Urinary p-cresol is elevated in small children with severe Autism Spectrum Disorder.

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Urinary *p*-cresol is elevated in small children with severe Autism Spectrum Disorder

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Abstract

Context: several studies have reported a frequent overgrowth of unusual gut bacterial strains in autistic patients, able to push the fermentation of tyrosine up to the formation of *p*-cresol.

Objectives: to compare urinary *p*-cresol levels in 59 autistic patients and 59 matched controls.

Materials and Methods: *p*-cresol was measured by HPLC-UV.

Results: urinary *p*-cresol is significantly elevated in autistic children below 8 years of age ($P<0.01$), more severely affected ($P<0.05$), and typically females ($P<0.05$).

Discussion: urinary cotinine measurements exclude smoking-related hydrocarbon contaminations.

Conclusion: urinary *p*-cresol may serve as a biomarker of autism liability in small children, especially in females.

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 and 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). *Pseudomonas stutzeri* forms *p*-cresol from toluene (Cafaro et al., 2005). Following intestinal absorption, *p*-cresol travels through the blood stream partly protein-bound, partly in free form; the latter is then filtered at the glomerular level and can be found in the urines 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 urines 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

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 non-syndromic autism using MRI, EEG, 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 ($\pm$ 1 yr) was applied to recruit 59 typically developing controls among the offspring of clinical/academic personnel. Cases and controls are all Caucasians of Italian ethnicity. All parents gave written informed consent for their children, using the consent form approved by the I.R.B. of University Campus Bio-Medico (Rome, Italy).

_P_-cresol measurement by HPLC

Urinary *p*-cresol concentrations were measured by HPLC-UV multiwavelenght DAD, adapting the method previously described by Birkett et al. (1995), and by King et al. (2009). Briefly, an aliquote of frozen urines was thawed, mixed and 30µL were transferred into a tube containing 60µL di HCl 6M and heated at 90°C for 60 minutes to hydrolyze glucuronide and sulfate conjugates. After cooling, *p*-cresol was extracted with 1 ml of diethyl ether; 300µL of the organic phase were then transferred into a tube with 20µL of NaOH 0.1N, and lyophilized. The residue was dissolved in 300µL of MilliQ H_2O/Acetonitrile 5% and 30 µl were injected for HPLC analysis (Dionex Ultimate 3000 HPLC system with variable wavelength detector, column Dionex Acclaim®120 C18 5µm 120 A° 4,6x150 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 minute, 0-50% B; 15-17 minute, 50-100% B; 17-20 minute 100% B; 20-21 minute 100%-50% B; 21-25 minute 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 (1mg/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.

Cotinine measurement by ELISA

Urinary cotinine levels were measured using the Cotinine ELISA kit (Calbiotech): 10 µl of standard, controls and specimens were pipetted into selected well in duplicate. 100 µl of the Enzyme Conjugate were added to each well. After incubation (60 minutes at room temperature, in the dark), the wells were washed and 100 µl of Substrate reagent was added to each well. After incubation (60 minutes at room temperature, in the dark), 100 µl of Stop Solution were added to

each well. The absorbance was read on 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 Zielińska-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 analysis

Cases and controls were contrasted using Student 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). Quantitative data are presented as mean $\pm$ S.E.M. Statistical significance is set at $P<0.05$.

Results

Urinary *p*-cresol concentrations are significantly higher among 59 autistic patients compared to 59 matched controls (123.5 $\pm$ 12.8 vs 91.2 $\pm$ 8.7 µg/ml, Student-t = 2.207, 116 df, $P<0.05$). This increase is interestingly restricted to ASD children younger than 8 years of age (134.1 $\pm$ 20.1 vs 70.3 $\pm$ 6.7 µg/ml, t = 2.922, 60 df, P=0.005), whereas no difference was found between patients and controls aged 8 or older (111.0 $\pm$ 14.5 vs 112.7 $\pm$ 15.5 µg/ml, t= -0.81, 54 df, P=0.936) (Fig.1). Before age 8, levels above 150 µg/ml were detected in 9/32 (28.1%) ASD patients vs 0/32 controls (P=0.002) (Fig. 2), while levels above 140 µg/ml were detected in 9/32 (28.1%) ASD patients vs 1/32 (3.1%) controls (P=0.0127). Urinary *p*-cresol displays no correlation with urinary cotinine levels (Kendall's τ =-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, non-smokers displayed higher, and not lower urinary *p*-cresol levels compared to light (4 ASD patients and 1 control) and heavy (2 controls) passive smokers (108.8 $\pm$ 8.2 vs 97.7 $\pm$ 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 do 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*sex interaction (P=0.02). In fact, 15 ASD females have 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, 30df, P<0.05), while female and male controls do not differ (70.6 ± 15.3 vs 98.2 ± 10.6 μg/ml). Among children aged 7 or below, 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, 12df, P=0.004), whereas differences between male autistics and controls do not reach statistical significance (104.7 ± 17.0 vs 75.1 ± 7.2 μg/ml, respectively; t-test=1.128, 46df, 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 below 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).

Interestingly, 8 of the 9 (88.9%) small children with urinary *p*-cresol above 150 μg/ml satisfy 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 display significantly higher rates of the less severe form, PDD-NOS (Fisher's exact test, P<0.05). Among children aged 7 or below, all fifteen CARS items measuring clinical severity are positively correlated with urinary *p*-cresol amounts, reaching statistical significance for imitation (item n. 2: τ=0.396, P=0.020, N=21), use of body (item n. 4: τ=0.330, P=0.036), verbal communication (item n. 11: τ=0.387, P=0.023), and general impression (item n. 15: τ=0.534, P=0.002). Also ADOS, ADI-R and VABS scores display correlation trends consistent with those found with the CARS, but P-values do 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, display non-significant trends (P<0.1) toward more frequent mental retardation [7/8 (87.5%) vs 8/17 (47.1%)], self-injurious behaviours [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 5 spoken words and of social abilities after initial acquisition. No significant correlations between urinary *p*-cresol and behavioural measures are present in ASD children older than 7 years of age.

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. This increase is present in approximately 30% of autistic children aged seven or younger, the majority being girls in our sample. Urinary excretion of *p*-cresol appears to normalize after age 7 (Fig. 1). 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 dosing urinary cotinine, the nicotine metabolite representing the best known marker of passive and active smoking (Zielińska-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 and 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 among autistic children, particularly enriched in *p*-cresol producing bacteria. 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 excretion through the urines in greater amounts, 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 urines 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 would conceivably stem from a maturation of the gut immune system, yielding greater control over clostridial overgrowth. 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 two probes (D'Eufemia, et al., 1996; De Magistris et al., 2010).

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. It will thus be important to assess in a rodent model whether and to what extent *p*-cresol can influence the function and/or the development of the central nervous system, 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). Another area of concern is represented by the competition exerted by *p*-cresol on hepatic sulfotransferases involved in the sulfation of many pharmacological agents, such as acetaminophen. Briefly, *p*-cresol

undergoes O-sulfonation by the same sulfotransferase which inactivates 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, which could render some ASD patients particularly prone to developing adverse side effects when administered pharmacological therapies.

Conclusions

Urinary amounts of the toxic compound *p*-cresol are significantly elevated in autistic children younger than age 8, especially in girls and in more severely affected patients. The present study thus replicates and extends previous reports indicating *p*-cresol among a host of compounds overrepresented in the urines of ASD patients (Yap et al., 2010). 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 behaviour, and personalized response to pharmacological treatments. 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.

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Declaration of interests

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Figure Legends

Figure 1. Urinary *p*-cresol concentrations by age group, in 59 ASD patients and in 59 age-, sex-, and ethnically-matched controls. 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.

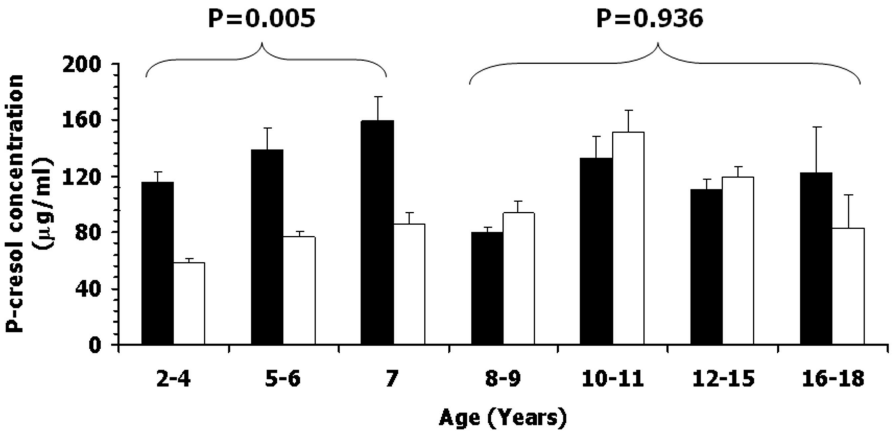
Figure 2. Urinary *p*-cresol concentrations in 32 ASD patient-control pairs, up to 7 years of age or younger. The nine patients highlighted by black circles are all above the maximum *p*-cresol concentration recorded in a typically developing child within this same age range (149.8 μg/ml, as highlighted by the hyphenated line).

Table 1. Demographic and clinical characteristics of the autistic sample. Total N=59, unless otherwise specified

	N	Mean/Median	Range
Age in yrs (mean± S.E.M.):	N = 59	8.29 ± 0.07	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) Stereotypies		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%
	PDD-NOS	18	30.5%
I.Q. (N=45):	*>70*	17	37.8%
	≤ 70	28	62.2%



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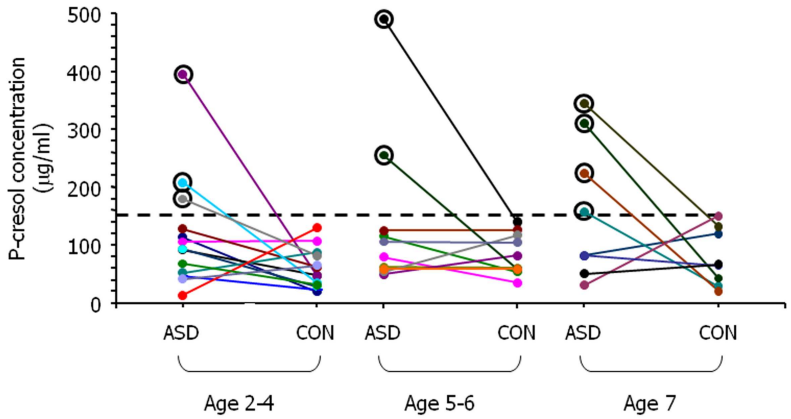

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Urinary *p*-cresol is elevated in small children with severe Autism Spectrum Disorder

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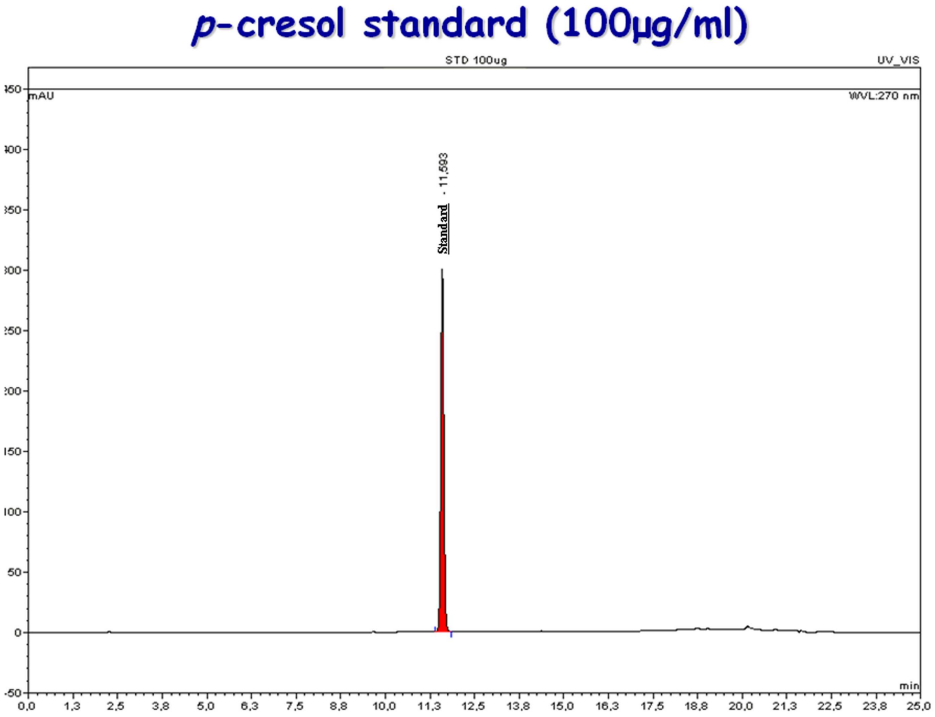
Supplementary Figures (for reviewers only)

Supplementary Figure S1. HPLC plot showing the peak recorded by injecting 100 μg/ml of *p*-cresol standard.

Supplementary Figure S2. HPLC plot of the urine sample from a 5 years old typically-developing male control. The *p*-cresol peak is highlighted in red.

Supplementary Figure S3. HPLC plot of the urine sample of a 4 years old autistic boy. The *p*-cresol peak is highlighted in red.



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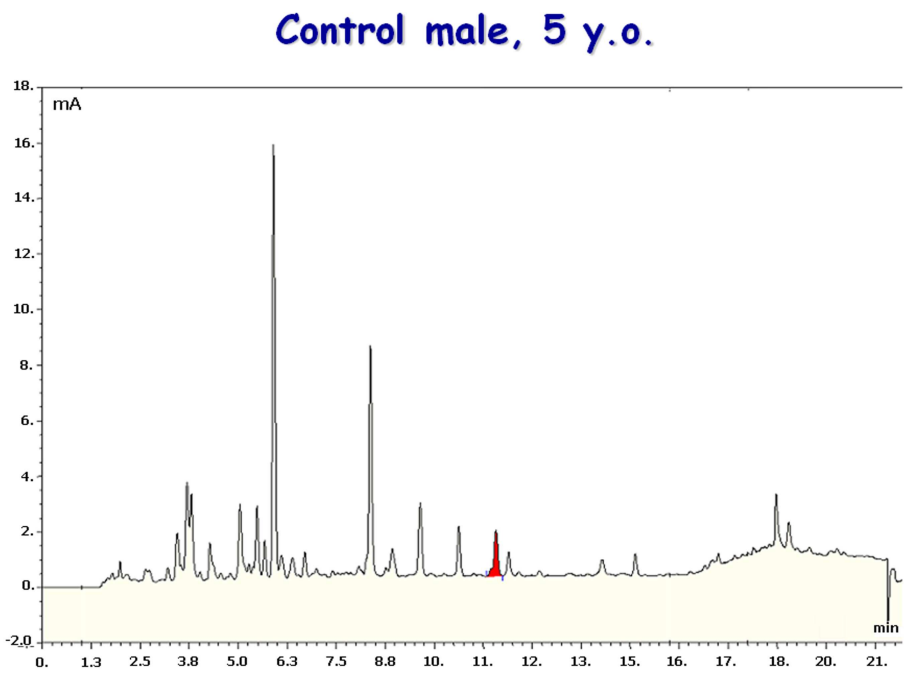

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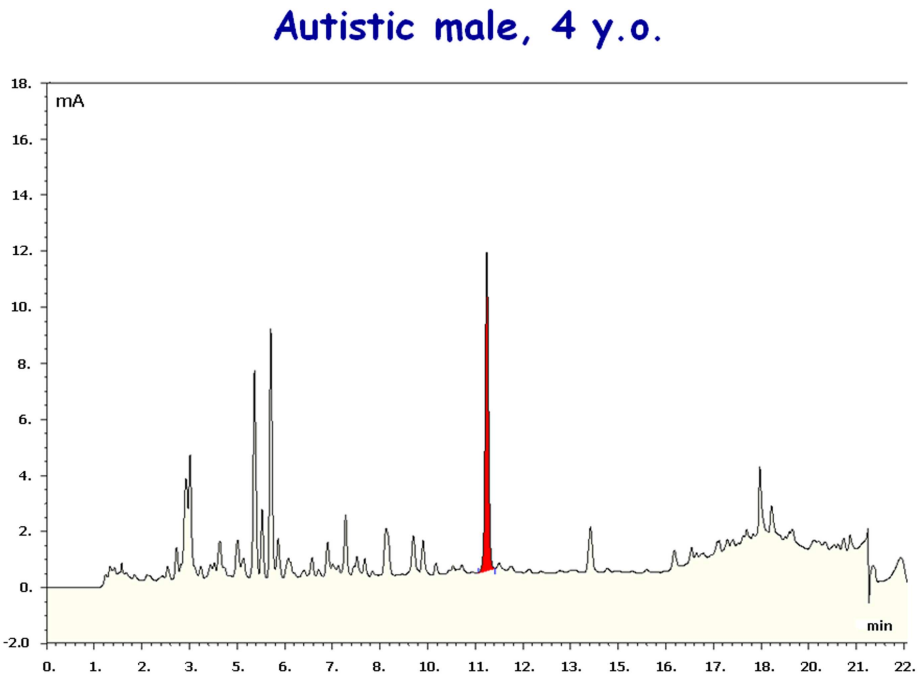

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