RESEARCH ARTICLE

Urinary p-cresol is elevated in young French children with autism spectrum disorder: a replication study

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Abstract

The aromatic compound p-cresol (4-methylphenol) has been found elevated in the urines of Italian autistic children up to 8 years of age. The present study aims at replicating these initial findings in an ethnically distinct sample and at extending them by measuring also the three components of urinary p-cresol, namely p-cresylsulfate, p-cresylglucuronate and free p-cresol. Total urinary p-cresol, p-cresylsulfate and p-cresylglucuronate were significantly elevated in 33 French autism spectrum disorder (ASD) cases compared with 33 sex- and age-matched controls (p < 0.05). This increase was limited to ASD children aged ≤8 years (p < 0.01), and not older (p = 0.17). Urinary levels of p-cresol and p-cresylsulfate were associated with stereotypic, compulsive/repetitive behaviors (p < 0.05), although not with overall autism severity. These results confirm the elevation of urinary p-cresol in a sizable set of small autistic children and spur interest into biomarker roles for p-cresol and p-cresylsulfate in autism.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impaired social interaction and communication, as well as by restricted patterns of interest and stereotyped behaviors (American Psychiatric Association, 2013). This is one of the most frequent disorders in child psychiatry, with an incidence of 1/68 newborns according to recent estimates by the Center for Disease Control (Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators and Centers for Disease Control and Prevention, 2014). It displays a broad variety of clinical signs, severity, developmental trajectory and treatment response. This striking clinical heterogeneity seemingly reflects an equally impressive etiological heterogeneity, where causes underlying ASD are complex and remain elusive in the majority of patients. Most ASD cases are compatible with a “multiple hit” model, whereby several inherited and/or de novo genetic variants, in combination with environmental factors especially active during prenatal life, yield abnormal neurodevelopment through “personalized” gene–gene and gene–environment interactions (Leblond et al., 2012; Persico & Napolioni, 2013). The contribution of environmental factors on top of genetic liability is supported by multiple lines of evidence, recently reviewed elsewhere (Persico & Merelli, in press). Developmental neurotoxicants and immune challenges have been linked to autistic behaviors following prenatal exposure typically occurring during the I and II trimester of pregnancy, respectively (Persico & Merelli, in press). Finally, an additional layer of pathophysiological complexity is conferred by the frequent involvement of other compartments outside the central nervous system, such as the immune and gastrointestinal (GI) systems (Jyonouchi et al., 2011; Wang & Kasper, 2014).

p-cresol (4-methylphenol), an organic molecule belonging to the cresol class of aromatic compounds (OECD, 2003), has recently become the object of our interest as an environmental factor possibly involved in ASD. Environmental exposure to p-cresol is relatively common and its absorption can occur through the skin, the GI system and the respiratory tract; however, the most common source of this compound is represented by some gut bacteria, such as Clostridium difficile, able to push the fermentation of tyrosine or tolune up to p-cresol, by means of synthetic enzymes not present in human cells (for review see Persico & Napolioni, 2012).
Traveling in the blood, \(p\)-cresol is mostly protein-bound, as a partially lipophilic compound (Bergé-Lefranc et al., 2010). Approximately 95% of total plasma \(p\)-cresol is metabolized to \(p\)-cresylsulfate through O-sulfonation, which occurs primarily in colon and liver; the remaining 3–4% is metabolized to \(p\)-cresylglucuronide through glucuronidation, which takes place only in the liver (De Bruin, 1976; Mandel, 1971; Ramakrishna et al., 1991). Only 0.5–1% of total plasma \(p\)-cresol is in free form. \(p\)-cresol and its conjugation derivates \(p\)-cresylsulfate and \(p\)-cresylglucuronide are then filtered from the blood stream at the glomerular level and can be found in the urines of all individuals in small amounts (Bone et al., 1976; Renwick et al., 1988).

We were interested in contrasting urinary \(p\)-cresol levels in autistic and typically developing children because ASD has been associated with enhanced frequencies of (1) excessive gut permeability, reported by at least some (D’Eufemia et al., 1996; De Magistris et al., 2010), though not all studies (Robertson et al., 2008) and (2) gut infection with creosol-producing \textit{C. difficile} (Finegold et al., 2002; De Angelis et al., 2013; Kesli et al., 2014; Selmer & Andrei, 2001; Wang et al., 2011). Our initial study reported significantly elevated amounts of urinary \(p\)-cresol in 59 Italian ASD children compared with 59 age- and sex-matched controls \((p < 0.05)\) (Altieri et al., 2011). In particular, urinary \(p\)-cresol was significantly elevated in ASD children only between the ages of 2 and 8 \((p < 0.01)\), more frequently in females \((p < 0.05)\, and in more severely autistic children regardless of sex \((p < 0.05)\). These initial findings suggested that urinary \(p\)-cresol could represent a potential biomarker defining a consistent subgroup of small ASD children, and may play a pathomorphologic role by increasing autism severity. The present study aims at replicating and extending our initial results in an independent and ethnically distinct sample of ASD patients and matched controls. We thus (a) performed high-performance liquid chromatography with fluorescence detector (HPLC-FLD) measurements of total urinary \(p\)-cresol and its derivates \(p\)-cresylsulfate and \(p\)-cresylglucuronate in an independent sample of 33 French ASD individuals and in 33 age- and sex-matched typically developing controls, and (b) assessed demographic as well as developmental, family history and clinical correlates of urinary \(p\)-cresol levels.

### Methods

#### Patient sample

A sample of 33 idiopathic ASD patients was recruited at the Center for Child and Adolescent Psychiatry of the Hôpital Bretonneau in Tours (France). Their demographic and clinical characteristics are summarized in Table 1. Tight sex- and age-matching \((± 1 \text{ yr})\) was applied to recruit 33 typically developing controls devoid of any overt ASD symptomatology among the offspring of clinical/academic personnel. Cases and controls were Caucasians of French ethnicity (with exception for eight cases of African, and four of mixed ethnicity), with mean age \((± \text{ S.E.M.})\) of 7.90 ± 0.57 and 7.60 ± 0.61 yrs, respectively \((\text{Student } t = 0.364, 64 \text{ df, } p = 0.717)\), and an M:F ratio of 7.2:1 for both. All parents gave written informed consent for their children, using the consent form approved by the Ethical Committee of University “Campus Bio-Medico” (Rome, Italy).

Diagnostic screening procedures used to exclude syndromic forms have been previously described (Sacco et al., 2010). Briefly, patients fulfilling Diagnostic and Statistical Manual IV (DSM-IV) diagnostic criteria for Autistic Disorder, Asperger Disorder or PDDNOS were screened for non-syndromic autism using MRI, EEG, audiometry, urinary aminoacid and organic acid measurements, cytogenetic and fragile-X testing. Patients with gross 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 Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2002), the Autism Diagnostic Interview – Revised (ADI-R) (Rutter et al., 2003), the Children Autism Rating Scales (CARS) (Schopler et al., 1986), the \textit{Évaluation des Comportements Autistiques – version révisée} (ECA-R) (Barthélemy et al., 1997), the \textit{Échelle d’évaluation des Comportements répétés et restreints} (EC2R) (Bourreau et al., 2009) and the \textit{Évaluation Fonctionnelle des Comportements – version révisée} (RFC-R) (Adrien et al., 2001). I.Q. was determined using the Griffith Mental Developmental Scales (Griffith, 1970), the Brunet–Lézin scales (Brunet & Lézin, 1976), the \textit{Echelles différencielles d’Efficience Intellectuelle} (EDEI-R) (Perron-Borrel, 1996), and the Wechsler Intelligence Scale for Children – Revised (WISC-R) (Wechsler, 2005). Developmental, clinical and family history variables were characterized using an updated version of the previously described questionnaire (Sacco et al., 2010). The complete list of all variables is reported in Supplementary Table S1.

#### Urine collection and \(p\)-cresol measurement by HPLC

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

### Table 1. Demographical and clinical characteristics of the French ASD sample.

<table>
<thead>
<tr>
<th>Age in yrs (mean ± S.E.M.)</th>
<th>(N)</th>
<th>Mean/median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td>33</td>
<td>7.90 0.57</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 29</td>
<td></td>
<td>29</td>
<td>87.9%</td>
</tr>
<tr>
<td>Female 4</td>
<td></td>
<td>4</td>
<td>12.1%</td>
</tr>
<tr>
<td>M/F ratio 7.2:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSM-IV diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autistic disorder 24</td>
<td></td>
<td>24</td>
<td>72.7%</td>
</tr>
<tr>
<td>Asperger syndrome 2</td>
<td></td>
<td>2</td>
<td>6.1%</td>
</tr>
<tr>
<td>PDD-NOS 7</td>
<td></td>
<td>7</td>
<td>21.2%</td>
</tr>
<tr>
<td>I.Q. ((N = 33))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;70 13</td>
<td></td>
<td>13</td>
<td>39.4%</td>
</tr>
<tr>
<td>(\leq 70) 20</td>
<td></td>
<td>20</td>
<td>60.6%</td>
</tr>
</tbody>
</table>

Total \(N = 33\).
Urinary p-cresol concentrations were measured by HPLC-FLD. Total urinary p-cresol concentrations were quantified after acid hydrolysis as previously described (Altieri et al., 2011). Urinary p-cresylsulfate levels were measured applying a selective hydrolysis of sulfate groups by using a sulfatase. Thus, 100 μl of urines were mixed with 5 μl of internal standard (t-butyl phenol 1 mg/ml, Sigma-Aldrich, St. Louis, MO), 5 μl of 2.5 M Tris HCl (pH 7.5) and 5 μl of a solution containing sulfatase from Aerobacter aerogenes (Sigma-Aldrich, St. Louis, MO). Urinary p-cresylglucuronide levels were measured applying a selective hydrolysis of glucuronate groups by using a β-glucuronidase. In this case, 100 μl of urines were mixed with 5 μl of internal standard (as above), 5 μl of 4M sodium acetate (pH 5.0), and 5 μl of a solution containing β-glucuronidase type B-10 from bovine liver (Sigma-Aldrich, St. Louis, MO). Free p-cresol levels were measured using 100 μl of urines treated with 100 μl of acetonitrile. Mixes were then incubated for 2 h at 37°C, kept on ice for 15 min and centrifuged at 12 000 g. Supernatants were transferred into fresh tubes and injected separately for HPLC analysis, using the Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC system with fluorescence detector (λex: 270 nm, λem: 305 nm), column Dionex Acclaim® 120 C18 5 μm 120 A° 4.6 × 150 mm, temperature at 28°C. The mobile phase consisted of (A): H2O/Acetonitrile (90/10)/trifluoroacetic acid (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 H2O/acetonitrile 5%, from a stock p-cresol solution (1 mg/ml, Sigma-Aldrich). 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), data were normalized by urinary specific gravity.

Statistical analyses

Cases and controls were contrasted using the Wilcoxon signed-rank test. Correlation analyses between continuous variables were performed using non-parametric Kendall’s τ test; categorical variables were tested using parametric Student t-tests or non-parametric Mann–Whitney U-tests depending on the normality of the distribution, as assessed using the Kolmogorov–Smirnov statistics. 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 ± S.E.M. Two-tail p values are reported throughout the manuscript and statistical significance is set at p < 0.05.

Results

Mean total urinary p-cresol concentration was significantly higher among 33 ASD children compared with 33 matched

Figure 1. (A) Total urinary p-cresol, (B) p-cresylsulfate, (C) p-cresylglucuronate and (D) free urinary p-cresol concentrations (μg/ml) in 33 French ASD patients and in 33 age-matched, sex-matched and ethnically matched controls. Data are presented as mean ± S.E.M. *p < 0.05.
controls (98.8 ± 17.3 versus 52.0 ± 7.8 µg/ml, Z = −2.743, p < 0.05) (Figure 1A). The three components, p-cresylsulfate, p-cresylglucuronate and free p-cresol, accounted for 94.99%, 4.93% and 0.08%, respectively, of total urinary p-cresol in ASD children and for 94.87%, 5.05% and 0.08% in controls. These distributions do not differ significantly (χ² = 0.002, 2 df, p = 0.99). p-cresylsulfate and p-cresylglucuronide derivatives were also significantly elevated in ASD children compared with controls (92.9 ± 16.8 versus 49.5 ± 7.8 µg/ml, Z = 2.564, p < 0.05; 4.8 ± 1.0 versus 2.7 ± 0.5 µg/ml, Z = −2.028, p < 0.05, respectively) (Figure 1B and C), whereas a non-significant trend was found with free urinary p-cresol (0.05 ± 0.13 versus 0.03 ± 0.13 µg/ml, Z = −1.202, p = 0.230) (Figure 1D), whose concentrations were close to the sensitivity threshold of our measurement system (see Discussion).

Age-specific analyses confirmed that elevated urinary p-cresol levels are exclusively found in a subset of small ASD children. Applying the age-threshold of <8 y.o. derived from our previous results (Altieri et al., 2011), total urinary p-cresol levels were found significantly increased in younger (p < 0.05), but not older (p = 0.210, n.s.) ASD children, compared with their matched controls (Figure S1). However, greatest sensitivity/specificity was obtained in the present data set applying a slightly higher age threshold, namely ≤8 y.o., whereby total urinary p-cresol concentrations are very significantly elevated in ASD children below (Z = −2.535, p < 0.01, N = 22 case-control pairs) (Figure 2), but not above this age cut-off (Z = −1.350, p = 0.17, N = 11 case-control pairs). In particular, two French affected children, aged 8y.–3m. and 8y–4m., display total urinary p-cresol levels at 243.5 and 453.3 µg/ml, respectively (Figure 3). Overall, 4/22 (18.2%) ASD children aged <8 years displayed total urinary p-cresol concentrations above and beyond the highest levels recorded in controls (185.0 µg/ml). One of these four children was a girl (M:F = 3:1), as compared with 3 girls among 18 children with levels within the control range (M:F = 5:1). Regression analyses confirmed a significant negative correlation of total urinary p-cresol concentration with age in ASD children (τ = −0.223, p < 0.05, linear R² = 0.030) (Figure 2), but not in controls (τ = 0.079, p = 0.51, linear R² = −0.030) (Figure S1).

No significant correlation was found between total urinary p-cresol, p-cresylsulphate or p-cresylglucuronate, and autism severity, based on ADOS, ADI-R, CARS, ECA, EC2-R and ECF scores (data not shown). Also no correlation was recorded here with the presence of mental retardation either in the whole ASD sample (U = 117.0, p = 0.65), or among ASD children ≤8 years old (U = 31.0, p = 0.71). The only clinical variables compatible with greater severity or with a pathomorphic effect of p-cresol and/or p-cresylsulfate are age at walking, as well as the observation of motor stereotypies and compulsive/repetitive behaviors at intake (Table 2).

### Discussion

The primary aim of this study was to replicate in an ethnically distinct sample of ASD children and controls our initial report, demonstrating: (a) significantly higher urinary p-cresol concentrations among Italian ASD children compared with matched controls, (b) limited to children smaller than 8 years of age, (c) more frequently females and (d) more severely affected (Altieri et al., 2011). The present study replicates in an independent French sample both the statistically significant elevation of total urinary p-cresol in ASD patients compared with controls (Figure 1), and the strong age effect, restricting this elevation to a subset of small ASD children, aged ≤8 years of age (Figures 2 and 3). Two

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**Figure 2.** Total urinary p-cresol concentrations by age group, in 33 ASD patients (grey bars) and in 33 age-matched, sex-matched and ethnically matched controls (white bars). Data are presented as mean ± S.E.M. Numbers inside each column represent sample sizes. **p < 0.01 for global case-control contrasts in 22 pairs aged 3–8.

**Figure 3.** Total urinary p-cresol concentrations are negatively correlated with age (τ = −0.223, p < 0.05, linear R² = 0.030) in (A) 33 French ASD children, but not in (B) 33 age- and sex-matched typically developing controls (τ = 0.079, p = 0.51, linear R² = −0.030).
Table 2. Correlation between total urinary (A) p-cresol or (B) p-cresylsulfate levels and clinical characteristics in 33 French ASD children.

<table>
<thead>
<tr>
<th></th>
<th>Total urinary p-cresol</th>
<th>Urinary p-cresylsulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean ± S.E.M.</td>
</tr>
<tr>
<td>Compulsive/repetitive behaviors at intake</td>
<td>Present</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>19</td>
</tr>
<tr>
<td>Motor stereotypies at intake</td>
<td>Present</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>19</td>
</tr>
<tr>
<td>Self-aggressive or self-injurious behavior at intake</td>
<td>Present</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>27</td>
</tr>
<tr>
<td>History of neuropsychiatric disorders in first-degree relatives</td>
<td>Present</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>8</td>
</tr>
<tr>
<td>Age at walking, in months</td>
<td>33</td>
<td>0.336</td>
</tr>
<tr>
<td>Age at first social smile, in months</td>
<td>33</td>
<td>−0.279</td>
</tr>
</tbody>
</table>

Only (A) categorical and (B) continuous variables reaching a nominal $p<0.05$ are listed.

children with particularly elevated p-cresol concentrations here support sliding the age cut-off from 8 to 8 years and a half (Figure 3A); however, also maintaining the <8 y.o. cut-off derived from Altieri et al. (2011), p values are still <0.05 and 0.21 for children below and above this age threshold, respectively (Figure S1). Hence, the association of elevated urinary p-cresol with autism and the age effect are indeed replicated. Also, a trend toward females being more prone to developing high urinary p-cresol levels is present here, although the French sample includes only four female case-control pairs and is thus not fit for addressing sex effects.

The association between urinary p-cresol levels and autism severity found in the Italian sample (Altieri et al., 2011) represents the only correlation which has not been replicated in the French sample, although high urinary p-cresol and p-cresylsulfate concentrations are associated here with compulsive/repetitive behaviors, motor stereotypies at intake and age at walking onset (Table 2). The association between urinary p-cresol levels and autism severity found in Italian, but not in French ASD children, can conceivably be interpreted as spurious, dose-dependent, or due to the combined action of more than one gut-derived compound. A spurious association in our original study is indeed possible, given the number of clinical variables analyzed and the lack of statistical control for multiple testing. Possible dose-dependent effects receive some support from two pieces of evidence: (a) mean urinary p-cresol concentrations in Italian cases and controls were 123.5 and 91.2 μg/ml, whereas in French cases and controls they are 98.8 and 52.0 μg/ml, respectively (compare Figure 1 in Altieri et al. 2011 with Figure 1 here). Mean p-cresol levels in French ASD children are practically superimposable to those of Italian controls, while urinary p-cresol is clearly highest among Italian autistic children; (b) acute administration of high (10 mg/kg i.v.), but not low (1 mg/kg i.v.) p-cresol, produces autistic-like behaviors in Black and Tan Brachyury (BTBR) mice, one of the most reliable rodent models of human ASD (Pascucci & Persico, unpublished manuscript). Hence, dose-dependent effects of p-cresol on behavior cannot be dismissed. Finally, not only p-cresol, but also several urinary metabolites, either derived from gut bacteria or from environmental sources, result unbalanced in ASD (Emond et al., 2013; Mavel et al., 2013; Yap et al., 2010). Further metabolomic profiling of our ASD sample will thus be critical to understand whether and to what extent different combinations of gut-derived compounds including, but perhaps not limited to p-cresol, may collectively produce clinically relevant effects in ASD children from different ethnic backgrounds.

The present study extends our initial findings by measuring, in addition to total urinary p-cresol, also its three fractions, namely p-cresylsulfate, p-cresylglucuronate and free p-cresol. Our results confirm the approximate 95:4:1 ratio for p-cresol derivatives previously reported in most studies (Bone et al., 1976; Renwick et al., 1988). Controls display a slightly larger share of p-cresylsulfate compared with ASD cases, in line with previously documented limitations in sulfation capacity among autistic individuals (Alberti et al., 1999). In the present study, we observed a statistically significant increase in urinary p-cresylsulfate and p-cresylglucuronate in ASD children, whereas free p-cresol showed a non-significant trend (Figure 1B–D). Given the detection threshold of our methodology (0.07 μg/ml), results on free p-cresol should be seen with caution. Most importantly, p-cresylsulfate is the most abundant p-cresol derivative also among autistic individuals and it could conceivably represent the “true” toxin, as proposed in recent years for uremic toxicity initially attributed to free p-cresol. In particular, studies on uremic toxicity have demonstrated that p-cresylsulfate impairs functional mechanisms only partly overlapping with those affected by p-cresol. For example, p-cresol reduces oxygen-derived free radical production by granulocytes in vitro (De Smet et al., 2003), whereas p-cresylsulfate activates free radical production by leukocytes, boosting oxidative stress (Meert et al., 2012; Schepers et al., 2007). Elevated p-cresylsulfate levels in chronic kidney disease, as well as in diabetic nephropathy, have been associated with poor clinical outcome, due to endothelial damage and vascular calcifications eventually leading to coronary heart disease (Chiu et al., 2010; Llaubeuf et al., 2010; Meijers et al., 2008; Wang et al., 2010, 2013). The elevated urinary concentrations of p-cresol and p-cresylsulfate found in a subset of small Italian and French autistic children...
are indeed within the range previously found to play clinically significant roles in patients with kidney failure (Liauf et al., 2010). Altogether, a greater involvement of p-cresylsulfate rather than of free p-cresol in autism is more compatible with our results. Their clinical and pathological implications specifically within the context of small autistic children will merit further investigation.

In humans, urinary p-cresol originates from environmental sources and/or it is synthesized from tyrosine by some gut bacterial strains, primarily belonging to the Clostridium and Pseudomonas species (Cafaro et al., 2005; Selmer & Andrei, 2001; for review see Persico & Napolioni, 2012). A similar compound also linked to autism is represented by propionic acid, a behaviorally active short chain fatty acid also of environmental origin or produced in the gut by anaerobic bacteria, such as Clostridia and Propionibacteria (Al-Lahham et al., 2010; MacFabe et al., 2011). Another bacterial compound, 4-ethylphenylsulfate (4EPS), detected at high plasma levels in mouse models of ASD and sharing a surprising homology in chemical structure with p-cresol, promotes anxiety-like behaviors which are completely reverted by treatment with B. fragilis in rodents (Hsiao et al., 2013). This evidence, while supporting pathomorphological roles for gut-derived compounds in ASD or, at a minimum, their potential as biomarkers in small autistic children, does not address the origin of their accumulation. Conceivably, enhanced urinary p-cresol amounts could reflect either environmental exposure or gut infection with cresol-producing bacteria, or both. Within this framework, the difference in baseline urinary p-cresol between French and Italian typically developing children (52.0 ± 7.8 versus 91.1 ± 8.7 µg/ml, Student t = 2.994, 90 df, p < 0.01) could stem from differences in environmental exposure, since the Italian sample was entirely drawn from large cities (Rome and Naples), while the French sample was recruited in the less densely populated and more rural Loire region. On the other hand, Ethnic differences in baseline p-cresol excretion could also reflect differences in gut flora composition, presumably due to ethnic-specific nutrient intake (Holmes et al., 2008). Distinct dietary habits can in fact shape the gut microbiome (de Wouters et al., 2012) and could thus indirectly influence urinary p-cresol levels. In comparison to typically developing individuals, autistic children display additional levels of complexity: environmental exposure may be especially relevant to “picky eaters”, who could absorb greater amounts of this compound by selecting cresol enriched foods; some parents could prefer administering food enriched in cresol antioxidants to their children, although this practice is relatively uncommon in Italy; autistic children with co-morbid intellectual disability may be more prone to bringing to their mouth objects covered with cresol-containing gloss. Meanwhile, also differences in gut bacterial composition between autistic and typically developing children may well yield enhanced synthesis of p-cresol in the gut lumen, resulting in more abundant urinary excretion of the compound. Within this framework, the age effect could be due to the maturation of the GI immune system and to its increasing ability to control the overgrowth of cresol-producing bacterial strains (Ashwood et al., 2006; Jyonouchi et al., 2005; Persico & Napolioni, 2012). The present replication of elevated urinary p-cresol levels in an ethnically independent sample of autistic children encourages further investigations aimed at assessing the incidence of cresol-producing gut infections in our cohort and the mechanism boosting p-cresol levels in some small ASD children.

Interest into urinary p-cresol and/or its conjugated derivative forms is spurred not only by their toxicant effects, but also by their potential inclusion into a multi-biomarker panel for ASD in small children. Urinary p-cresol beyond highest control levels was recorded in 9/32 (28.1%) and in 4/22 (18.2%) Italian and French ASD children aged <8 years, respectively (Figure 3 in Altieri et al., 2011, and Figure 3A here). Given the heterogeneity of ASD and the specific etiopathogenic underpinnings present in each patient, multiple-biomarker panels able to foster earlier diagnoses and to predict clinical prognosis as well as treatment response will be very useful in clinical practice (Walsh et al., 2011). ASD complexity requires sex- and age-specific panels, including biomarkers from different biological and physiological domains (Ruggeri et al., 2014). Furthermore, some racial and ethnic groups may require specific biomarker panels, accounting not only for genetic markers conferring ASD vulnerability, but also for differences in gut-derived compounds (Persico & Napolioni, 2012). Preliminary results indicate that p-cresol may represent one of several compounds able to, collectively, distinguish small autistic children from controls with promising reliability (Neri et al., unpublished manuscript).

Conclusions

The present and previous results (Altieri et al., 2011), confirm that urinary amounts of the toxic compound p-cresol and of its derivatives, especially p-cresylsulfate, are significantly elevated in a sizable subgroup of small autistic children. These results were replicated in two case-control samples belonging to distinct ethnic groups, recruited in different geographical areas in Europe and screened at two independent clinical sites. Unbiased metabolomic and microbiomic approaches will have to define the degree of connection between elevated urinary p-cresol, skewed urinary metabolomic profiles and gut flora composition in our ASD patients. Clinical studies involving large cohorts will also be needed to conclusively define possible dose-dependent influences on the spectrum and severity of clinical signs and symptoms of ASD, as well as on endophenotypic subgroupings. Finally, perspective studies of high-risk infant siblings will be instrumental in determining the potential of urinary p-cresol and/or p-cresylsulfate as biological markers for an ASD diagnosis in small children and for predicting developmental trajectories.

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Declaration of interest

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Supplementary material available online

Supplementary Table S1 and Figure S1