	$\chi^2=2.55$, 2 df, $P=0.27$, n.s.		$\chi^2=6.84$, 2 df, $P=0.032$, n.s.			$\chi^2=0.61$, 1 df, $P=0.43$, n.s.		**$\chi^2=6.33$, 1 df, $P<0.025$**	

Caucasian-American controls are from Brophy *et al*.[27]
Statistically significant differences are displayed in bold.

in the Caucasian-American sample, where the presence of one or two R alleles conferred an odds ratio for affected status of 1.73 (CI 1.12–2.69) (Table 2).

Male and female controls did not differ in genotype, allele, or haplotype distributions (data not shown), excluding a potential stratification bias introduced by sex, as males tend to be over-represented in autistic patient samples (M : F ratio = 7.86 and 1.08 in Italian patients and controls, respectively). Italian and Caucasian-American fathers, mothers, patients, unaffected siblings, and controls each displayed no significant deviation from Hardy–Weinberg equilibrium, except for Italian mothers at C−108T ($\chi^2 = 9.510$, 1 df, $P < 0.01$) and Italian patients at Q192R ($\chi^2 = 5.557$, 1 df, $P < 0.025$), both showing an excess of heterozygous individuals whose genotypes were confirmed.

Family-based linkage/association study

Positive case–control associations can potentially stem from ethnically biased sampling or from chance. We thus performed TDT analyses on 177 Italian and 107 Caucasian-American complete trios, confirming the preferential transmission of PON1 R192 alleles from heterozygous Caucasian-American, but not Italian, parents to affected offspring, with PON1 T−108 and L55 alleles displaying a nonsignificant trend (Table 3). We found no evidence for parent-of-origin effects (maternal transmissions: −108C/T = 16/24, 55L/M = 23/17, 192Q/R = 12/23; paternal transmissions: −108C/T = 19/22, 55L/M = 22/12, 192Q/R = 11/22), and no transmission disequilibrium from heterozygous Caucasian-American parents to unaffected offspring (transmissions: Q/R = 23/27, TDT $\chi^2 = 0.32$, $P = 0.57$, n.s.).

FBAT analyses confirmed an association between PON1 alleles and autism in Caucasian-American, but not Italian, families (Table 4). The enhanced power of FBAT yielded a statistically significant association with the Q192R and L55M markers in Caucasian-American families ($P < 0.025$). No association was found in Italian families or in the unaffected siblings from the same Caucasian-American families (Table 4). Haplotype analyses employing these two markers confirmed the preferential transmission of chromosomes carrying the L55 and R192 alleles to Caucasian-American affected offspring (Table 5).

Caucasian-American, but not Italian, patients carrying at least one copy of the R192 allele seemingly display a peculiar biochemical and morphological phenotype, including significantly lower serotonin (5-HT) blood levels, a trend toward lower urinary peptide excretion rates, and more homogeneously enlarged head circumference (see semi-IQR in Table 6).

Linkage disequilibrium at the PON1 locus

LD at the PON1 locus was assessed for Caucasian-Americans and Italians, to determine whether differences in LD structure could explain the presence of genetic association only in one of the two ethnic groups. Assessments of both unrelated individuals and pedigrees provided converging estimates of total normalized LD coefficients (D'), which are summarized in Table 7. Our Italian controls, Italian patients, and Caucasian-American patients all display essentially superimposable LD structures, with highly significant D' coefficients between the C−108T and L55M, and the L55M and Q192R SNPs, and relatively little LD between C−108T and Q192R.

Mutational analysis by DHPLC

In addition to known polymorphisms, a mutational screening of the nine exons flanking intronic sequences and 5′/3′ UTRs in 58 autistic patients unveiled one novel missense mutation (A112G), resulting in an amino-acid change (N19D). This mutation was found in one patient from an Italian nuclear family, and in another patient from a Caucasian-American multiplex family, where the other brother with autism and an unaffected sister were also heterozygous carriers. The mutation was paternally transmitted from a heterozygous father in both families. No N19D mutations were found in 160 chromosomes from 80 Italian controls.

Table 3 TDT of PON1 C−108T, L55M, and Q192R alleles, performed on complete trios and including a single trio per multiplex family

Markers	*Italian families*		*Caucasian-American families*	
PON −108	*N = 176 complete trios*		*N = 107 complete trios*	
C transmitted	95	$\chi^2 = 0.086$ (1df), $P = 0.77$, n.s.	51	$\chi^2 = 1.071$ (1df), $P = 0.30$, n.s.
T transmitted	91		62	
PON 55	*N = 155 complete trios*		*N = 104 complete trios*	
L transmitted	85	$\chi^2 = 0.006$ (1 df), $P = 0.94$, n.s.	56	$\chi^2 = 2.67$ (1 df), $P = 0.10$, n.s
M transmitted	84		40	
PON 192	*N = 175 complete trios*		*N = 107 complete trios*	
Q transmitted	70	$\chi^2 = 0.007$ (1df), $P = 0.93$, n.s.	35	**$\chi^2 = 5.26$ (1df), $P < 0.025$**
R transmitted	69		57	

Statistically significant differences are displayed in bold.

Table 4 Family-based association tests of PON1 C−108T, L55M, and Q192R alleles, under an additive model[38]

Marker	*Allele*	*No. of families*	*S*	*E(S)*	*Var(S)*	*Z*	*P*
Caucasian-American patients							
PON1 C−108T	C	82	100.000	101.833	34.972	−0.310	0.756551
	T	82	108.000	106.167	34.972	0.310	
PON1 L55M	L	74	116.000	103.000	28.500	2.435	**0.014887**
	M	74	66.000	79.000	28.500	−2.435	
PON1 Q192R	Q	73	99.000	111.000	27.444	−2.291	**0.021985**
	R	73	79.000	67.000	27.444	2.291	
Caucasian-American unaffected siblings							
PON1 C−108T	C	48	56.000	56.167	18.472	−0.039	0.969067
	T	48	62.000	61.833	18.472	0.039	
PON1 L55M	L	40	54.000	54.500	14.750	−0.130	0.896417
	M	40	42.000	41.500	14.750	0.130	
PON1 Q192R	Q	41	63.000	62.500	15.194	0.128	0.897935
	R	41	37.000	37.500	15.194	−0.128	
Italian patients							
PON1 C−108T	C	130	135.000	133.000	47.500	0.290	0.771671
	T	130	127.000	129.000	47.500	−0.290	
PON1 L55M	L	118	141.000	140.500	40.250	0.079	0.937183
	M	118	97.000	97.500	40.250	−0.079	
PON1 Q192R	Q	107	138.000	138.000	35.000	0.000	1.000000
	R	107	78.000	78.000	35.000	0.000	

Statistically significant differences are displayed in bold.

Table 5 Haplotype frequency distributions for the PON1 L55M and Q192R markers, estimated by the HBAT program[39] in 106 Caucasian-American trios

Haplotype	*Frequency*	*No. of families*	*S*	*E(S)*	*Var(S)*	*Z*	*P*
L-Q	0.341	69	67.854	68.034	26.463	−0.035	0.972126
L-R	0.286	63	69.146	57.466	23.099	2.430	**0.015092**
M-Q	0.352	69	61.146	71.466	25.845	−2.030	0.042352
M-R	0.022	15	2.854	4.034	1.672	−0.912	0.361536

Statistically significant differences are displayed in bold.

Table 6 Serotonin blood levels, urinary peptide excretion rates, and head circumference of Italian and Caucasian-American individuals with autism by presence/absence of the PON1 R192 allele

	Italian patients			*Caucasian-American patients*		
	R192 present	*R192 absent*	*Statistics*	*R192 present*	*R192 absent*	*Statistics*
Serotonin blood levels (ng/ml)	235.04±17.9 *(54)*	225.8±13.9 *(53)*	$t=-0.407$, 112 df, $P=0.50$, n.s.	121.04±23.1 *(24)*	246.70±33.37 *(20)*	**$t=3.175$, 46 df, $P<0.01$**
Urinary peptides (AUC in μm^2)	276.5±90.0 *(70)*	314.0±98.5 *(65)*	$U=1939.5$, $P=0.14$, n.s.	404.0±126.0 *(23)*	566.0±288.0 *(19)*	$U=139.0$, $P=0.04$, n.s.
Head circumference (percentile)	75.0±23.8 *(60)*	82.5±23.8 *(54)*	$U=1499.5$, $P=0.49$, n.s.	95.0±7.5 *(32)*	92.5±35.9 *(16)*	$U=210.0$, $P=0.30$, n.s.

Data are expressed as mean±SEM for serotonin blood levels, and median±semi-IQR for amounts of urinary peptides and head circumference; two-tail *P*-values are reported. Sample sizes are shown in italics.
Statistically significant differences are displayed in bold.

Table 7 Total normalized disequilibrium coefficients (D') at the PON1 locus, estimated from (a) genotype data of unrelated individuals using the 3LOCUS program,[40] and (b) family-based association data for Italian and Caucasian-American individuals with autism only (italics), using the HBAT program[39]

Markers	*Italian controls (N = 178)*	*Italian patients (N = 168)*	*American patients (N = 106)*
−108/55	0.520**	0.479**	0.477*
		0.520	*0.500*
55/192	0.842**	0.877**	0.884**
		0.780	*0.830*
−108/192	0.213	0.102	0.246
		0.020	*0.080*

*$P < 0.01$.
**$P < 0.001$.
P-values refer to the G test statistic provided by the 3LOCUS program, compared with a simulated null distribution including 1000 replications.[40]

Gene–gene interactions between PON1 and RELN
Logistic regression performed on PON1 and RELN allelic data from 262 informative meioses of Caucasian-American maternal and paternal origin excluded the cosegregation of long RELN alleles and PON1 R192 alleles from the same parent to the affected offspring (Wald = 0.000, 1 df, $P = 0.985$, n.s.; OR = 0.99, 95% CI 0.40–2.45). Instead, analyses performed on genotypic data in 133 Caucasian-American patients disclosed a trend toward the possible convergence of long RELN alleles and PON1 R192 alleles from different parents to an affected individual, with long RELN alleles found in 11.5% (6/52) *vs* 19.8% (16/81) Caucasian-American patients carrying the QQ *vs* QR + RR genotypes, respectively (Fisher's exact test, one-tail $P = 0.157$, n.s.). In order to achieve the necessary statistical power, an additional sample of 252 individuals with autism recruited by the ASD-CARC and previously genotyped at the RELN locus[19] was typed at the PON1 Q192R SNP, yielding superimposable results, that is, long RELN alleles present in 12.5% (13/104) *vs* 20.9% (31/148) patients with the QQ *vs* QR + RR genotypes, respectively (Fisher's exact test, one-tail $P = 0.057$, n.s.). Merging of the two samples provided statistically significant evidence of gene–gene interactions. Coincident long RELN and PON1 R192 alleles in autistic patients were found at a significantly higher frequency than expected by chance, with long RELN alleles present in 12.2% (19/156) *vs* 20.5% (47/229) of patients carrying the QQ *vs* QR + RR genotypes (Fisher's exact test: one-tail $P < 0.025$).

Discussion

This study reports genetic evidence that in our Caucasian-American sample, but not in our Italian sample, autism vulnerability is conferred by the PON1 L55/R192 gene variant, which codes for a paraoxonase isoform less active *in vitro* on the OP diazinon as compared to the M55/Q192 isoform. Caucasian-American, but not Italian, patients also display a nonsignificant trend toward increased allele frequencies and transmission rates of the T−108 allele, which is associated with approximately 50% decrease in paraoxonase serum levels (Tables 2 and 3).[27,41] The consistency of our case–control and intrafamilial association analyses, the preferential transmission of R192 alleles to Caucasian-American patients and not to their unaffected siblings, the homogenous genetic structure of Italians and Caucasian-Americans at the PON1 locus, and the constellation of biochemical and morphological features, which surprisingly characterizes R192 allele carriers, all strongly support the reliability and biological significance of these findings.

The presence of an association between autism and PON1 alleles in Caucasian-American, but not in Italian, patients does not reflect a population-genetic artifact produced by interethnic differences in LD patterns and allelic frequencies. This potential bias can be excluded on the basis of LD analyses presented here (Table 7), also when including markers located in the nearby PON2 locus (data not shown). This conclusion is in accordance with prior findings, showing a relatively homogeneous genetic structure within Caucasian ethnic groups at this locus, in the face of large inter-racial differences between Caucasians, African-Americans, Chinese, Japanese, Asian-Indians, and Hispanics.[42] It also fits with our Italian controls displaying genotypic and allelic distributions very similar to those of the large sample of Caucasian-American controls reported by Brophy *et al*,[27] and employed here as ethnically matched controls for our Caucasian-American patients (Table 2). Therefore, this genetic association, unless due to chance, must be explained at some pathophysiological level.

The polymorphisms assessed in this study could in principle either mark through LD alleles carrying a functionally relevant mutation within PON1 or a nearby locus, or they could confer autism vulnerability by directly affecting gene expression or protein function. In reference to PON1, the novel missense mutation found in one Caucasian-American and in one Italian patient, although not present in 80 controls and interesting in many respects, can neither account for nor largely contribute to the genetic association described here. Among other loci located in proximity to PON1, acetylcholinesterase (ACHE) represents an appealing candidate, both as a direct target of OP compounds and for its involvement in brain development. However, the distance of approximately 3 Mb separating ACHE and PON1 makes significant LD between the two loci unlikely, although we cannot currently exclude this possibility. Instead, the SNPs assessed in this study have been shown to account for a significant proportion of interindividual variability in paraoxonase activity. In

particular, the replacement of Gln with Arg at position 192 significantly affects enzyme activity against diazinon, while exerting no influence on paraoxonase serum levels.[26,29,41] In contrast, the T−108 allele is associated with approximately 50% mean reductions in serum paraoxonase levels, but exerts no effect on affinity for specific substrates.[27,41] These phenotypic correlates of PON1 genotypes are even more prominent in neonates than in adults, and this trend may likely extend into prenatal life.[43] Hence, although we cannot exclude the existence of mutations in noncoding regions of PON1 or in nearby loci, the most parsimonious explanation for our findings is that a reduction in paraoxonase activity and/or amount produced by these SNPs may by itself confer autism vulnerability in one ethnic group only.

PON1 encodes the human HDL-associated paraoxonase, which, in addition to hydrolyzing OP compounds, physiologically reduces LDL and HDL oxidation, and hydrolyzes platelet-activating factor (PAF).[28,29,44] It also modulates immune responses in such a way that paraoxonase deficits would be expected to result in immune response alterations similar to those found in autism.[28,45] The gene cluster encompassing the PON1, PON3, and PON2 genes, in a centromeric-to-telomeric order, is located in human chromosome 7q21.3–22.1, a region showing an maximum LOD score (MLS) of 3.37.[46] There is, however, no evidence for direct paraoxonase involvement in CNS development and function: unless immune-mediated effects play a primary role in autism pathogenesis, the genetic association reported in this study is quite unlikely to reflect a direct link between paraoxonase gene variants and autism. Thus, the functional SNPs assessed in this study may likely confer autism vulnerability through an indirect mechanism.

Within the framework of our gene–environment interaction model, this indirect link would be provided by prenatal exposure to low doses of OP compounds in individuals carrying a genetic vulnerability seemingly involving, among others, specific alleles at either the PON1 or the RELN locus, or both. In this scenario, OP doses would obviously be much lower than those administered in experimental bioassays, and would be expected to produce consequences different from those typical of acute OP toxicity. It is particularly interesting that our Caucasian-American sample displays an association with the R192 allele, which is less active *in vitro* against diazinon, in contrast to the Q192 allele, which confers enhanced *in vitro* sensitivity to chlorpyrifos.[29] Until very recent action by the US Environmental Protection Agency (EPA) to phase out residential use of these compounds, as many as 80–90% of American households employed pesticides, and as much as 75% of diazinon and 50% of chlorpyrifos use in the USA were devoted to residential pest control.[14,18,47] An impressive toxicological screening of 20 meconium samples collected in the New York City area revealed metabolites of chlorpyrifos and diazinon in all, while metabolites of OPs used as pesticides in agriculture were detected only sporadically.[48] Prospective studies monitoring OP exposure in 316 pregnant African-American and Dominican women from New York City found many of them (35%) reporting at least one intervention by an exterminator during their pregnancy, all of them (100%) showing detectable levels of diazinon, chlorpyrifos, and carbamate propoxur in maternal and cord blood, and in maternal ambient air, and some of them ($9/230 = 3.9\%$) with documented exposures exceeding the health-based reference dose (RfD) set by the EPA.[49,50] Interestingly, of the three compounds, diazinon alone was involved in all nine cases of overexposure.[50] This evidence of maternal exposure is even more relevant to the fetus, since human serum paraoxonase reaches adult levels only at approximately 12–18 months of postnatal life, leaving the fetus generally less protected regardless of PON1 genotypic status.[51] Another instance of environmental exposure continuously exceeding for several days the RfD set for diazinon was documented in a preschool child, whose household had received a pesticide application 3 days prior to toxicological screening.[52] Therefore, epidemiological studies clearly show that exposure to OPs can and indeed does occur in American households after spraying for pest control, and does involve pregnant women and small children. This is much less likely to occur in Europe, where OP use has been steadily decreasing over the past decade while remaining equally intensive in the USA during the same period of time.[14,15] The difference between North America and Europe is especially broad for household use, conceivably due to differences in pest diffusion, climate, and housing construction materials and techniques (Persico *et al*, manuscript in preparation). Noticeably, exposure to low-dose pesticides in the Italian general population is not unusual,[16] but it has been shown to almost entirely stem from OP ingestion with wine and fresh foods, such as vegetables, milk, and water,[16,17] while inhalation of OP insecticide is more prevalent in North America.[18]

The present data also provide initial support for possible gene–gene interactions between RELN and PON1 alleles. Carriers of both 'long' GGC RELN alleles and R192 PON1 variants may display increased probability of affection status. The identical results obtained in our sample and in the North American sample recruited by the ASD-CARC are consistent with reduced production of Reelin protein, and reduced protection against OP exposure, acting as two independent risk factors conferring vulnerability toward Reelin enzymatic activity falling below the threshold necessary for correct neuronal migration under the effect of prenatal toxic exposure. We are currently in the process of studying this pathogenetic chain of events by assessing the behavioral and neurobiological consequences of low-dose diazinon administration at different time periods during gestation to hz reeler/hz PON1 knockout mice, used as

animal models of humans carrying 'long' GGC RELN alleles and 'low-function' PON1 gene variants. Reelin is a particularly interesting candidate due to its neurodevelopmental roles, functional genetic variants, decreased gene expression documented in autistic brains, linkage/association with autism, and enzymatic activity with a specific pharmaco-toxicological spectrum.[11–13,25] Nonetheless, low-dose exposure to OPs can affect a tremendous variety of developmentally relevant targets, ranging from acetylcholinesterase, to DNA synthesis and serotoninergic neurotransmission.[53–56] These toxins could thus affect brain growth and maturation in genetically vulnerable individuals through several different mechanisms, in addition to the Reelin pathway.

The major limitation of this study is that it draws information exclusively from the genetic level and not from direct measurements of serum enzyme activity.[57,58] Unfortunately, paraoxonase activity is calcium dependent and cannot be measured in the platelet-rich plasma we have collected from our entire sample. We have thus begun assessing paraoxonase, diazoxonase, and arylesterase activity in all 217 serum samples currently available from the AGRE collection,[30] including 40 families with one or more autistic patients and their first-degree relatives. Preliminary data indicate that most of these samples fall within the predicted enzymatic activity range, and three families for which both PON1 genotyping and serum paraoxonase status are available confirm the consistency between Q192R genotypes and measures of enzymatic activity. Some samples, however, do fall outside the predicted range and these results are currently under scrutiny.

Configuring a plausible explanation for the biochemical characteristics associated with the R192 variant in autism is neither simple nor straightforward, since the pathophysiology of these endophenotypes is not entirely known. Enhanced urinary peptide excretion rates in autism have been interpreted as possibly stemming from deficits in peptidase activities and/or transulfation;[35] the latter deficit has been described in some low-functioning individuals with autism,[59] but its direct link with peptiduria has not been investigated. Our most robust finding consists of decreased 5-HT blood levels in patients carrying the R192 allele. 5-HT blood levels, elevated in at least 25% of autistic patients but also decreased in a subgroup of patients, represent a consistent endophenotype in autism and possibly even a marker for familial forms of the disease.[34,60,61] Altered 5-HT blood levels in autism typically stem from changes in the density of functionally active 5-HT transporters (5-HTT) on platelet membranes, with no change either in 5-HTT affinity for 5-HT or in free 5-HT plasma level.[62–64] To our knowledge, no direct interaction between paraoxonase activity and 5-HT transport has ever been described, whereas persistent sex- and timing-specific effects of prenatal OP administration on 5-HTT gene expression have been documented in the developing brain.[55,65] Moreover, subacute, but not acute, exposure to chlorpyrifos yields a significant 35% decrease in platelet 5-HT uptake in adult rats.[66] Conceivably, similar effects on megakaryocytes occurring during critical periods in development could permanently affect platelet 5-HT levels. At this stage, however, there is no experimental evidence concerning this hypothesis.

The interpretation outlined above is based on a long series of *in vitro* studies correlating paraoxonase activity with PON1 allelic variants.[28,29] Initial *in vivo* assessments employing rodent models, however, suggest that the R192 variant may be slightly more, rather than less, active upon diazinon.[57,67] The discrepancy between *in vitro* and *in vivo* experiments may possibly stem from an inhibitory influence of high sodium chloride concentrations on the enzymatic activity of the R192 isoform *in vitro*.[57,67] *In vivo* studies indeed raise caution in drawing simplistic and unequivocal conclusions and, if confirmed, would point toward the existence of functionally relevant mutations at nearby loci.

In conclusion, our genetic and biochemical findings appear compatible with a gene–environment interactive model of autism pathogenesis, whereby the genetic vulnerability component is conferred at least in part by 'long' GGC alleles at the RELN locus yielding reduced amounts of Reelin protein, and by R192 alleles at the PON1 locus possibly decreasing paraoxonase activity against OP compounds such as diazinon. The environmental component consists of subacute exposure to OPs during critical periods of prenatal neurodevelopment. The cooccurrence of genetic liability and environmental exposure would then result in decreased Reelin proteolytic activity, altered neuronal migration, miswiring of neuronal circuits underlying social cognition, and autistic symptoms appearing once the child reaches a developmental stage requiring those circuits to begin functioning 'on-line'. The evidence provided by the present study and by our prior studies,[4,3] while converging and suggestive, is still only correlative and indirect, and it can in no way be regarded as conclusive proof that OPs contribute to precipitating autism in a subgroup of North American individuals. In addition, the relative weight of a single SNP or haplotype at a single locus must necessarily be small when put into the context of the complexity of autism genetics. Nonetheless, we feel that these results spur interest in possible environmental contributions to this largely genetically based disease and further endorse federal action by the US EPA aimed at phasing out residential use of diazinon and chlorpyrifos in the United States.

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Note added in proof

While this manuscript was in press, we have become aware of two papers indicating that human PON Q192 and R192 alloforms do not significantly differ in their catalytic efficiency for hydrolyzing diazoxon when injected in PON1 knockout mice: Furlong CE *et al*, *J Biochem Mol Toxicol* 2005; **19** 182–183 and Cole TB *et al.*, Pharmacogenet Genomics 2005, in press.

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