Slow Intestinal Transit Contributes to Elevate Urinary p-Cresol Level in Italian Autistic Children

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The uremic toxin p-cresol (4-methylphenol) is either of environmental origin or can be synthetized from tyrosine by cresol-producing bacteria present in the gut lumen. Elevated p-cresol amounts have been previously found in the urines of Italian and French autism spectrum disorder (ASD) children up until 8 years of age, and may be associated with autism severity or with the intensity of abnormal behaviors. This study aims to investigate the mechanism producing elevated urinary p-cresol in ASD. Urinary p-cresol levels were thus measured by High Performance Liquid Chromatography in a sample of 53 Italian ASD children assessed for (a) presence of Clostridium spp. strains in the gut by means of an in vitro fecal stool test and of Clostridium difficile-derived toxin A/B in the feces, (b) intestinal permeability using the lactulose/mannitol (LA/MA) test, (c) frequent use of antibiotics due to recurrent infections during the first 2 years of postnatal life, and (d) stool habits with the Bristol Stool Form Scale. Chronic constipation was the only variable significantly associated with total urinary p-cresol concentration ($P < 0.05$). No association was found with presence of Clostridium spp. in the gut flora ($P = 0.92$), augmented intestinal permeability ($P = 0.18$), or frequent use of antibiotics in early infancy ($P = 0.47$). No ASD child was found to carry C. difficile in the gut or to release toxin A/B in the feces. In conclusion, urinary p-cresol levels are elevated in young ASD children with increased intestinal transit time and chronic constipation. *Autism Res* 2015, 00: 000–000. © 2015 International Society for Autism Research, Wiley Periodicals, Inc.

**Keywords:** autism; autism spectrum disorder; biomarker; constipation; gut; intestinal transit; organic contaminants; neurotoxicity

**Introduction**

Autism spectrum disorder (ASD) is a neurodevelopmental condition with onset in early childhood, characterized by deficits in social interaction and communication, as well as by repetitive behaviors, rigid adherence to routines, restricted interests, and abnormal sensory processing [American Psychiatric Association, 2013; Persico, 2013]. The prominent clinical heterogeneity of ASD similarly reflects a remarkable etiological heterogeneity, encompassing several combinations of genetic, epigenetic, and environmental factors yielding abnormal neurodevelopment through “personalized” gene–gene and gene–environment interactions [Persico, 2013; Persico & Napolioni, 2013a,b; Persico & Merelli, 2015]. Not surprisingly, the exact causes underlying ASD remain elusive in the majority of patients. Moreover, the frequent involvement of other compartments outside the central nervous system (CNS), such as the immune and gastrointestinal (GI) systems, confers another layer of pathophysiological complexity [McElhanon, McCracken, Karpen, & Sharp, 2014; Onore, Careaga, & Ashwood, 2012].

New insights into ASD etiology are progressively arising from studies selecting subgroups of ASD patients sharing a heritable endophenotype, a set of biomarkers or some outlining clinical characteristics [Ruggeri, Sarkans, Schumann, & Persico, 2014; Sacco et al., 2010, 2012; Walsh, Elsabbagh, Bolton, & Singh, 2011]. For example, a consistent subgroup of ASD patients shows GI comorbidities, leading to a variety of symptoms including diarrhea, constipation, abdominal bloating, irritability, gastro-esophageal reflux/vomiting, and abdominal...
pains/discomfort [Adams, Johansen, Powell, Quig, & Rubin, 2011; Buie et al., 2010; McElhanon et al., 2014; Nikolov et al., 2009]. Many ASD children have also been shown to carry abnormalities in GI physiology, including: (a) increased intestinal permeability reported by some [D’Eufemia et al., 1996; De Magistris et al., 2010], although not all studies [Robertson et al., 2008], (b) overall microbiota alterations [De Angelis et al., 2013; Finegold et al., 2002, 2010; Mulle, Sharp, & Cubells, 2013; Parracho, Bingham, Gibson, & McCartney, 2005; Wang et al., 2011], and (3) gut infection with cresol-producing Clostridium difficile [Elsden, Hilton, & Waller, 1976; Finegold et al., 2002; Keşli, Gökçen, Buluş, & Terzi, 2014; Parracho et al., 2005; Selmer & Andrei, 2001; Song, Liu, & Finegold, 2004; Wang et al., 2011].

Gut bacterial-derived molecules are well known to pass from the gut into the blood stream, exert systemic effects on a variety of systems including the CNS and then be filtered at glomerular level into urines in variable amounts [Halm, Franke, Ashburn, Hebshi, & Willkens, 2008; Krueger, 1990]. Several gut-derived toxicants have been potentially linked to ASD in recent years, including propionic acid (PPA) and p-cresol [Persico & Napolioni, 2013a,b]. The aromatic compound p-cresol (4-methylphenol) can either be synthesized in the gut lumen by cresol-producing bacteria, especially C. difficile [Elsden et al., 1976; Selmer & Andrei, 2001], or can stem from environmental exposure following intestinal, respiratory, or skin absorption [Persico & Napolioni, 2013a,b]; differently from PPA, it does not derive from human metabolism, as humans do not express p-hydroxy-phenylacetate decarboxylase, the enzyme necessary to bring tyrosine metabolism down to the final end product [Selmer & Andrei, 2001]. P-cresol and especially its conjugated derivative p-cresylsulfate, represent some of the best-characterized uremic toxins, shown to negatively impact on several systems in individuals with chronic renal disease [Liabeuf et al., 2010; Persico & Napolioni, 2013a,b]. Our initial study reported significantly elevated amounts of urinary p-cresol in 59 Italian ASD children compared to 59 sex- and age-matched controls [Altieri et al., 2011]. This increase was strictly limited to young children up to 8 years of age and was associated with greater severity of ASD symptoms [Altieri et al., 2011]. These findings were remarkably replicated in an independent and ethnically-distinct sample of 33 French ASD children and matched controls, where urinary levels of p-cresol and its derivate p-cresylsulfate were found to be significantly increased in ASD children, again up to 8 years of age [Gabriele et al., 2014]. These converging results strengthen the potential role of urinary p-cresol as an important player in shaping ASD severity and/or as a biomarker of ASD in young children.

The present study was undertaken to investigate potential factors contributing to elevated urinary p-cresol levels in ASD. We thus measured total urinary p-cresol in a newly-recruited sample of 53 Italian ASD children and assessed their correlation with: (a) presence of Clostridium spp. in the gut and especially C. difficile, (b) intestinal permeability, (c) frequent use of antibiotics due to recurrent infections during the first 2 years of postnatal life, and (d) stool habits. Our results support a primary role for slow intestinal transit in linking ASD to elevated urinary p-cresol, which seemingly characterizes most autistic children with chronic constipation.

**Methods**

**Patient Sample**

A sample of 53 idiopathic ASD patients was recruited at the Department of Physical and Mental Health and Preventive Medicine, Second University of Naples (Naples, Italy). All patients were Caucasians of Italian ethnicity.

Demographic and clinical characteristics are summarized in Table 1. 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, 2012]. Briefly, patients fulfilling DSM-IV diagnostic criteria for Autistic Disorder, Asperger Disorder, or Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) were screened for nonsyndromic autism using Magnetic Resonance Imaging (MRI), Electroencephalography (EEG), audiometry, urinary amino-acid 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. I.Q.
was determined using the Griffith Mental Developmental Scales [Griffiths, 1970].

Clinical history of recurrent use of antibiotics due to recurrent infections during the first two years of life was assessed at intake by the clinician with both parents and tabulated into a bicategorical variable (presence/absence) for statistical analysis.

**Lactulose/Mannitol Test**

Intestinal permeability was assessed using the lactulose/mannitol (LA/MA) test, as previously described [D'Eufemia et al., 1996]. Briefly, 5 g of lactulose (LA) and 2 g of mannitol (MA) were orally administered to fasting subjects and urine samples were collected during the following 5 hr (for urine collection methods, see section “P-cresol measurement by high performance liquid chromatography [HPLC]” below). Saccharide probes were measured by High-performance anion exchange chromatography with pulsed amperometric detection, as described [Generoso et al., 2003]. Intestinal permeability was expressed as the ratio of the recovered percentage of lactulose over mannitol (LA/MA). The cut-off value for the normal range was set at LA/MA <0.030 [D’Eufemia et al., 1996]. The test was administered once to all recruited patients.

**Fecal Stool Test for Clostridium Species Detection**

For *Clostridium* species detection, fecal samples were incubated at 100°C for 10 min and immediately placed on ice to destroy all bacteria in the vegetative form and promote sporulation of any spore-forming bacteria. A small amount of sample was then inoculated in 10 mL culture medium mix of Chocolate (Vitox) general culture medium for detection of fastidious microorganisms, and 10 mL of Brazier’s *C. difficile* selective modified medium. Cultures were plated and incubation was performed under anaerobic conditions, in appropriate envelopes (Compact Plastic Pouches, OXOID AGS) added of a CO2 generator (AnaeroGen Compact, OXOID AGS). Pouches were incubated in a stove thermostat at 36 ± 1°C for 48–72 hr. At the end of incubation, direct count of colonies grown on both media [expressed as colony-forming units/mg (CFU/mg)] was performed. Colonies were stained with Gram Stain, isolated and transferred into fresh medium for the final incubation prior to strain identification.

**Clostridium Difficile Toxin A/B Test**

The direct and qualitative detection of *C. difficile* Toxin A and/or B in human fecal specimens was performed using the Remel Xpect *C. difficile* toxin A/B in vitro immunochromatographic test (Remel Europe, Ltd.), according to the manufacturer’s instructions.

**Bristol Stool Form Scale**

Stool habits were assessed collecting a specific gastroenterologic patient history by means of a modified Pediatric Questionnaire for Gastrointestinal Symptoms based on the Rome III Criteria [Caplan, Walker, & Rasquin, 2005], with the aid of the Bristol Stool Form Scale [Lewis & Heaton, 1997]. Parents retrospectively reported the usual appearance of stool for a period of 6 months prior to intake, according to the seven point Bristol scale, ranging from separate lumps (Type I) of slow transit to watery stools (Type VII) (Supporting Information Table S1). For correlation analysis, Bristol scale types were grouped into three broader categories, as in the study by O’Donnell, Virjee, & Heaton [1990], where the distribution of mean stool form scores with respect to whole gut transit time defined three distinct groups of individuals, each one representative of a different stool habit: constipation (types I–II), regular stool (types III–V), and diarrhea (types VI–VII) (Supporting Information Table S1). A fourth category, “alternating,” was added to define cases that periodically switched from “constipation” to “diarrhea” and back, without ever achieving a regular stool pattern (for example, 3 weeks of constipation alternating with 1 week of diarrhea every month). For regression analysis between total urinary p-cresol amounts and intestinal transit time, cases were ranked in order of Bristol stool form level whereby increasing rank values correspond to decreasing intestinal transit time (i.e. levels I-to-VIII are equivalent of “maximum constipation-to-maximum diarrhea”).

**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. Skin adhesive pediatric urine collection bags were utilized for younger children not yet toiled trained, following instructions provided by the manufacturer. Urine samples were then frozen, shipped in dry ice, and stored at −80°C until analysis.

Total urinary p-cresol concentrations were measured by High performance liquid chromatography with fluorescence detector and quantified after acid hydrolysis as previously described [Altieri et al., 2011; Gabriele et al., 2014]. The 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. As creatinine excretion may be abnormally reduced in ASD children [Whiteley et al., 2006], data were normalized by urinary specific gravity.

**Statistical Analyses**

Kendall’s τ statistics was used for correlation analysis; categorical variables were tested using parametric
Student \( t \)-tests, \( \chi^2 \) statistics, or nonparametric Mann–Whitney \( U \)-tests or Wilcoxon signed-rank test depending on the normality of the distribution, as assessed using the Kolmogorov–Smirnov statistics. Regression analysis between total urinary \( p \)-cresol amounts and intestinal transit time was performed ranking cases in order of constipation level, where increasing rank values corresponded to decreasing degree of constipation, in accordance with Bristol scale types. Quantitative data are presented as mean ± S.E.M. Two-tail \( P \)-values are reported throughout the manuscript and statistical significance is set at \( P < 0.05 \), following correction for multiple testing using the Benjamini–Hochberg procedure [Benjamini, Drai, Elmer, Kafkafi, & Golani, 2001].

Results

Total urinary \( p \)-cresol levels among the 53 ASD patients recruited for this study are superimposable to those found in 59 ASD patients from our previous study [Altieri et al., 2011] (138.6 ± 18.2 and 123.5 ± 12.8 \( \mu \)g/mL, respectively), and again significantly higher compared to the previously-assessed 59 controls (91.2 ± 8.7 \( \mu \)g/mL, Student \( t = -2.425 \), 110 df, \( P < 0.01 \)). This increase in urinary \( p \)-cresol levels is not correlated with frequent use of antibiotics to treat recurrent infections during the first two years of life (Student \( t = -0.730 \), 46 df, \( P = 0.47 \)) (Fig. 1A). Similarly, urinary levels of \( p \)-cresol appear unrelated to the presence of gut flora positive for \( C. \) perfringens or other \( Clostridium \) species (\( F = 0.081 \), 2 df, \( P = 0.92 \)) (Fig. 1B). Importantly, none of the 53 ASD children recruited in this study was positive for gut infection with \( C. \) difficile, assessed both by bacterial growth or by detection of toxin A/B. Also intestinal permeability does not seem to influence urinary \( p \)-cresol concentrations (Student \( t = 1.365 \), 45 df, \( P = 0.18 \)) (Fig. 1C). Instead, stool habits represent the only parameter yielding statistical significance even after controlling for multiple testing (\( \chi^2 = 10.162 \), 3 df, \( P = 0.017 \)) (Fig. 1D). In particular, urinary \( p \)-cresol amounts show a gradient ranging from highest among 17 chronically constipated ASD children (218.2 ± 40.8 \( \mu \)g/mL), down to lowest in seven chronically diarrhoeic ASD children (88.6 ± 27.1 \( \mu \)g/mL), as compared to 17 ASD children with regular stools (97.9 ± 20.5 \( \mu \)g/mL) (Fig. 1D). Also nominally significant
tridium species, some of the major producers of \[\text{Persico & Napolioni, 2013a,b}\]. Gut infection with \[\text{Persico & Napolioni, 2013a,b}\]. However, the most important source of respiratory tracts is relatively common \[\text{Persico & Napolioni, 2013a,b}\]. Respiratory infections triggered by several bacteria in the gut lumen, increased absorption through the gut wall, and more abundant urinary excretion of the compound. However, in contrast with previous reports suggesting an association between ASD and gut infection with \[\text{C. perfringens}\] spp, especially \[\text{C. difficile}\] \[\text{Elsden et al., 1976; Finegold et al., 2002; Kešli et al., 2014; Parracho et al., 2005; Selmer & Andrei, 2001; Song et al., 2004; Wang et al., 2011]\]. In particular, the fecal flora of the ASD sample assessed here was mainly characterized by the presence of \[\text{C. perfringens}\], which does not produce \[\text{p-cresol}\] and whose presence here is indeed not associated with differences in urinary \[\text{p-cresol}\] amounts (Fig. 1B). A frequent and early use of antibiotics in ASD children with recurrent ear or upper airways infections could have conceivably either fostered an abnormal intestinal colonization by cresol-producing bacteria, such as \[\text{C. difficile}\] \[\text{Holmes, Li, Athanasiou, Ashrafian, & Nicholson, 2011; Kinross, Darzi, & Nicholson, 2011}\], or could have resulted from immune abnormalities possibly promoting this scenario, which is instead not supported by our data (Fig. 1A). Hence, collectively, the present results do not support a skewed selection of gut bacterial strains as the primary cause of elevated urinary \[\text{p-cresol}\] levels in ASD.

The increase in urinary \[\text{p-cresol}\] significantly correlated with only one variable, namely stool type which reflects intestinal transit time. In particular, higher levels of urinary \[\text{p-cresol}\] are linked to chronic constipation (Figs. 1D and 2), with no additional contribution coming from increased gut permeability (Fig. 1C). Interestingly, chronic constipation is one of the most frequent GI issues reported by parents with regard to their autistic children \[\text{Buie et al., 2010; McElhanon et al., 2014; Nikolov et al., 2009}\]. Moreover, fasting increases urinary \[\text{p-cresol}\] levels by slowing intestinal transit in rats \[\text{Kawakami, Kojima, Makino, Kato, & Onoue, 2007}\], whereas fiber-rich diets and probiotics yield the exact opposite effect on urinary \[\text{p-cresol}\] by stimulating intestinal transit \[\text{Cummings, Hill, Bone, Branch, & Jenkins, 1979; Kawakami, Makino, Asahara, Kato, & Onoue, 2005; Nakabayashi et al., 2011}\]. These results corroborate the hypothesis that, both in rodents and humans, slow intestinal transit time and chronic constipation increase the absorption of \[\text{p-cresol}\], resulting in its enhanced renal excretion. Hence, regardless of whether and to what extent \[\text{p-cresol}\] in each single patient has mainly an environmental or gut-bacterial origin, our results point toward intestinal transit time as the main variable determining the rate of \[\text{p-cresol}\] inflow into the body and consequently its urinary outflow rates. It will thus be extremely interesting to examine whether urinary \[\text{p-cresol}\] is specifically elevated in young children with ASD or whether this biochemical characteristic is nonspecifically present also in children with other pediatric conditions associated with chronic constipation.

The gut flora is a complex microbial ecosystem \[\text{Aru-mugam et al., 2011; Holmes et al., 2011; Kinross et al., 2011; Mulle et al., 2013}\]. Several studies have assessed

![Figure 2. Linear regression analysis between total urinary p-cresol concentrations (µg/mL) and degree of constipation assessed in 53 Italian ASD patients.](image)
the fecal flora of autistic individuals, reporting an over-
growth of potentially pathogenic gut microbial species in a sizable subgroup of patients, some of these known to produce \textit{p}-cresol \cite{De Angelis et al., 2013; Elsdon et al., 1976; Finegold et al., 2002, 2010; Kesli et al., 2014; Muller et al., 2013; Parracho et al., 2005; Selmer \\& Andrei, 2001; Song et al., 2004; Wang et al., 2011}. The main limitation of this study is the use of microbiological cultivation methods focused on clostridial strains, which may have possibly missed other more infrequent cresol-producing phyla of microbiota, although the absence of \textit{C. difficile} was also confirmed by the lack of specific A/B toxins. An unbiased profile of the complete fecal flora by pyrosequencing will be necessary to conclusively exclude an infection with nonclostridial cresol-producing gut bacteria as a relevant source of \textit{p}-cresol in ASD. Moreover, a dysbiotic gut bacterial community could also produce an accumulation of other toxic metabolites, in addition to \textit{p}-cresol. For example, PPA, a short fatty acid produced in the gut by anaerobic bacteria yields ASD-like behavioral abnormalities when administered intracerebroventricularly to young rats \cite{Al-Lahham, Peppelenbosch, Roelofs, Vonk, \\& Venema, 2010; MacFabe, Cain, Boon, Ossenkopp, \\& Cain, 2011}. The tryptophan derivative indoxyl sulfate passes the blood-brain barrier using the organic anion transporter 3 (OAT3). Influx of indoxyl sulfate through OAT3 has been shown in rat brain to significantly reduce the efflux of various neurotransmitter metabolites through the same transporter, leading to their accumulation \cite{Ohtsuki et al., 2002}. Together with \textit{p}-cresylsulfate, indoxyl sulfate is frequently found elevated in patients with chronic kidney disease (CKD) and represents a risk factor for CKD progression \cite{Meijers \\& Evenepoel, 2011} and for cognitive impairment \cite{Watanabe, Watanabe, \\& Nakayama, 2014}. Urinary metabolomics profiles in our ASD patients may be able to better define the degree of relationship between \textit{p}-cresol/\textit{p}-cresylsulfate and these other uremic compounds endowed with comparable toxicity.

The patient history questionnaire used in this study is focused on the frequency of antibiotic use and on recurrent infections during the first 2 years of postnatal life. This is a critical time just prior to and/or concomitant with the onset of clear behavioral abnormalities, when many children who will develop ASD suffer from recurrent infections, especially affecting middle ear, upper airways, or gut \cite{Sacco et al., 2010, 2012}. ASD children treated with vancomycin for \textit{Clostridium} infections have been reported to show significant improvements in behavioral symptoms, which regress approximately two weeks after discontinuation of antibiotic treatment \cite{Sandler et al., 2000}. The temporary elimination of some gut microbial species could conceivably be responsible for these beneficial effects. We cannot exclude that our results may have been influenced by very recent antibiotic use, which was not explored in this study, although this instance should have occurred in very few cases, at most.

Finally, the total \textit{p}-cresol measured here is actually the sum of free \textit{p}-cresol and its two conjugated derivatives, namely \textit{p}-cresylsulfate and \textit{p}-cresylglucuronate \cite{Gabriele et al., 2014}. Mounting evidence points toward \textit{p}-cresyl-


cresol statistically acts as an additive component to the behavioral consequences of abdominal discomfort conceivably due to chronic constipation in ASD; (d) the effects of gut mobilization on urinary \textit{p}-cresol levels and on autistic behaviors in chronically constipated ASD children, and (e) the effects of \textit{p}-cresol and \textit{p}-cresylsulfate in cellular and rodent models of ASD, eventually providing a link, if present, between these compounds and ASD pathophysiology. These studies will be needed to conclusively clarify the existence of possible dose-dependent influences by \textit{p}-cresol and \textit{p}-cresylsulfate on the spectrum and severity of autistic symptoms, to verify whether chronic

Conclusions

The present results reveal that elevated urinary amounts of the toxic compound \textit{p}-cresol in ASD are significantly influenced by intestinal transit time, as reflected by stool habits in a sizable subgroup of young autistic children reporting chronic constipation. We have recently found urinary \textit{p}-cresol levels correlated with autism severity \cite{Altieri et al., 2011} and with behavioral abnormalities \cite{Gabriele et al., 2014}, possibly implicating a neuroactive role for this metabolite in ASD pathophysiology and/or its potential as biological marker of ASD in small children. The present results spur interest into further addressing: (a) the specificity of elevated urinary \textit{p}-cresol in ASD, as compared to other pediatric conditions associated with chronic constipation, (b) intestinal transit time, as measured directly by performing a Sitzmark colonic transit study \cite{Eltringham et al., 2008}; (c) the correlation between chronic constipation, urinary \textit{p}-cresol and intensity of autistic behaviors, to assess whether \textit{p}-cresol statistically acts as an additive component to the behavioral consequences of abdominal discomfort conceivably due to chronic constipation in ASD; (d) the effects of gut mobilization on urinary \textit{p}-cresol levels and on autistic behaviors in chronically constipated ASD children, and (e) the effects of \textit{p}-cresol and \textit{p}-cresylsulfate in cellular and rodent models of ASD, eventually providing a link, if present, between these compounds and ASD pathophysiology. These studies will be needed to conclusively clarify the existence of possible dose-dependent influences by \textit{p}-cresol and \textit{p}-cresylsulfate on the spectrum and severity of autistic symptoms, to verify whether chronic
constipation indeed represents a real endophenotype or is at least as predictive of ASD severity, to define whether and to what extent evidence-based gut mobilizing and/or probiotic therapies may ameliorate behavioral symptoms in ASD, and to identify the subset of autistic patients which may ultimately benefit from these targeted approaches.

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References


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Table S1. Bristol Stool Form Scale types and derived stool categories, as used in our statistical analyses (adapted from Lewis and Heaton, 1997).