Biomarkers, 2011; 16(3): 252–260

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2010.548010

informa
healthcare

RESEARCH ARTICLE

Urinary *p*-cresol is elevated in small children with severe autism spectrum disorder

Laura Altieri[1,2], Cristina Neri[2,3], Roberto Sacco[1,2], Paolo Curatolo[4], Arianna Benvenuto[4], Filippo Muratori[5], Elisa Santocchi[5], Carmela Bravaccio[6], Carlo Lenti[7], Monica Saccani[7], Roberto Rigardetto[8], Marina Gandione[8], Andrea Urbani[2,3], and Antonio M. Persico[1,2]

[1]Laboratory of Molecular Psychiatry and Neurogenetics, University Campus Bio-Medico, Rome, Italy, [2]Department of Experimental Neurosciences, I.R.C.C.S. "Fondazione S. Lucia," Rome, Italy, [3]Department of Internal Medicine, University "Tor Vergata," Rome, Italy, [4]Department of Child Neuropsychiatry, University "Tor Vergata," Rome, Italy, [5]Department of Child Neurology and Psychiatry, I.R.C.C.S. "Stella Maris" and University of Pisa, Pisa, Italy, [6]Department of Pediatrics, University "Federico II," Naples, Italy, [7]Department of Child Neuropsychiatry, University of Milan, Milan, Italy, and [8]Department of Child Neuropsychiatry, University of Turin, Turin, Italy

Abstract

Several studies have described in autistic patients an overgrowth of unusual gut bacterial strains, able to push the fermentation of tyrosine up to the formation of *p*-cresol. We compared levels of urinary *p*-cresol, measured by high-performance liquid chromatography–ultraviolet, in 59 matched case-control pairs. Urinary *p*-cresol was significantly elevated in autistic children smaller than 8 years of age ($p < 0.01$), typically females ($p < 0.05$), and more severely affected regardless of sex ($p < 0.05$). Urinary cotinine measurements excluded smoking-related hydrocarbon contaminations as contributors to these differences. Hence, elevated urinary *p*-cresol may serve as a biomarker of autism liability in small children, especially females and more severely affected males.

Keywords: Clostridium, cotinine, gut flora, organic contaminants, pervasive developmental disorders

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments of variable entity in social interaction and communication, associated with restricted patterns of interest and stereotyped behaviors (Filipek et al., 1999). ASD encompasses several distinct disorders currently listed in the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV) (American Psychiatric Association, 1994), namely autistic disorder, Asperger disorder and pervasive developmental disorder not otherwise specified (PDDNOS). Family and twin studies have shown that ASD has a significant genetic component, which follows a complex inheritance pattern likely reflecting gene–gene and gene–environment interactions (Veenstra-VanderWeele and Cook, 2004; Persico and Bourgeron, 2006; Freitag, 2007). Among environmental factors possibly contributing to clinical heterogeneity, several reports have documented in a sizable subgroup of autistic patients the overgrowth of unusual gut bacterial strains, most consistently represented by clostridial species (Finegold et al., 2002, 2010; Song et al., 2004; Parracho et al., 2005). One of these species, *Clostridium difficile*, expresses a *p*-hydroxyphenylacetate decarboxylase, able to push the fermentation of tyrosine up to the formation of *p*-cresol (Selmer and Andrei, 2001). Also *Pseudomonas stutzeri* forms *p*-cresol from toluene (Cafaro et al., 2005). Following intestinal

Address for Correspondence: Antonio M. Persico, Department of Child Neuropsychiatry, Laboratory of Molecular Psychiatry and Neurogenetics, University "Campus Bio-Medico", Via Alvaro del Portillo 21, I-00128 Rome, Italy. Tel. +39-06-225419155, Fax +39-06-501703333. E-mail: a.persico@unicampus.it

(Received 05 October 2010; revised 10 December 2010; accepted 11 December 2010)

absorption, *p*-cresol travels through the blood stream partly protein-bound, partly in free form (De Smet et al., 2003); the latter is then filtered at the glomerular level and can be found in the urine of all individuals in small amounts. Whenever too abundant, as occurs in uremic patients, *p*-cresol has been convincingly shown to exert toxic effects, such as hampered phagocytic activity and enhanced endothelial permeability (Vanholder et al., 1995; De Smet et al., 2003; Cerini et al., 2004).

Two recent studies have documented elevated concentrations of compounds presumably derived from clostridial strains or other gut flora in the urine of autistic individuals (Shaw, 2010; Yap et al., 2010). To begin addressing possible pathophysiological roles of the gut in autism (White, 2003), we have measured urinary *p*-cresol concentrations in 59 autistic individuals and in 59 sex- and age-matched controls. Clinical and demographic correlates of urinary *p*-cresol levels were assessed. Since urinary *p*-cresol can also stem from hydrocarbon contamination, the most common form being active/passive smoking, urinary amounts of the nicotine metabolite, cotinine, were also measured.

Methods

Patient sample and urine collection

The demographic and clinical characteristics of 59 idiopathic ASD patients recruited in Central and Northern Italy are summarized in Table 1. Demographic and clinical characteristics, as well as diagnostic screening procedures used to exclude syndromic forms, have been previously described (Lintas et al., 2009). Briefly, patients fulfilling DSM-IV diagnostic criteria for autistic disorder, Asperger disorder or PDDNOS were screened for nonsyndromic autism using magnetic resonance imaging, electroencephalogram, audiometry, urinary aminoacid and organic acid measurements, cytogenetic and fragile-X testing. Patients with dysmorphic features were excluded even in the absence of detectable cytogenetic alterations. Patients with sporadic seizures (i.e., <1 every 6 months) were included; patients with frequent seizures or focal neurological deficits were excluded. Autistic behaviors were assessed using the official Italian version of the Autism Diagnostic Observation Schedule (ADOS; Lord et al., 2002), and of the Autism Diagnostic Interview—Revised (ADI-R; Rutter et al., 2003), as well as the Children Autism Rating Scales (CARS; Schopler et al., 1986); adaptive functioning was assessed using the Vineland Adaptive Behavior Scales (VABS) (Sparrow et al., 1984); I.Q. was determined using either the Griffith Mental Developmental Scales, the Coloured Raven Matrices, the Bayley Developmental Scales or the Leiter International Performance Scale. Tight sex- and age-matching (±1 year) was applied to recruit 59 typically developing controls devoid of any overt ASD symptomatology among the offspring of clinical/academic personnel. Cases and controls were all Caucasians of Italian ethnicity, with mean age (±SEM) of 8.29 ± 0.56 and 8.46 ± 0.59 years, respectively (Student $t = -0.210$, 116 df, $p = 0.834$), and an M:F ratio of 44:15 for both. All parents gave written informed consent for their children, using the consent form approved by the IRB of University Campus Bio-Medico (Rome, Italy).

First-morning urine were collected at home by parents using sterile containers and were brought to each clinical centre the same morning in wet ice. Urine samples were then frozen, shipped in dry ice and stored at –80°C until analysis.

p-Cresol measurement by HPLC

Urinary *p*-cresol concentrations were measured by high-performance liquid chromatography (HPLC)–ultraviolet ultraviolet diode array detection (UV–DAD), adapting the method previously described by Birkett et al. (1995), and by King et al. (2009). Briefly, an aliquot of frozen urine was thawed and mixed, and 30 µl was transferred into a tube containing 60 µl of 6 M HCl and heated at 90°C for 60 min to hydrolyze glucuronide and sulfate conjugates. After cooling, *p*-cresol was extracted with 1 ml of diethyl ether; 300 µl of the organic phase was then transferred into a tube with 20 µl of NaOH 0.1 N, and dried under a gentle flow of nitrogen. The residue was dissolved in 300 µl of MilliQ H_2O/acetonitrile 5%, and 30 µl was injected for HPLC analysis (Dionex Ultimate 3000 HPLC system with variable wavelength detector, column Dionex Acclaim®120 C18 5 µm 120 A° 4.6 × 150 mm, temperature at 28°C and detection wavelength at 270 nm). The mobile phase consisted of A) H_2O/acetonitrile (90/10)/TFA 0.05% and B) acetonitrile/TFA 0.05%. The gradient elution program was 0-15 min, 0-50% B; 15-17 min, 50-100% B; 17-20 min, 100% B; 20-21 min, 100-50% B; 21-25 min, 0% B; the flow rate was 1 ml/min. Spiked samples were run to determine the efficiency of *p*-cresol recovery. Standard solutions at various *p*-cresol concentrations were made in MilliQ H_2O/acetonitrile 5%, from a stock *p*-cresol solution (1 mg/ml, Sigma-Aldrich, Gillingham, UK). Correlation coefficient of the calibration straight lines was always >0.999. The limit of detection, calculated as three times the height of baseline long-term noise, was 20 ng/ml, and the limit of quantification was 70 ng/ml. Since creatinine excretion may be abnormally reduced in ASD children (Whiteley et al., 2006), d*ata were normalized by urinary specific gravity.*

Cotinine measurement by ELISA

Urinary cotinine levels were measured using the Cotinine ELISA kit (Calbiotech Inc., Spring Valley, CA): 10 µl of standard, controls and specimens were pipetted into selected wells in duplicate. The enzyme conjugate, 100 µl, was added into each well. After incubation (60 min at room temperature, in the dark), wells were washed and 100 µl of substrate reagent was added to each well. After

Table 1. Demographic and clinical characteristics of the autistic sample.

			N	Mean/median	Range
Age in yrs (mean± SEM):			*N* = 59	8.29 ± 0.56	2-18
Median ADOS scores:			*N* = 32		
1) Language and communication				5.0	0-10
2) Social interactions				10.0	4-13
3) Play and imagination				3.0	0-5
4) Stereotypes				2.5	0-6
5) Abnormal behaviors				0.0	0-2
Median ADI scores:			*N* = 18		
A) Reciprocal social interactions				23.0	5-34
B) Language/Communication				16.0	9-24
C) Restricted, repetitive and stereotyped behaviors and interests				7.0	1-12
D) Behavioral abnormalities at or prior to 36 months of age				4.0	0-5
Median CARS scores:			*N* = 32		
1) Social relationship				2.75	1.0-4.0
2) Imitation				2.75	1.0-4.0
3) Emotional response				2.50	1.5-4.0
4) Use of body				2.50	1.0-4.0
5) Use of objects				3.00	1.0-4.0
6) Mental and behavioral flexibility				2.00	1.0-3.5
7) Visual response				2.25	1.0-3.5
8) Hearing response				2.00	1.0-3.5
9) Use of senses				2.00	1.0-4.0
10) Fear and anxiety				2.00	1.0-4.0
11) Verbal communication				3.00	1.5-4.0
12) Non verbal communication				2.50	1.0-4.0
13) Activity level				2.00	1.0-3.5
14) Cognitive level				2.25	1.0-3.5
15) General impression				3.00	1.5-4.0
Median VABS scores:			*N* = 25		
Communication				88.0	25-124
Daily living skills				100.0	20-180
Socialization				93.0	30-123
Motor skills				104.5	60-120
Composite				90.0	23-123
			N	Percent	
Gender:	Male		44	74.6%	
	Female		15	25.4%	
	M/F ratio		2.9:1		
Family type:	Simplex		54	91.5%	
	Multiplex		5	8.5%	
DSM-IV Diagnosis:	Autistic disorder		37	62.7%	
	Asperger syndrome		4	6.8%	
	PDDNOS		18	30.5%	
I.Q. (*N* = 45):		>70	17	37.8%	
		≤70	28	62.2%	

Total *N* = 59, unless otherwise specified.
ADI, Autism Diagnostic Interview; ADOS, Autism Diagnostic Observation Schedule; CARS, Childhood Autism Rating Scale; PDDNOS, pervasive developmental disorder not otherwise specified; VABS, Vineland Adaptive Behavior Scales.

incubation (60 min at room temperature, in the dark), 100 µl of stop solution was added to each well. The absorbance was read on an ELISA reader at 450 nm. The lower limit of detection of this assay is 5 ng/ml, whereas the upper limit is 100 ng/ml. Smoking status was determined as in Zielinska-Danch et al. (2007): 0-50 ng/ml = non-smoker; 51-170 ng/ml = light passive smoker; 171-550 ng/ml = heavy passive smoker; >550 ng/ml = active smoker.

Statistical analyses

Cases and controls were contrasted using analysis of variance (ANOVA) for paired data, Student's *t*-test, Fisher's exact test or the χ^2 statistics; Kendall's τ statistics was employed for correlation analysis; regression analysis was always performed on the entire sample ($N = 118$). Given the exploratory nature of analyses involving clinical variables and their relative non-independence (Sacco et al., 2010), no correction for multiple testing was applied. Quantitative data are presented as mean ± SEM. Statistical significance is set at $p < 0.05$.

Results

Urinary *p*-cresol concentrations were significantly higher among 59 autistic patients compared to 59 matched controls (123.5 ± 12.8 vs. 91.2 ± 8.7 μg/ml, Student $t = 2.207, 116$ df, $p < 0.05$) (Figure 1). This increase was interestingly age-dependent (Figure 2: ANOVA for paired data $t = 2.089$, 58 df, $p < 0.05$). Increases in urinary *p*-cresol levels were in fact restricted to ASD children younger than 8 years of age (134.1 ± 20.1 vs. 70.3 ± 6.7 μg/ml, $t = 2.922$, 60 df, $p = 0.005$). No difference was found between patients and controls aged 8 or older (111.0 ± 14.5 vs. 112.7 ± 15.5 μg/ml, $t = -0.81$, 54 df, $p = 0.936$), nor were there significant age-related changes in urinary *p*-cresol concentrations among controls between 2 and 18 years old ($F = 2.0$; 6.58 df; $p = 0.082$) (Figure 2). Before age 8, levels above 150 μg/ml were detected in 9/32 (28.1%) ASD patients versus 0/32 controls ($p = 0.002$) (Figure 3), while levels above 140 μg/ml were detected in 9/32 (28.1%) ASD patients versus 1/32 (3.1%) controls ($p = 0.0127$). Urinary *p*-cresol displayed no correlation with urinary cotinine levels (Kendall's $\tau = -0.005$ for the entire sample, −0.035 for ASD patients and −0.009 for controls, $p = 0.939$, 0.700 and 0.922, respectively). We found no evidence of active smoking in our sample; on average, nonsmokers displayed higher, and not lower, urinary *p*-cresol levels compared to light (four ASD patients and one control) and heavy (two controls) passive smokers (108.8 ± 8.2 vs. 97.7 ± 25.4 vs. 48.4 ± 35.0 μg/ml, respectively; $F = 1.385$; 2.58 df, $p = 0.259$). Hence elevated urinary *p*-cresol amounts did not stem from active/passive smoking.

Urinary *p*-cresol concentrations were not significantly influenced by geographical region ($p = 0.261$), or by sex ($p = 0.624$), whereas significant effects were detected for diagnostic status ($p = 0.001$), and for a status x sex interaction ($p = 0.02$). In fact, 15 ASD females displayed significantly higher *p*-cresol amounts compared to 44 ASD males (188.2 ± 35.9 vs. 101.5 ± 10.9 μg/ml, respectively; *t*-test = −2.484, 30 df, $p < 0.05$), while female and male controls did not differ (70.6 ± 15.3 vs. 98.2 ± 10.6 μg/ml). Among children aged ≤7, urinary *p*-cresol concentrations were vastly higher among female autistics compared to female controls (222.2 ± 53.4 vs. 51.2 ± 16.6 μg/ml, respectively; *t*-test = 3.590, 12 df, $p = 0.004$), whereas differences between male autistics and controls did not reach statistical significance (104.7 ± 17.0 vs. 75.1 ± 7.2 μg/ml, respectively; *t*-test = 1.128, 46 df, $p = 0.265$). In numerical terms, if urinary *p*-cresol had been used as a diagnostic marker in our sample (positive when *p*-cresol >150 μg/ml), among children aged 7 or younger the test would have been positive in 5/8 (62.5%) ASD females and in 4/24 (16.7%) ASD males (Fisher's exact test, $p < 0.05$).

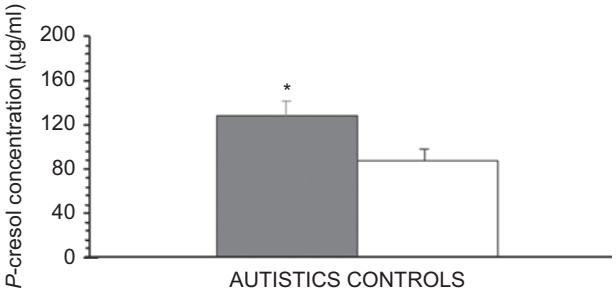

Figure 1. Urinary *p*-cresol concentrations (μg/ml) in 59 ASD patients and in 59 age-, sex- and ethnically matched controls. Data are presented as mean ± S.E.M. *$p < 0.05$.

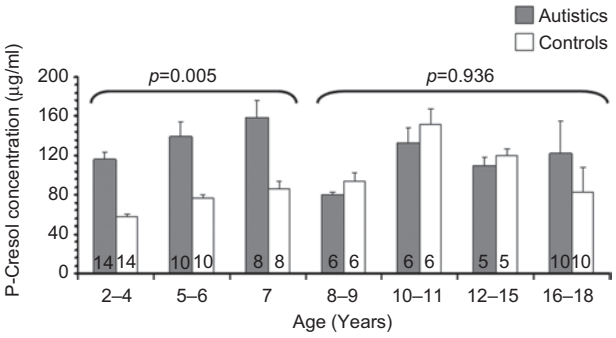

Figure 2. Urinary *p*-cresol concentrations by age group, in 59 ASD patients (grey bars) and in 59 age-, sex- and ethnically matched controls (white bars). Data are presented as mean ± S.E.M. *p* Values refer to case-control contrasts in 32 pairs aged 2–7, and in 27 pairs 8–18 years old. Numbers inside each column represent sample sizes.

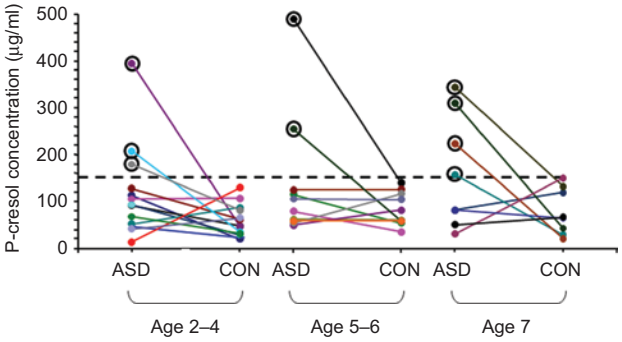

Figure 3. Urinary *p*-cresol concentrations in 32 ASD patient-control pairs, up to 7 years of age or younger. The nine patients highlighted by black circles are all above the maximum *p*-cresol concentration recorded in a typically developing child within this same age range (149.8 μg/ml, as highlighted by the hyphenated line).

Interestingly, eight of the nine (88.9%) small children with urinary *p*-cresol above 150 μg/ml satisfied DSM-IV diagnostic criteria for the most severe form of ASD, autistic disorder, compared to only 11/23 (47.8%) of the remaining children in the same age interval, who displayed significantly higher rates of the less severe form, PDDNOS (Fisher's exact test, $p<0.05$). Among children aged 7 or younger, all 15 CARS items measuring clinical severity were positively correlated with urinary *p*-cresol amounts, reaching statistical significance for imitation (item no. 2: $\tau=0.396$, $p=0.020$, $N=21$), use of body (item no. 4: $\tau=0.330$, $p=0.036$), verbal communication (item no. 11: $\tau=0.387$, $p=0.023$) and general impression (item no. 15: $\tau=0.534$, $p=0.002$). Also ADOS, ADI-R and VABS scores displayed correlation trends consistent with those found with the CARS, but *p* values did not reach statistical significance due to low statistical power in our current sample. Small children with urinary *p*-cresol above 150 μg/ml, compared to children with normal *p*-cresol amounts, showed nonsignificant trends ($p<0.1$) toward more frequent mental retardation [7/8 (87.5%) vs. 8/17 (47.1%)], self-injurious behaviors [4/9 (44.4%) vs. 3/18 (16.7%)] and a history of regression [6/9 (66.7%) vs. 7/23 (30.4%)], reported by parents as loss of language skills after acquisition of more than five spoken words and of social abilities after initial acquisition. No significant correlations between urinary *p*-cresol and behavioral measures were present in ASD children older than 7 years of age. Urinary *p*-cresol levels were not correlated with body mass index in our entire sample ($\tau=-0.153$, $p=0.159$), nor in females ($\tau=-0.158$, $p=0.460$), or males ($\tau=-0.250$, $p=0.06$) analyzed separately.

The M:F ratio in ASD is approximately 4:1, but it goes down to 2:1 in the presence of severe autism and mental retardation (Fombonne, 2002). As described above, urinary *p*-cresol levels were higher among our female, but not male, cases compared to sex-matched controls. We thus performed male-only analyses to exclude spurious links between urinary *p*-cresol and clinical severity merely reflecting a skewed sex ratio. In 24 males aged 7 or younger, all CARS items were again positively correlated with urinary *p*-cresol levels, reaching statistical significance for verbal communication (item no. 11: $\tau=0.383$, $p=0.033$), cognitive level (item no. 14: $\tau=0.398$, $p=0.030$) and general impression (item no. 15: $\tau=0.454$, $p=0.015$) (Figure 4). Vineland scores were negatively correlated with urinary *p*-cresol (i.e., worse adaptive level with increasing *p*-cresol concentrations), while ADOS and ADI-R scores were positively correlated with *p*-cresol, although none reached statistical significance. Female-only analyses were not meaningful due to small sample size.

Discussion

The present study demonstrates a significant increase in urinary *p*-cresol concentrations among 59 Italian ASD patients compared to an equal number of age-, sex-, and ethnically matched controls (Figure 1). This increase was present in approximately 30% of autistic children aged 7 or younger, the majority being girls in our sample. Urinary excretion of *p*-cresol appeared to normalize after age 7 (Figure 2). Importantly, urinary *p*-cresol does not derive from human metabolism: it can either stem from the presence of gut bacteria, such as *Clostridium difficile* and *Pseudomonas stutzeri*, or from contamination with petroleum hydrocarbon mixtures containing *p*-cresol. The most common source of petroleum hydrocarbon contamination, active or passive smoking, has been excluded as a cause of elevated urinary *p*-cresol in our sample by measuring urinary cotinine, the nicotine metabolite representing the best known marker of passive and active smoking (Zielinska-Danch et al., 2007). Other sources of environmental exposure to petroleum-derived *p*-cresol cannot be ruled out (Bright and Healey, 2003). However, a selective exposure of autistic, and not of control children, appears unlikely. Based on previously published reports (Finegold et al., 2002, 2010; Song et al., 2004; Parracho et al., 2005), a more plausible explanation would envision increased urinary *p*-cresol as originating from an abnormal gut flora, particularly enriched in *p*-cresol producing bacteria among autistic children. An excess of these microorganisms would result in increased *p*-cresol formation, followed by absorption of *p*-cresol through the gut, filtration by the renal glomeruli and greater excretion in urine, compared to typically developing age-matched children. This scenario has been recently proposed in two other studies, documenting elevated concentrations of compounds presumably derived from clostridial strains or other gut flora, in the urine of autistic individuals (Shaw, 2010; Yap et al., 2010). Based on this hypothesis, the normalization of urinary *p*-cresol excretion around 8 years of age could reflect an ASD-specific variation in gut microbial composition, which normally does change with age but not prominently in its clostridial components (Hopkins et al., 2002). Conceivably, an age-dependent maturation of the gut immune system could yield greater control over clostridial overgrowth after 7 years of age, with ASD-specific developmental trajectories stemming from the complex array of immune abnormalities frequently encountered in ASD children (Jyonouchi et al., 2005; Ashwood et al., 2006). Also genetic and hormonal liability could play an important role, with females particularly prone to developing elevated urinary *p*-cresol, but normalizing at an age when sex hormone secretion begins to increase. Alternatively, or in association with clostridial infections, excessive gut permeability could facilitate *p*-cresol absorption through the gut wall. The existence of a "leaky gut" in a subgroup of ASD patients is controversial and deserves further scrutiny according to a recent consensus report (Buie et al., 2010). Nonetheless, at least some studies document abnormally elevated ratios of urinary lactulose/mannitol following a standardized oral isomolar load of these

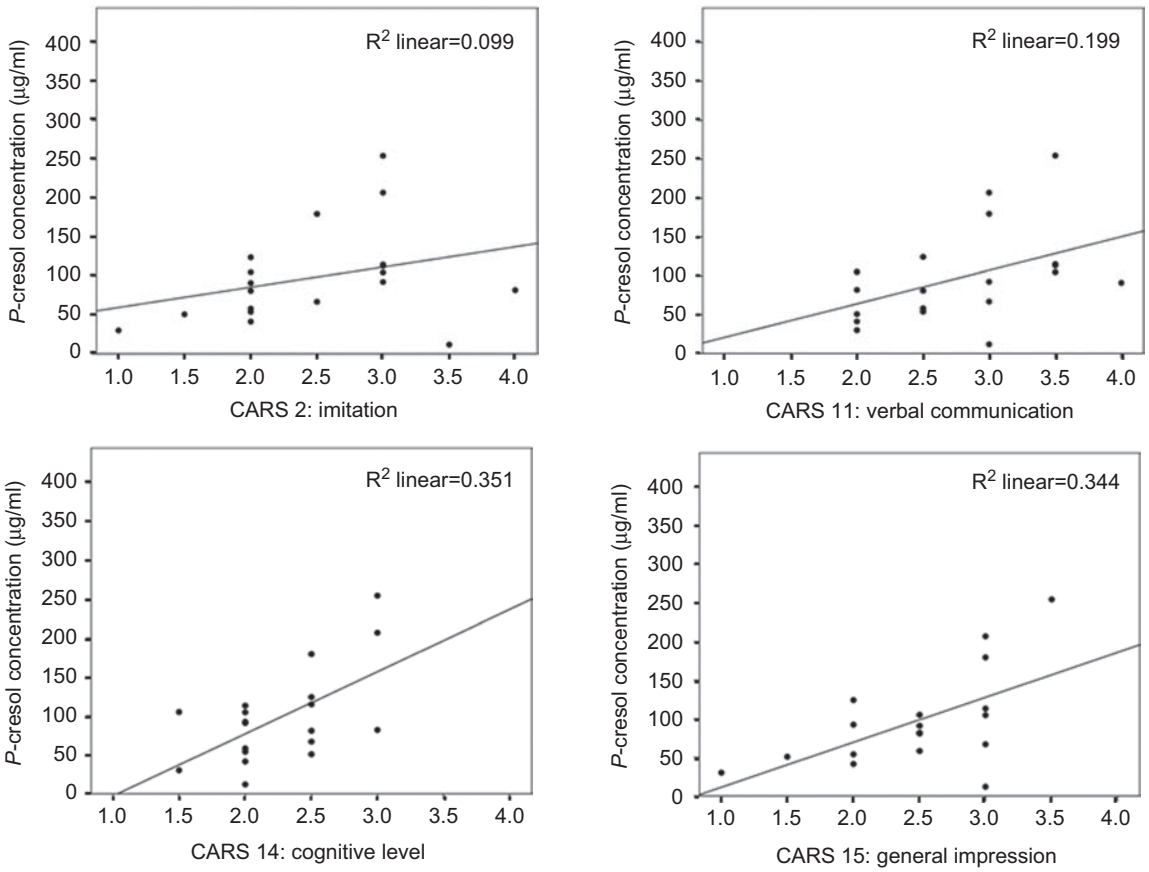

Figure 4. Male-only analyses: scatter plots showing the correlation between urinary p-cresol concentrations and autism severity in ASD males, up to 7 years of age or younger. CARS scores for items n. 2 (imitation), n. 11 (verbal communication), n. 14 (cognitive level), and n. 15 (general impression) are plotted. R^2 values are displayed in the top right corner of each graph. All *p* Values are <0.05, except for item n. 2 ($p = 0.067$).

two probes (D'Eufemia et al., 1996; de Magistris et al., 2010).

Once produced by gut bacteria and absorbed through the colon, approximately 85-90% *p*-cresol binds to albumin in plasma. Importantly, only the remaining 10-15% free *p*-cresol is toxicologically active, as it is known to cause some signs and symptoms of uremic toxicity, especially increased frequency of infections requiring hospitalization (De Smet et al., 2003). In fact, the capacity of *p*-cresol to impair free radical production by granulocytes *in vitro* is correlated positively with *p*-cresol concentrations and negatively with albumin concentrations in the culture medium (De Smet et al., 2003). Knowing that only free *p*-cresol undergoes glomerular ultrafiltration, assuming that *p*-cresol is neither reabsorbed nor excreted by the renal tubule, and setting creatinine clearance at 100 ml/min and urinary volume at 1.0 ml/min, urinary concentrations of 150 mg/l, 200 mg/l and 300 mg/l can be estimated to correspond to 1.5 mg/l, 2.0 mg/l and 2.5 mg/l free *p*-cresol in blood. These values recorded in our autistic patients are clearly in the upper range of concentrations recorded in symptomatic uremic patients, displaying free *p*-cresol blood levels ranging between 0.3 and 2.8 mg/l (De Smet et al., 2003). From a methodological standpoint, on one hand urinary *p*-cresol concentrations have the limitation of not immediately reflecting production of *p*-cresol in the gut, since urinary and fecal *p*-cresol concentrations are not correlated (Birkett et al., 1995). On the other hand, however, they have the advantage of more faithfully reflecting plasma concentrations of toxicologically active free *p*-cresol, accounting also for possible fluctuations in albumin plasma levels.

Another area of concern is represented by potential pharmacokinetic interactions between *p*-cresol and pharmacological drug therapies in autistic children. In particular, *p*-cresol undergoes O-sulfonation by the same sulfotransferase that inactivates many therapeutic drugs, such as acetaminophen; interestingly, urinary *p*-cresol levels are negatively correlated with liver capacity to sulfonate acetaminophen (Clayton et al., 2009). A reduction in liver sulfation capacity, specifically tested using acetaminophen, has been recorded in low functioning autistic individuals (Alberti et al., 1999). Our results spur interest into the potential role of *p*-cresol in these sulfation deficits; competition of *p*-cresol for hepatic sulfotransferases, paired with competition for albumin, could decrease drug clearance

while increasing free drug plasma levels, respectively, rendering a subset of ASD children particularly prone to developing adverse side effects when administered pharmacological therapies. ASD patients indeed show nonsignificantly decreased (and not increased) urinary concentrations of *p*-cresol sulphate (Yap et al., 2010). In this study, the hydrolysis of glucuronide and sulfate conjugates by HCl yields a single *p*-cresol peak, encompassing both conjugated and unconjugated *p*-cresol into a single cumulative measure (see Methods). A standard *p*-cresol sulfate solution is being commercially synthesized, in order to specifically measure *p*-cresol sulfate in these same urine samples. Should we confirm decreased *p*-cresol sulfate urinary concentrations in ASD children compared to controls, as reported by Yap et al. (2010), the elevated urinary *p*-cresol concentrations recorded here would even more prominently derive from excessive plasma levels of free *p*-cresol, further raising the probability of pharmacokinetically and toxicologically relevant effects exerted by *p*-cresol in autistic children, as previously demonstrated for uremic patients (De Smet et al., 2003).

Regardless of the mechanisms underlying elevated urinary *p*-cresol, this compound may have multiple negative consequences on the clinical course and management of a consistent subgroup of ASD children. The positive correlation between urinary *p*-cresol and clinical severity reported in the present study, as well as the correlation with a clinical history of regression, are especially intriguing, given the striking similarities in chemical structure between *p*-cresol and substances as toxic as phenol, or uncoupling agents like 2,4-dinitrophenol. A single report provides initial support to possible uncoupling effects, as *o-, m-, and p*-cresol were found to inhibit the respiratory chain without negatively affecting oxidative phosphorylation in mitochondria (Kitagawa, 2001). These results are highly compatible with the aforementioned impairment in free radical production exerted by *p*-cresol on granulocytes *in vitro* (De Smet et al., 2003). It will thus be important to assess at the cellular level whether and to what extent *p*-cresol can influence mitochondrial function, neurite growth and synaptogenesis, whereas rodent models should unveil systemic effects on the function and/or the development of the central nervous system, exerted by *p*-cresol either directly or through its negative influence on immune parameters and endothelial permeability (Vanholder et al., 1995; De Smet et al., 2003; Cerini et al., 2004).

This study presents several limitations, which must be duly acknowledged. First, its exploratory nature requires caution, until our data are independently replicated. Second, the unforeseen role played by age actually brings our sample size from 59 down to 32 case-control pairs, reducing our power to detect significant differences and hampering attempts to analyze both sexes separately. Third, we have no information on dietary habits, which could conceivably influence gut microflora, for example, through low soluble fiber content and recent fasting, both able to increase *p*-cresol production and/or absorption (Kawakami et al., 2007). We also have no information on recent use of antibiotics, which could change the gut microflora and give rise to an environment that favors *p*-cresol producing bacteria. Nonetheless, in our cultural context the use of antibiotics in small children is relatively limited and unlikely to represent the sole cause of our findings. Despite these limitations and the need for replication in larger and more thoroughly characterized samples, our results do provide initial evidence of excessive urinary *p*-cresol levels in a sizable subgroup of younger and severely affected autistic children.

Conclusions

Urinary amounts of the toxic compound *p*-cresol were significantly elevated in autistic children younger than age 8, especially in girls and, regardless of sex, in more severely affected patients. Follow-up mechanistic studies will have to define the degree of overlap between elevated urinary *p*-cresol, gut flora composition and enhanced intestinal permeability in ASD patients, as well as their potential relationship with gastrointestinal symptoms, abnormal behavior and personalized response to pharmacological treatments. Further studies will also be needed to define more precisely sex-specific correlations between urinary *p*-cresol and clinical variables, as well as gene-environment interactions involving *p*-cresol. Finally, replication of these findings in larger samples should spur interest into possible uses of urinary *p*-cresol as a biological marker of disease. In conjunction with other genetic and biochemical markers, *p*-cresol could contribute to estimate autism risk or to support a clinical diagnosis of ASD in small children, especially young girls.

Acknowledgements

The authors gratefully acknowledge all the families who participated in this study.

Declaration of interest

This work was supported by the Italian Ministry for University, Scientific Research and Technology (PRIN no. 2006058195 and no. 2008BACT54_002), the Italian Ministry of Health (RFPS-2007-5-640174), and the Autism Speaks Foundation (Princeton, NJ).

References

Alberti A, Pirrone P, Elia M, Waring RH, Romano C. (1999). Sulphation deficit in "low-functioning" autistic children: a pilot study. *Biol Psychiatry* 46:420–424.

American Psychiatric Association. (1994). *Diagnostic and Statistical Manual of Mental Disorders*. 4th ed. Washington, DC: American Psychiatric Press.

Ashwood P, Wills S, Van de Water J. (2006). The immune response in autism: a new frontier for autism research. *J Leukoc Biol* 80:1–15.

Birkett AM, Jones GP, Muir JG. (1995). Simple high-performance liquid chromatographic analysis of phenol and p-cresol in urine and feces. *J Chromatogr B, Biomed Appl* 674:187–191.

Bright DA, Healey N. (2003). Contaminant risks from biosolids land application: contemporary organic contaminant levels in digested sewage sludge from five treatment plants in Greater Vancouver, British Columbia. *Environ Pollut* 126:39–49.

Buie T, Campbell DB, Fuchs GJ 3rd, Furuta GT, Levy J, Vandewater J, Whitaker AH, Atkins D, Bauman ML, Beaudet AL, Carr EG, Gershon MD, Hyman SL, Jirapinyo P, Jyonouchi H, Kooros K, Kushak R, Levitt P, Levy SE, Lewis JD, Murray KF, Natowicz MR, Sabra A, Wershil BK, Weston SC, Zeltzer L, Winter H. (2010). Evaluation, diagnosis, and treatment of gastrointestinal disorders in individuals with ASDs: a consensus report. *Pediatrics* 125(Suppl 1):S1–18.

Cafaro V, Notomista E, Capasso P, Di Donato A. (2005). Mutation of glutamic acid 103 of toluene o-xylene monooxygenase as a means to control the catabolic efficiency of a recombinant upper pathway for degradation of methylated aromatic compounds. *Appl Environ Microbiol* 71:4744–4750.

Cerini C, Dou L, Anfosso F, Sabatier F, Moal V, Glorieux G, De Smet R, Vanholder R, Dignat-George F, Sampol J, Berland Y, Brunet P. (2004). P-cresol, a uremic retention solute, alters the endothelial barrier function *in vitro*. *Thromb Haemost* 92:140–150.

Clayton TA, Baker D, Lindon JC, Everett JR, Nicholson JK. (2009). Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. *Proc Natl Acad Sci USA* 106:14728–14733.

D'Eufemia P, Celli M, Finocchiaro R, Pacifico L, Viozzi L, Zaccagnini M, Cardi E, Giardini O. (1996). Abnormal intestinal permeability in children with autism. *Acta Paediatr* 85:1076–1079.

de Magistris L, Familiari V, Pascotto A, Sapone A, Frolli A, Iardino P, Carteni M, De Rosa M, Francavilla R, Riegler G, Militerni R, Bravaccio C. (2010). Alterations of the intestinal barrier in patients with autism spectrum disorders and in their first-degree relatives. *J Pediatr Gastroenterol Nutr* 51:418–424.

De Smet R, Van Kaer J, Van Vlem B, De Cubber A, Brunet P, Lameire N, Vanholder R. (2003). Toxicity of free p-cresol: a prospective and cross-sectional analysis. *Clin Chem* 49:470–478.

Filipek PA, Accardo PJ, Baranek GT, Cook EH Jr, Dawson G, Gordon B, Gravel JS, Johnson CP, Kallen RJ, Levy SE, Minshew NJ, Ozonoff S, Prizant BM, Rapin I, Rogers SJ, Stone WL, Teplin S, Tuchman RF, Volkmar FR. (1999). The screening and diagnosis of autistic spectrum disorders. *J Autism Dev Disord* 29:439–484.

Finegold SM, Molitoris D, Song Y, Liu C, Vaisanen ML, Bolte E, McTeague M, Sandler R, Wexler H, Marlowe EM, Collins MD, Lawson PA, Summanen P, Baysallar M, Tomzynski TJ, Read E, Johnson E, Rolfe R, Nasir P, Shah H, Haake DA, Manning P, Kaul A. (2002). Gastrointestinal microflora studies in late-onset autism. *Clin Infect Dis* 35:S6–S16.

Finegold SM, Dowd SE, Gontcharova V, Liu C, Henley KE, Wolcott RD, Youn E, Summanen PH, Granpeesheh D, Dixon D, Liu M, Molitoris DR, Green JA 3rd. (2010). Pyrosequencing study of fecal microflora of autistic and control children. *Anaerobe* 16:444–453.

Fombonne E. (2002). Epidemiological trends in rates of autism. *Mol Psychiatry* 7(Suppl 2):S4–S6.

Freitag CM. (2007). The genetics of autistic disorders and its clinical relevance: a review of the literature. *Mol Psychiatry* 12:2–22.

Hopkins MJ, Sharp R, Macfarlane GT. (2002). Variation in human intestinal microbiota with age. *Dig Liver Dis* 34(Suppl 2):S12–S18.

Jyonouchi H, Geng L, Ruby A, Zimmerman-Bier B. (2005). Dysregulated innate immune responses in young children with autism spectrum disorders: their relationship to gastrointestinal symptoms and dietary intervention. *Neuropsychobiology* 51:77–85.

Kawakami K, Kojima K, Makino I, Kato I, Onoue M. (2007). Fasting enhances p-Cresol production in the rat intestinal tract. *Exp Anim* 56:301–307.

King RA, May BL, Davies DA, Bird AR. (2009). Measurement of phenol and p-cresol in urine and feces using vacuum microdistillation and high-performance liquid chromatography. *Anal Biochem* 384:27–33.

Kitagawa A. (2001). Effects of cresols (o-, m-, and p-isomers) on the bioenergetic system in isolated rat liver mitochondria. *Drug Chem Toxicol* 24:39–47.

Lintas C, Sacco R, Garbett K, Mirnics K, Militerni R, Bravaccio C, Curatolo P, Manzi B, Schneider C, Melmed R, Elia M, Pascucci T, Puglisi-Allegra S, Reichelt KL, Persico AM. (2009). Involvement of the PRKCB1 gene in autistic disorder: significant genetic association and reduced neocortical gene expression. *Mol Psychiatry* 14:705–718.

Lord C, Rutter M, DiLavore PC, Risi S. (2002). ADOS, Autism Diagnostic Observation Schedule. Los Angeles, CA: *Western Psychological Services* [Italian version by Tancredi R, Saccani M, Persico AM, Parrini B, Igliozzi R, Faggioli R, editors (2005). Florence, Italy: Organizzazioni Speciali].

Parracho HM, Bingham MO, Gibson GR, McCartney AL. (2005). Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. *J Med Microbiol* 54:987–991.

Persico AM, Bourgeron T. (2006). Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. *Trends Neurosci* 29:349–358.

Rutter M, Le Couter A, Lord C. (2003). ADI-R, Autism Diagnostic Interview—Revised. Los Angeles, CA: *Western Psychological Services* [Italian version by Faggioli R, Saccani M, Persico AM, Tancredi R, Parrini B, Igliozzi R, Eds. (2005). Florence, Italy: Organizzazioni Speciali].

Sacco R, Curatolo P, Manzi B, Militerni R, Bravaccio C, Frolli A, Lenti C, Saccani M, Elia M, Reichelt KL, Pascucci T, Puglisi-Allegra S, Persico AM. (2010). Principal pathogenetic components and biological endophenotypes in autism spectrum disorders. *Autism Res* 3:237–252.

Schopler E, Reichler RJ, Rochen Renner BR. (1986). *The Childhood Autism Rating Scale for diagnostic screening and classification of autism*. New York, NY: Irvington.

Selmer T, Andrei PI. (2001). p-Hydroxyphenylacetate decarboxylase from Clostridium difficile. A novel glycyl radical enzyme catalysing the formation of p-cresol. *Eur J Biochem* 268:1363–1372.

Shaw W. (2010). Increased urinary excretion of a 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA), an abnormal phenylalanine metabolite of Clostridia spp. in the gastrointestinal tract, in urine samples from patients with autism and schizophrenia. *Nutr Neurosci* 13:135–143.

Song Y, Liu C, Finegold SM. (2004). Real-time PCR quantitation of clostridia in feces of autistic children. *Appl Environ Microbiol* 70:6459–6465.

Sparrow SS, Balla DA, Cicchetti DV. (1984). *Vineland Adaptive Behavior Scales—Survey Form*. Circle Pines, MN: American Guidance Service Inc.

Vanholder R, De Smet R, Waterloos MA, Van Landschoot N, Vogeleere P, Hoste E, Ringoir S. (1995). Mechanisms of uremic inhibition of phagocyte reactive species production: characterization of the role of p-cresol. *Kidney Int* 47:510–517.

Veenstra-VanderWeele J, Cook EH Jr. (2004). Molecular genetics of autism spectrum disorder. *Mol Psychiatry* 9:819–832.

White JF. (2003). Intestinal pathophysiology in autism. *Exp Biol Med (Maywood)* 228:639–649.

Whiteley P, Waring R, Williams L, Klovrza L, Nolan F, Smith S, Farrow M, Dodou K, Lough WJ, Shattock P. (2006). Spot urinary creatinine excretion in pervasive developmental disorders. *Pediatr Int* 48:292–297.

Yap IK, Angley M, Veselkov KA, Holmes E, Lindon JC, Nicholson JK. (2010). Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls. *J Proteome Res* 9:2996–3004.

Zielinska-Danch W, Wardas W, Sobczak A, Szoltysek-Boldys I. (2007). Estimation of urinary cotinine cut-off points distinguishing non-smokers, passive and active smokers. *Biomarkers* 12:484–496.