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Di-(2-ethylhexyl) phthalate and autism spectrum disorders

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ABSTRACT

ASDs (autism spectrum disorders) are a complex group of neurodevelopment disorders, still poorly understood, steadily rising in frequency and treatment refractory. Extensive research has been so far unable to explain the aetiology of this condition, whereas a growing body of evidence suggests the involvement of environmental factors. Phthalates, given their extensive use and their persistence, are ubiquitous environmental contaminants. They are EDs (endocrine disruptors) suspected to interfere with neurodevelopment. Therefore they represent interesting candidate risk factors for ASD pathogenesis. The aim of this study was to evaluate the levels of the primary and secondary metabolites of DEHP [di-(2-ethylhexyl) phthalate] in children with ASD. A total of 48 children with ASD (male: 36, female: 12; mean age: 11 ± 5 years) and age- and sex-comparable 45 HCs (healthy controls; male: 25, female: 20; mean age: 12 ± 5 years) were enrolled. A diagnostic methodology, based on the determination of urinary concentrations of DEHP metabolites by HPLC-ESI-MS (HPLC electrospray ionization MS), was applied to urine spot samples. MEHP [mono-(2-ethylhexenyl) 1,2-benzenedicarboxylate], 6-OH-MEHP [mono-(2-ethyl-6-hydroxyhexyl) 1,2-benzenedicarboxylate], 5-OH-MEHP [mono-(2-ethyl-5-hydroxyhexyl) 1,2-benzenedicarboxylate] and 5-oxo-MEHP [mono-(2-ethyl-5-oxohexyl) 1,2-benzenedicarboxylate] were measured and compared with unequivocally characterized, pure synthetic compounds (>98%) taken as standard. In ASD patients, significant increase in 5-OH-MEHP (52.1%, median 0.18) and 5-oxo-MEHP (46.0%, median 0.096) urinary concentrations were detected, with a significant positive correlation between 5-OH-MEHP and 5-oxo-MEHP ($r_s=0.668$, $P<0.0001$). The fully oxidized form 5-oxo-MEHP showed 91.1% specificity in identifying patients with ASDs. Our findings demonstrate for the first time an association between phthalates exposure and ASDs, thus suggesting a previously unrecognized role for these ubiquitous environmental contaminants in the pathogenesis of autism.

Key words: autism, di-(2-ethylhexyl) phthalate, endocrine disrupters, HPLC-ESI-MS urine analysis, secondary metabolites.

INTRODUCTION

ASDs (autism spectrum disorders), also known as PDDs (pervasive developmental disorders), are a group of complex neurodevelopment disorders, characterized by social impairments, communication difficulties, and restricted, repetitive and stereotyped patterns of behaviour.

A dramatic increase in frequency of ASDs has been reported over the last 20 years (Weintraub, 2011; Kim et al., 2011). Nevertheless, it is difficult to determine how much of this increase may be due to actual increase in the incidence or to increased awareness and diagnosis. The aetiology is unknown, but it is believed to result from disruption of normal neurobiological mechanisms primarily in the prenatal period (Nelson, 1991). Although it is widely recognized that ASDs may

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Abbreviations: ASD, autism spectrum disorder; CARS, childhood autism rating scale; CI, confidence interval; DEHP, di-(2-ethylhexyl) phthalate; ED, endocrine disruptor; HC, healthy control; HPLC-ESI-MS, HPLC electrospray ionization MS; MEHP, mono-(2-ethylhexenyl) 1,2-benzenedicarboxylate; 5-OH-MEHP, mono-(2-ethyl-5-hydroxyhexyl) 1,2-benzenedicarboxylate; 6-OH-MEHP, mono-(2-ethyl-6-hydroxyhexyl) 1,2-benzenedicarboxylate; OS, oxidative stress; 5-oxo-MEHP, mono-(2-ethyl-5-oxohexyl) 1,2-benzenedicarboxylate; QC, quality control; RP-HPLC-ESI-MS, reverse-phase HPLC-ESI-MS; ROC, receiver operating characteristic; SPE, solid phase extraction; PPARα, peroxisome-proliferator-activated receptor α; TH, thyroid hormone.

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have a strong genetic component (Risch et al., 1999; Anney et al., 2011). To the best of our clinical experience, not more than 5% of all autism cases appear to be due primarily to single gene mutations. In particular, syndromic ASD accounts for 15–20% of ASDs, and other complex genetic factors appear to play a major role in non-syndromic forms of autism. World literature evidence seems to indicate that autism has a strong genetic component (10–20%, Geschwind, 2011) which, by itself, does not fully explain the prevalence of the disorder exponentially increasing overthe last two decades. It is quite likely that exposure to potential environmental factors has differential effects depending on genetic background.

According to our observation, the focus of autism research is shifting from purely genetic influences to multifactorial diseases in which complex set of genes should be associated with relevant environmental factors (Larsson et al., 2009; Herbert, 2010). To date, no information is available on the potential role in the pathogenesis of autism for phthalates, ubiquitous contaminants (Griffiths et al., 1985; Bauer and Herrmann, 1997) used as plasticizers, solvents and additives in many consumer products, i.e. vinyl flooring, wall coverings, food containers and cosmetics (Schettler, 2006; Wormuth et al., 2006). In particular, DEHP [di-(2-ethylhexyl) phthalate] represents one of the most commonly used plasticizer in pharmaceutical and medical devices (U.S. FDA 2011). DEHP is primarily metabolized to monoester, further oxidized in ω and ω-1 positions leading to a pool of secondary metabolites (Albro, 1986; Koch et al., 2003, 2006), and it can be hypothesized that all DEHP metabolites, derived from a hydrolytic/oxidative pathway could, after glucuronidation, be excreted in urine (Figure 1).

Children are known to be exposed to DEHP levels twice as high as those for adults (Hellerstedt et al., 2008; Wittassek et al., 2009). Foetuses and neonates are highly sensitive to physiologically active agents because they are exposed during critical periods of human development (Latini et al., 2003; Kim et al., 2009; Cho et al., 2010; Suzuki et al., 2010). Furthermore, in the case of intensive therapy procedures, neonates can have higher exposure to phthalates and to their toxic monoesters supposed to be EDs (endocrine disruptors) (Silva et al., 2003a, 2003b; Latini et al., 2004). EDs may also be transferred from the mother to the developing foetus across the placenta or to the newborn through breast-feeding (Hines et al., 2009; Latini et al., 2009). For these reasons, the French National Assembly has required the ban of phthalates and parabens (Bill of Law adopted by French National Assembly on 3 May 2011). Moreover, very recently, prenatal phthalate exposure has been reported to decrease child mental/motor development, and increase internalizing behaviour during the preschool years (Whyatt et al., 2011), thus consolidating the concept of an adverse effect of these contaminants on function of the CNS (central nervous system).

The aim of the present study was to evaluate the levels of the primary and secondary metabolites of DEHP in autistic children by using innovative chemical reverse approach (Alcaro et al., 2009; Papini, 2009).

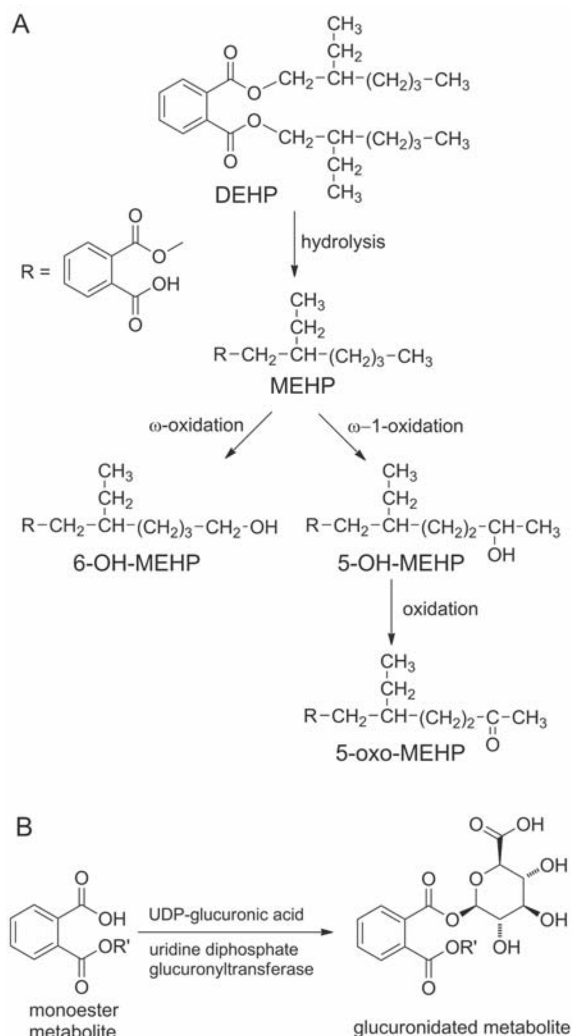

Figure 1 Metabolism of DEHP
(**A**) Hydrolytic/oxidative pathway leading to MEHP and secondary metabolite formation. (**B**) Formation of glucuronic conjugates of DEHP metabolites.

MATERIALS AND METHODS

Subject population

A total of 48 children with ASD (male: 36, female: 12; age at examination: 11.0 $\pm$ 5 years) were recruited from the staff of Children Neuropsychiatric Department, Siena, Italy. All the 48 patients with ASD, diagnosed by DSM IV (Diagnostic and Statistical Manual of mental disorders) and evaluated using ADOS (autism diagnostic observation schedule), ABC (autism behaviour checklist) and CARS (childhood autism rating scale) scores entered the study. Patients with Rett syndrome, X-fragile syndrome, inborn errors of metabolism, 21 trisomy, tuberous sclerosis and gene microdeletions were excluded from the present study. Informed consent from the parents or

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tutors was obtained, and institutional review board approval was obtained for the study. Mean age at diagnosis was between 30 and 36 months. However, the diagnosis for the examined ASDs population go back to procedures employed several years ago (today, mean age at the diagnosis for infantile autism is about 18–24 months). Forty-five gender- and age-comparable HCs (healthy controls) [male: 25, female: 20; age at examination: 12 ± 5 years] were randomly chosen from outpatients who had no pathological symptoms.

Urine from children with ASD and HCs were collected in polypropylene specimen cups, divided into aliquots (1.0 ml) and frozen at −20℃ until analysis. Field blanks consisted in purified water collected in polypropylene tubes and frozen at −20℃.

Determining secondary metabolites in urine

The metabolites measured in this study included: MEHP [mono-(2-ethylhexenyl) 1,2-benzenedicarboxylate] and 6-OH-MEHP [mono-(2-ethyl-6-hydroxyhexyl) 1,2-benzenedicarboxylate], 5-oxo-MEHP [mono-(2-ethyl-5-oxohexyl) 1,2-benzenedicarboxylate] and 5-OH-MEHP [mono-(2-ethyl-5-hydroxyhexyl) 1,2-benzenedicarboxylate]. All these metabolites were synthesized in the Laboratory of Peptide and Protein Chemistry and Biology (PeptLab) following their previously described procedure (Nuti et al., 2005). The unequivocally characterized synthetic metabolites were used as pure analytical standards (>98% purity) for quantitative determination in urine from ASD children and HCs.

All the solvents (acetonitrile, water, buffers, etc.), labware (polypropylene vials containing urine, solvent bottles, SPE (solid phase extraction) cartridge, Teflon capped-glass bottles, pipettes) and instrumentation used during SPE procedure and the HPLC-ESI-MS (HPLC electrospray ionization MS) analytical process, to detect MEHP and/or secondary metabolites in ASD patients and HCs, were verified to be MEHP– and secondary oxidative metabolites-free. The internal standards were prepared in acetonitrile and were used as reported in the literature (Blount et al., 2000; Kato et al., 2004).

First morning urine specimens were collected. All specimens displayed urinary creatinine in the children reference value range, dependent on age and lean body mass (0.5–4.0 mg/kg per 24 h). For creatinine measurement, we used a Synchron AS/ASTRA clinical analyser (Beckman Instruments). We used values (μg/l) for creatinine and (g/l) for dilution correction in the analyses.

For SPE treatment of urine, we prepared the following buffers: ammonium acetate buffer 1 M, pH 6.5; acid buffer, pH 2.0 by preparing a solution of NaH_2PO_4 (0.14 M) and 1% of 85% H_3PO_4; basic buffer was prepared by adding concentrated ammonium hydroxide (1 ml of 30% NH_3 solution) to a 50:50 acetonitrile/water (200 ml). All buffers were stored in sealed bottles at room temperature (20℃): basic buffer was discarded after 1 week, acid buffer after 1 month.

Human urines (1 ml) were defrosted, sonicated, mixed and dispensed in glass tubes. Then ammonium acetate buffer (250 μl, pH 6.5) was added. Incubation with β-glucoronidase (5 μl, 200 units/ml, Roche Biochemical) was performed at 37℃ for 90 min, resulting in quantitative glucuronide hydrolysis of phthalates and metabolites. *Escherichia coli* K12 β-glucuronidase has excellent glucuronidase activity and no measurable lipase activity on phthalate diesters (Blount et al., 2000; Kato et al., 2004). After deconjugation, samples were treated with two steps of SPE, using SPE cartridge (3 ml/60 mg of Oasis HBL, Waters) to remove any contamination of biological matrix, following the procedure described by Blount et al. (2000). The first cartridge was used to retain hydrophobic compounds while the phthalate metabolites were eluted. The second cartridge was helpful in removing residual salts. Analytes were finally eluted with acetonitrile and ethyl acetate, concentrated, re-suspended in water and transferred into vials. All the samples were analysed by RP-HPLC-ESI-MS (reverse-phase HPLC-ESI-MS). One blank and one QC (quality control) sample were included in each batch of samples. The QC sample was spiked with pooled urine and MEHP and secondary oxidative metabolite standards in known concentration (200 ng/ml). When urine analysis resulted in values for metabolite concentrations exceeding the linear range of the analytical method, we subjected a new aliquot of the same sample to the entire process (deconjugation and SPE) and we analysed again. Before analysis, known concentration samples were treated by SPE and analysed by RP-HPLC-ESI-MS to test SPE efficiency. In our study the efficiency of SPE procedure is in accordance with the literature (Mazzeo et al., 2007). Treated urine samples could be stored at 4℃ without degradation. Storage of untreated urines at −40℃ for 6 months showed no decrease in phthalate monoester levels.

The analytical methodology adapted for measuring MEHP and secondary oxidative metabolites in urine had already been described in the literature (Blount et al., 2000; Silva et al., 2003a, 2003b; Kato et al., 2005).

In particular, we used an HPLC tandem MS, RP-HPLC-ESI-MS (Waters, Alliance 2695, Waters, Micromass ZQ) equipped with phenyl column (Betasil, 5 μm, 50 mm × 3 mm, Keystone Scientific) and with a Waters 2996 Photodiode Array Detector. All reagents were of at least analytical reagent grade. The lower LOQs (limits of quantification) were 0.042 μg/l MEHP, 0.048 μg/l 5-OH-MEHP, 0.049 μg/l 5-oxo-MEHP and 0.008 μg/l 6-OH-MEHP. In urine, the LODs (limits of detection) were 0.014 μg/l MEHP, 0.016 μg/l 5-OH-MEHP, 0.016 μg/l 5-oxo-MEHP and 0.002 μg/l 6-OH-MEHP. The chromatographic separations of metabolites were resolved using a linear gradient from 3 to 60% B in 10 min (solvent system A: 0.1% acetic acid in water; B: 0.1% acetic acid in acetonitrile). The flow rate was 0.6 ml/min. The column temperature was 32℃. A guard column (XBridgeTm Phenyl 3.5 μm, 3.0 × 20 mm) was used to prevent column degradation. Column eluates were monitored at 215, 230 and 254 nm. The mass-specific detection was achieved using a Waters, Micromass ZQ ESI in positive ion mode. The product ion with higher signal intensity was selected for the quantitative analysis for each of the four phthalates. The

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Table 1 ROC for DHEP secondary metabolite urinary excretion and infantile autism
AUC, area under curve; 95% CI, 95% confidence intervals for the AUC; Sens., sensitivity; spec., specificity; +PV, positive value; −PV, negative value; ALL, total sum of all the examined di(2-ethylhexyl)phthalate secondary metabolites (5-OH-MEHP+5-oxo-MEHP+6-OH-MEHP+MEHP).

Urinary phthalate	Cut-off	AUC±S.E.M.	95% CI	*P*-value	Sens (%)	Spec. (%)	+PV (%)	−PV (%)
5-OH-MEHP	>0.177	0.638±0.057	0.531–0.735	0.0154	52.1	75.6	69.4	59.6
5-Oxo-MEHP	>0.142	0.666±0.055	0.561–0.759	0.0028	46.0	91.1	85.2	60.3
MEHP	>0.01	0.631±0.058	0.524–0.730	0.0233	79.2	44.2	61.3	65.5
ALL	>0.724	0.671±0.055	0.568–0.764	0.0021	39.2	97.8	95.2	58.7

optimal MS parameters were as follows: the source and desolvation temperature were 120 and 400℃ respectively; the capillary voltage was 3.24 kV; cone voltage 30 kV, nitrogen gas was used as desolvatation gas and as cone gas as well; the cone gas and the desolvatation flow was 60 and 800 l/h respectively; the collision gas was argon with a flow of 0.60 ml/min. Data were acquired and processed using MassLynx™ software (Waters).

Calibration curves for the quantitative urine analysis were calculated for all analytes plotting peak area average (*y*) against concentration of standards (*x*). Five standard solutions (linear range: 2.5–2500 ng/ml) for calibration curve plotting, were prepared for all the metabolites. Curves with correlation coefficients (r^2) greater than 0.998 were generated (MEHP, 5-OH-MEHP, 5-oxo-MEHP 0.999 and 6-OH-MEHP 0.998).

Statistical analysis

All variables were tested for normal distribution (D'Agostino-Pearson test) and data were presented as means with 95% CI (confidence interval) for normally distributed variables or medians means and with 95% CI for non-normally distributed data. Differences between groups were evaluated using independent-sample *t* test (continuous normally distributed data), Mann–Whitney rank sum test (continuous non-normally distributed data), $\chi 2$ statistics (categorical variables with minimum number of cases per cell ⩾5) of Fisher's exact test (categorical variables with minimum number of cases per cell <5). Associations between variables were tested by unvaried regression analysis. The efficiency of urinary phthalates metabolites in discriminating ASD patients from HCs were evaluated using ROC (receiver operating characteristic) curve analyses. All analyses were considered to be statistically significant for *P*-values <0.05. Correction for multiple comparisons was made (Bonferroni's correction). The MedCalc version 9.5.2.0 statistical software package (MedCalc Software) was used.

RESULTS

Among the four metabolites (MEHP and secondary oxidative metabolites) measured in urines of ASDs, we could detect urinary concentration with median values for MEHP (0.055 μg/ml), 5-OH-MEHP (0.18 μg/ml), 6-OH-MEHP (0.017 μg/ml) and 5-oxo-MEHP (0.096 μg/ml). By contrast, in urine from HCs we found the following values for MEHP (0.028 μg/ml), 5-OH-MEHP (0.04 μg/ml), 6-OH-MEHP (0.019 μg/ml) and 5-oxo-MEHP (0.04 μg/ml). However, the data illustrated in Table 2 are without urinary creatinine correction. Differences between groups by using creatinine-adjusted data were comparable with those employing raw data (data not shown). The statistical significance of variables was independent of effects of urinary creatinine concentration. In fact, it is well known that urine concentration/dilution can affect the results of measurements of urinary metabolites.

We found detectable levels of MEHP, 5-OH-MEHP and 5-oxo-MEHP in 79.2, 52.1 and 46.0% of ASD patients respectively (Table 1). Interestingly, 5-OH-MEHP was the major metabolite in terms of urine concentrations, followed by 5-oxo-MEHP and MEHP. Urinary excretion of 5-oxo-MEHP (P=0.005), 5-OH-MEHP (P=0.0224) and MEHP (P=0.0312) was significantly higher in autistic patients compared with gender- and age-comparable HCs (Table 2). By contrast, 6-OH-MEHP (P=0.9305) was not able to discriminate ASDs. High levels of 5-OH-MEHP were detected in 26.6% of HCs. A significant positive correlation between 5-OH-MEHP and 5-oxo-MEHP could be observed only in autistic children (r_s=0.668, P<0.0001), but not in the control population (r_s=0.125, P=0.5565) (data not shown). The completely oxidized form of the metabolite pathway, corresponding to 5-oxo-MEHP, had a specificity of 91.1% in identifying autistic children (Figure 2).

The efficiency of urinary secondary metabolites in discriminating ASD patients from HCs was evaluated using ROC curve analyses (Figure 3, Tables 1 and 2).

Table 2 Comparisons of urinary excretion of secondary metabolites for DEHP in autistic patients (n=48) versus HCs (n=45) in μg/ml
Values are medians (95% confidence intervals of the median).

	ASD healthy	Controls	*P*-value*
5-OH-MEHP	0.18 (0.037–0.399)	0.04 (0–0.124)	**0.0224**
5-Oxo-MEHP	0.096 (0.04–0.17)	0.04 (0.015–0.079)	**0.005**
6-OH-MEHP	0.017 (0.01–0.034)	0.019 (0–0.043)	0.9305
MEHP	0.055 (0–0.11)	0.028 (0–0.059)	**0.0312**

* Mann–Whitney test for independent samples; statistically significant differences are highlighted in bold.

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Finally, we analysed the correlations between total CARS (childhood autism rating scale) score and levels of the different metabolites. Total CARS scores were (means $\pm$ S.D.) 44 $\pm$ 7.4 (range: 31–60). A positive correlation between CARS scores and urinary MEHP levels was observed (ρ=0.429, P=0.0033), whereas no significant relationships with the levels of the other examined metabolites were found (total CARS versus 5-OH-MEHP: ρ=0.120, P=0.4298; total CARS versus 5-oxo-MEHP: ρ=0.127, P=0.3931; total CARS versus 6-OH-MEHP: ρ=0.0085, P=0.9529).

Moreover, we compared ASDs with n=10 patients with Rett syndrome (a genetic form of autism due in up to 95% of cases to mutation of a gene, i.e. MeCP2), urinary phthalate metabolites appear to be, once again, significantly elevated in ASD patients (Table 3). Actual behaviour for most patients with Rett syndrome is not autistic even if all of them do show autistic traits during stage 2 of the disease. Therefore 5-oxo-MEHP urinary excretion appears to be lower in Rett syndrome (P=0.0344), whereas 5-OH-MEHP (P=0.0601), 6-OH-MEHP (P=0.2246) and MEHP (P=0.7098) excretion is comparable with that observed in HCs, despite the heavy medication needed in this multi-system genetic disease. These results indicate that medications are likely not the main cause for the increased urinary phthalate excretion evidenced in ASDs, while suggesting the likely intervention of environmental factors in the pathogenesis of this pervasive development disorder.

DISCUSSION

As ASDs are disorders of brain development, any factors that regulate brain development and are known to be altered in autism should be considered as possibly contributing to the phenotype.

Our findings, for the first time, demonstrate an association between phthalates and ASDs. It is interesting to note that, in 87% of the ASD urine samples, we detected MEHP and 5-oxo-MEHP levels higher than the levels recently reported, by Cho et al. (2010). In that study Cho et al. reported a possible relationship between environmental phthalate exposure (external contamination) and the intelligence level of 667 school-age children randomly recruited from nine elementary schools in five South Korean cities. The reported median values were for MEHP (0.055 μg/ml), 5-OH-MEHP (0.18 μg/ml) and 5-oxo-MEHP (0.096 μg/ml) respectively. As spot urines are among the most commonly used samples in clinical toxicology, we showed the data unadjusted for creatinine. In fact, in epidemiological studies it is not practical to collect 24 h urine samples or, when young children are involved, even first morning voids. In particular, spot urines have also been used in a widely promoted paper relating maternal urinary-phthalate metabolite excretion in mothers to psychomotor development in their children at age 3, recently published (Whyatt et al., 2011). Likewise in ASDs, MEHP was found to be the minor urinary metabolite compared with the corresponding oxidized metabolites as in some adult metabolic diseases (Wittassek and Angerer, 2008). It is possible to hypothesize that, in the case of ASDs, a high MEHP urinary excretion does not correspond to external contamination, but rather MEHP could likely act as an ED.

From our data it is possible to speculate that phthalate metabolites as EDs may play an important and previously not recognized role either in the neurotransmitter system and/or in neurodevelopment, possibly increasing the risk of ASDs. In any case, our findings indicate for the first time that specific DEHP metabolites are statistically significantly increased in ASDs. It is intriguing that urinary levels of the most oxidized form of the DEHP metabolites (5-oxo-MEHP) is able to efficiently discriminate ASDs from HCs (Figure 2). Nevertheless, it has already

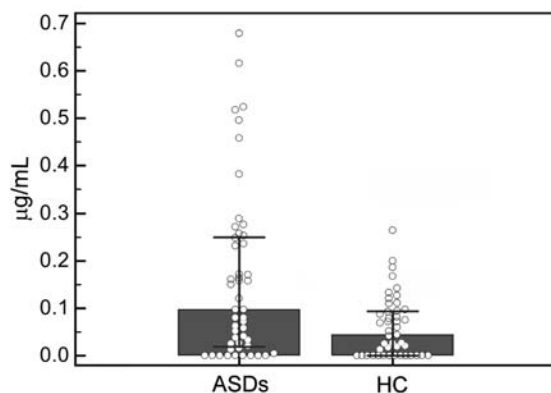

Figure 2 Differences between groups: scatterplots for 5-oxo-MEHP values in ASDs urine samples (ASDs, n=48; HC, n=45)
Data are medians and inter-quartile range.

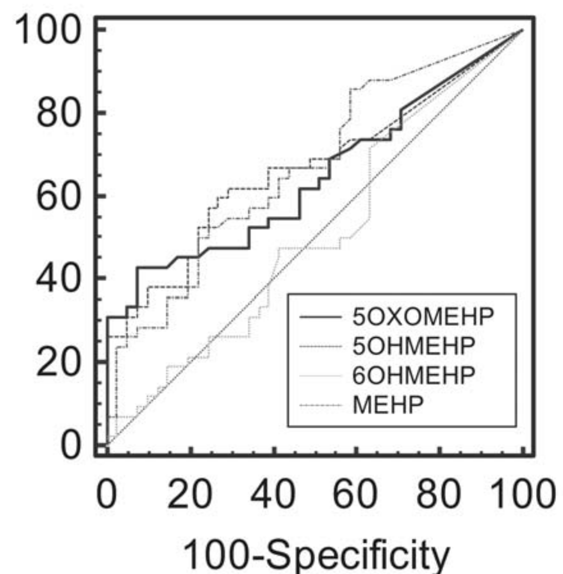

Figure 3 ROC curve analysis (ASD patients versus HC) for DEHP metabolites

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Table 3 **Comparisons of urinary excretion of secondary oxidative metabolites of DEHP in ASDs with $n=10$ patients with Rett syndrome in μg/ml**
Data are expressed as medians and 95% CI; phthalate metabolite concentrations are adjusted to creatinine and are calculated per 100 mg of urinary creatinine. Statistically significant differences are highlighted in bold.

Urinary phthalate metabolite	ASD Rett	Syndrome	*P-value*
5-Oxo-MEHP	0.0997 (0.0397–0.1708)	0 (0–0.0755)	**0.0344**
5-OH-MEHP	0.1740 (0.0311–0.35)	0 (0–0.0091)	**0.0601**
6-OH-MEHP	0.0107 (0–0.0261)	0 (0–0.0832)	0.2246
MEHP	0.0287 (0.0159–0.0847)	0 (0–0.1344)	**0.7098**

been reported that the most oxidized DEHP metabolites have longer half-life of excretion in urine (Koch et al., 2006). Therefore it is possible that environmental factors and OS (oxidative stress) imbalance may interact in leading to increased ASD risk, although further investigation is needed in dissecting this interaction *in vivo*.

A link between oxidative brain damage and ASD has been previously reported by several authors (James et al., 2004; Villagonzalo et al., 2010; Sajdel-Sulkowska et al., 2011). Over the last few years, our team has demonstrated that the biological 'duo' hypoxia–OS is a key player in modulating genotype–phenotype expression in Rett syndrome, a well-established genetic form of ASD (De Felice et al., 2009; Pecorelli et al., 2011; Signorini et al., 2011).

Moreover, in previous reports, a correlation between OS and ASD has been widely explored by measuring different molecules, possibly coming from oxidative pathway as metabolic biomarkers of OS, in biological fluids (Chauhan et al., 2004; James et al., 2004).

To the best of our knowledge, a strict relationship between OS entity and the *in vivo* presence of oxidized forms of DEHP has not been reported before. Therefore we could speculate that, in susceptible subjects, DEHP exposure, when finding OS conditions, can lead to *in vivo* accumulation of toxic metabolites (i.e. the oxidized phthalate metabolites), possibly acting as EDs. Considering that urinary 5-oxo-MEHP reported in our study as increased in ASDs compared with HCs, we can state that our findings are in agreement with an increased OS environment.

Moreover, phthalates are known as EDs, but it is unclear how they could be involved in aetiology of neurodevelopment disorders. Phthalate metabolites activate α-PPARα (peroxisome proliferator-activated receptor α), interfering with cellular proliferation and lipid metabolism. Activation of PPARα by phthalates may alter lipid metabolism in the brain (Clark-Taylor and Clark-Taylor, 2004). Recently the signal transduction pathway of PPARα was correlated with progression of neurodegenerative and psychiatric diseases.

Phthalates also interfere with the TH (thyroid hormone) system by inducing hypothyroidism. Recent studies correlated *in utero* hypothyroxinaemia to decreased intellectual capacity, mental retardation and ASD (Roman, 2007), thus reinforcing our speculation. Moreover, exposure to DBP (di-*n*-butyl phthalate) seems to affect thyroid activity in pregnant women, thus leading to adverse effects on the foetus (Huang et al., 2007).

On the other hand, transient intra-uterine deficits of THs have been shown to result in permanent alterations of cerebral cortex similar to those found in brains of children with autism (Roman, 2007). As a consequence, the current surge of this disease could be related to transient maternal hypothyroxinemia resulting from exposure to anti-thyroid environmental contaminants.

For the first time in this study we correlated different levels of the primary and secondary metabolites with ASDs compared with HC children. These data generate the idea that either current exposure is higher in children with ASD, or alternatively, and more likely, ASD children may differ in their ability to metabolize phthalates.

In conclusion, our findings generate the idea that prenatal plus postnatal phthalate exposure may have synergistic and cumulative actions affecting brain development, thus possibly contributing to the ASD phenotype, according to the general concept generated by Herbert (2010) of 'environmentally vulnerable physiology'. Therefore a prenatal/postnatal screening for urinary DEHP metabolites in the population at high risk for ASDs could have an important social impact.

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