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Di(2-ethylhexyl)phthalate and Autism Spectrum Disorders
Running title: Secondary Metabolites detection in autism

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Abbreviations:
ASDs - Autism Spectrum Disorders
PDDs - Pervasive Developmental Disorders
DEHP - di(2-ethylhexyl)phthalate
FDA – Food and Drug Administration
EDs - Endocrine Disruptors
RP-HPLC-ESI-MS, Reverse Phase-High Liquid Chromatography Electrospray Ionization Mass Spectrometry
DMS - Diagnostic and Statistical Manual of mental disorders
ADOS - Autism Diagnostic Observation Schedule
ABC - Autism Behaviour Checklist
CARS - Childhood Autism Rating Scalescores
HC - Healthy Control
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
5-oxo-MEHP - mono(2-etil-5-oxoesil) 1,2- benzenedicarboxylate
ACN - Acetonitrile
SPE - Solid-Phase Extraction
QC – Quality Control
AUC - Area Under the Curve
CNS - Central Nervous System
O.S. - Hypoxia-Oxidative Stress
α-PPAR - Peroxisome Proliferator-Activated Receptor
DBP - di-n-butyl phthalate

THs - Thyroid Hormones.
LOD - Limit of Detection
LOQ - Limit of Quantification
ROC - Receiver Operating Characteristic

Abstract

Autism spectrum disorders (ASDs) 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 endocrine-disrupting chemicals suspected to interfere with neurodevelopment. Therefore, they represent interesting candidate risk factors for ASDs pathogenesis.

Aim of this study was to evaluate the levels of the primary and secondary metabolites of the di(2-ethylhexyl)phthalate (DEHP) in children with ASDs.

A total of 48 children with ASDs [M: 36, F: 12; mean age: 11 years ± 5 years] and 45 age and sex comparable healthy controls (HCs, M: 25, F: 20; mean age: 12 years± 5 years) were enrolled. A diagnostic methodology, based on the determination of urinary concentrations of DEHP metabolites by HPLC-ESI-MS, was applied to urine spot samples. MEHP, 6-OH-MEHP, 5-OH-MEHP, and 5-oxo-MEHP were measured and compared to unequivocally characterised, pure synthetic compounds (>98%) taken as standards.

In ASDs patients, significantly increased 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 oxidised 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.

Introduction

Autism spectrum disorders (ASDs), also known as Pervasive Developmental Disorders (PDDs), 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). 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 have a strong genetic component (Anney et al. 2011, Risch et al. 1999). To the best of our clinical experience no more than 5% of all autism cases appear to be due primarily to a 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 over the 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 to relevant environmental factors (Herbert 2010, Larsson et al. 2009). To date, no information is available on the potential role in the pathogenesis of autism for phthalates, ubiquitous contaminants (Bauer et al. 1997, Griffiths et al. 1985) used as plasticizers, solvents, and additives in many consumer products, i.e. vinyl flooring, wall coverings, food containers, cosmetics (Schettler 2006, Wormuth et al. 2006). In particular, di-(2-ethylhexyl) phthalate (DEHP) 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 ω1 positions leading to a pool of secondary metabolites (Albro 1986, Koch et al. 2006), and it can be hypothesized that all DEHP metabolites,

derived from a hydrolytic/oxidative pathway could, after glucuronidation, be excreted in urines (Figure 1).

Children are known to be exposed to DEHP twice as high as compared to adult subjects (Wittassek et al. 2009, Hellerstedt et al. 2008). Foetuses and neonates are highly sensitive to physiologically active agents because they are exposed during critical periods of human development (Cho et al. 2010, Kim et al. 2009, Latini et al. 2003, 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 Endocrine Disruptors (EDs) (Latini et al. 2004, Silva et al. 2003). EDs may also be transferred from the mother to the developing foetus across the placenta, or to the newborn through breastfeeding (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 May 3, 2011). Moreover, very recently, prenatal phthalate exposure has been reported to decrease child mental/motor development, and increase internalizing behaviors during the preschool years (Whyatt et al. 2011), thus consolidating the concept of an adverse effect of these contaminants on function of the central nervous system.

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).

Materials and methods Subjects' population

A total of 48 children with ASDs [M: 36, F: 12; age at examination: 11.0 ± 5 years] were recruited from the staff of Children Neuropsychiatric Department, Siena, Italy. All the 48 patients with ASDs, 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 Scalescores) 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 tutors was obtained as well as institutional review board approval for the study. Mean age at diagnosis was between 30-36 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 month). 45 gender and age-comparable healthy controls (HC) [M: 25, F: 20; age at examination: 12 ± 5] were randomly chosen from outpatients who had no pathological symptoms.

Urines from children with ASDs and healthy controls were collected in polypropylene specimen cups, shared in aliquots (1.0 mL) and frozen at –20 °C until analysis. Field blanks consisted in purified water collected in polypropylene tubes and frozen at –20 °C.

Determining secondary metabolites in urine

The metabolites measured in this study included: mono(2-ethylhexenyl) 1,2-benzenedicarboxylate (MEHP) and mono(2-ethyl-6-hydroxyhexyl) 1,2-benzenedicarboxylate (6-OH-MEHP), mono(2-etil-5-oxohexyl) 1,2-benzenedicarboxylate (5-oxo-MEHP), mono(2-ethyl-5-hydroxyhexyl) 1,2-benzenedicarboxylate (5-OH-MEHP). All these metabolites were synthesised in the Laboratory of Peptide & Protein Chemistry & 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 urines from ASDs children and healthy controls.

All the solvents (ACN, water, buffers, etc.), labware (polypropylene vials containing urines, solvent bottles, SPE cartridge, Teflon capped-glass bottles, pipets), and instrumentation used during SPE procedure and the HPLC-ESI-MS analytical process, to detect MEHP and/or secondary metabolites in ASDs patients and healthy controls, were verified to be MEHP- and secondary oxidative metabolites-free. The internal standards were prepared in ACN and were used as reported in literature (Blount et al. 2000 and 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/24 h). For creatinine measurement, we used a Beckman Synchron AS/ASTRA clinical analyzer (Beckman Instruments, Inc., Brea, CA). We used value (micrograms per liter)/creatinine (grams per liter) for dilution correction in the analyses.

For SPE treatment of urine, we prepared the following buffers: buffer ammonium acetate 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 30% NH_3 solution) to a 50:50 acetonitrile/water (200 mL). All buffers were stored in sealed bottles at room temperature: basic buffer was discarded after one week, acid buffer after one month.

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

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

In particular we used an high-performance liquid chromatography tandem mass spectrometry RP-HPLC -ESI-MS (Waters, Alliance 2695, Waters, Micromass ZQ) equipped with phenyl column (Betasil, 5 µm, 50 mm x 3 mm, Keystone Scientific, Bellefonte, PA) and with a Waters 2996 Photodiode Array Detector. All reagents were of at least analytical reagent grade. The lower limits of quantification (LOQs) 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 limit of detection (LODs) 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 ACN). The flow rate was 0.6 mL/min. The column temperature was 32 °C. A guard column (XBridgeTm Phenyl 3.5µm, 3.0x20 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 Electrospray ionization (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 optimal MS parameters were as follows: the source and desolvation temperature were 120 °C and 400 °C 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 L/h

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 to 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; 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% confidence intervals (95% C.I.) for normally distributed variables or medians means and with 95% C.I. 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-square 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 ASDs patients from healthy controls were evaluated using Receiver Operating Characteristic (ROC) 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, Mariakerke, Belgium) 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), 5-oxo-MEHP (0.096 µg/mL). On the contrary, in urine from healthy controls 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), 5-oxo-MEHP (0.04 µg/mL). Although the data illustrated in Table 2 are without urinary creatinine-correction. Differences between groups by using creatinine-adjusted data were comparable to 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 ASDs 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 to gender- and age-comparable healthy controls (Table 2). On the contrary, 6-OH-MEHP ($p = 0.9305$) was not able to discriminate ASDs. High levels of 5-OH-MEHP were detected in 26.6% of healthy controls. 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 oxidised form of the metabolites 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 ASDs patients from healthy controls was evaluated using ROC curve analyses (Figure 3, Table 1 and Table 2).
Finally, we analyzed the correlations between total Childhood Autism Rating Scale (CARS) score and levels of the different metabolites. Total CARS scores were (means ± SD) 44 ± 7.4 (range: 31-60). A positive correlation between CARS scores and urinary MEHP levels was observed (rho = 0.429, $p = 0.0033$), whereas no significant relationships with the levels of the other examined

metabolites were found (total CARS vs. 5-OH-MEHP: rho=0.120, p=0.4298; total CARS vs. 5-oxo-MEHP: rho=0.127, p=0.3931; total CARS vs. 6-OH-MEHP: rho=0.0085, p=0.9529).

Moreover, we compare 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 ASDs patients (Table 3). Actual behavior for the majority of 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 to that observed in healthy controls, despite the heavy medications 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 which 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 ASDs urine samples we detected MEHP and 5-oxo-MEHP levels higher than the levels recently published, by Cho et al. (Cho et al. 2010). In that study Cho et al. report 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 medians values was for MEHP (0.055 µg/mL), 5-OH-MEHP (0.18 µg/mL), 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 epidemiologic studies it is not practical to collect 24-hr 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 metabolites excretion in mothers to psychomotor development in their children at age 3, just published on a top-rated toxicology journal such as Environmental Health Perspectives (Whyatt et al, 2011). Likewise in ASDs, MEHP was found to be the minor urinary metabolite compared to the corresponding oxidised metabolites as in some adult metabolic diseases (Wittassek et al. 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 endocrine disrupter.

From our data it is possible to speculate that phthalates metabolites as endocrine disrupters 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 Healthy Controls (Figure 2). Nevertheless, it has already 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 oxidative stress unbalance may interact in leading to increased ASDs risk, although further investigation is needed in dissecting this interaction in vivo.

A link between oxidative brain damage and ASDs has been previously reported by several authors (James et al. 2004, Sajdel-Sulkowska et al. 2011, Villagonzalo et al. 2010). Over the last years, our team has demonstrated that the biological "duo" hypoxia-oxidative stress (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 ASDs 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 oxidised 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 oxidised phthalates metabolites), possibly acting as EDs. Considering that urinary 5-oxo-MEHP reported in our study as increased in ASDs compared to healthy controls, 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 etiology of neuro development disorders. Phthalate metabolites activate peroxisome proliferator-activated receptor (α-PPAR), interfering with cellular proliferation and lipid metabolism. Activation of α-PPAR by phthalates may alter lipid metabolism in the brain (Clark-Taylor et al. 2004). Recently signal transduction pathway of α-PPAR was correlated to progression of neurodegenerative and psychiatric diseases.
Phthalates also interfere with the thyroid hormone system by inducing hypothyroidism. Recent studies correlated in *utero* hypothyroxinemia to decreased intellectual capacity, mental retardation, and ASDs (Roman 2007). Thus reinforcing our speculation. Moreover, exposure to di-n-butyl phthalate (DBP) seems to affect thyroid activity in pregnant women, thus leading to adverse effects in the foetus (Huang et al. 2007).
On the other hand, transient intrauterine deficits of thyroid hormones 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 antithyroid environmental contaminants.
For the first time in this study we correlated different levels of the primary and secondary metabolites with ASDs compared to HC children. These data generate the idea that either current exposure is higher in children with ASDs, 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 ASDs phenotype, according to the general concept generated by Martha Herbert of "environmentally vulnerable physiology" (Herbert 2010). 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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Table 1. ROC curve for di(2-ethylhexyl)phthalate secondary metabolites urinary excretion and infantile autism.

Urinary phthalates	Cut-off	AUC±SE	95% CI	P-value	Sens (%)	Spec. (%)	+PV (%)	-PV (%)
5-OH-MEHP	>.177	.638± .057	.531-.735	.0154	52.1	75.6	69.4	59.6
5-oxo-MEHP	>.142	.666± .055	.561-.759	.0028	46.0	91.1	85.2	60.3
MEHP	>.01	.631± .058	.524-.730	.0233	79.2	44.2	61.3	65.5
ALL	>.724	.671± .055	.568-.764	.0021	39.2	97.8	95.2	58.7

AUC: Area under the Curve; SE: Standard error; 95% CI: 95% confidence intervals for the Area under the Curve; Sens.: sensitivity: spec.: specificity; +PV: positive value; -PV: negative value; ALL: total sum of all the examined di(2-ethylhexyl)phthalate secondary metabolites (5-0H-MEHP + 5-oxo-MEHP + 6-OH- MEHP+ MEHP).

Table 2. Comparisons of urinary excretion of secondary metabolites for di(2-ethylhexyl)phthalate in autistic patients (N = 48) vs. healthy controls (N = 45) in (µg/mL).

	ASDs 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**

Values are medians (95% confidence intervals of the median); * Mann-Whitney test for independent samples; statistically significant differences are highlighted in bold.

Table 3. Comparisons of urinary excretion of secondary oxidative metabolites of DEHP in ASDs with n=10 patients with Rett syndrome in (µg/mL).

Urinary Phthalate Metabolite	ASDs Rett syndrome	p-value
5-oxo-MEHP	0.0997 (0.0397-0.1708) vs 0 (0 -0.0755)	**0.0344**
5-OH-MEHP	0.1740 (0.0311-0.35) vs 0 (0 -0.0091)	**0.0601**
6-OH-MEHP	0.0107 (0-0.0261) vs 0 (0 -0.0832)	0.2246
MEHP	0.0287 (0.0159-0.0847) vs 0 (0 -0.1344)	**0.7098**

(data are expressed as medians and 95% confidence intervals; phthalate metabolite concentrations are adjusted to creatinine and are calculated per 100 mg of urinary creatinine)

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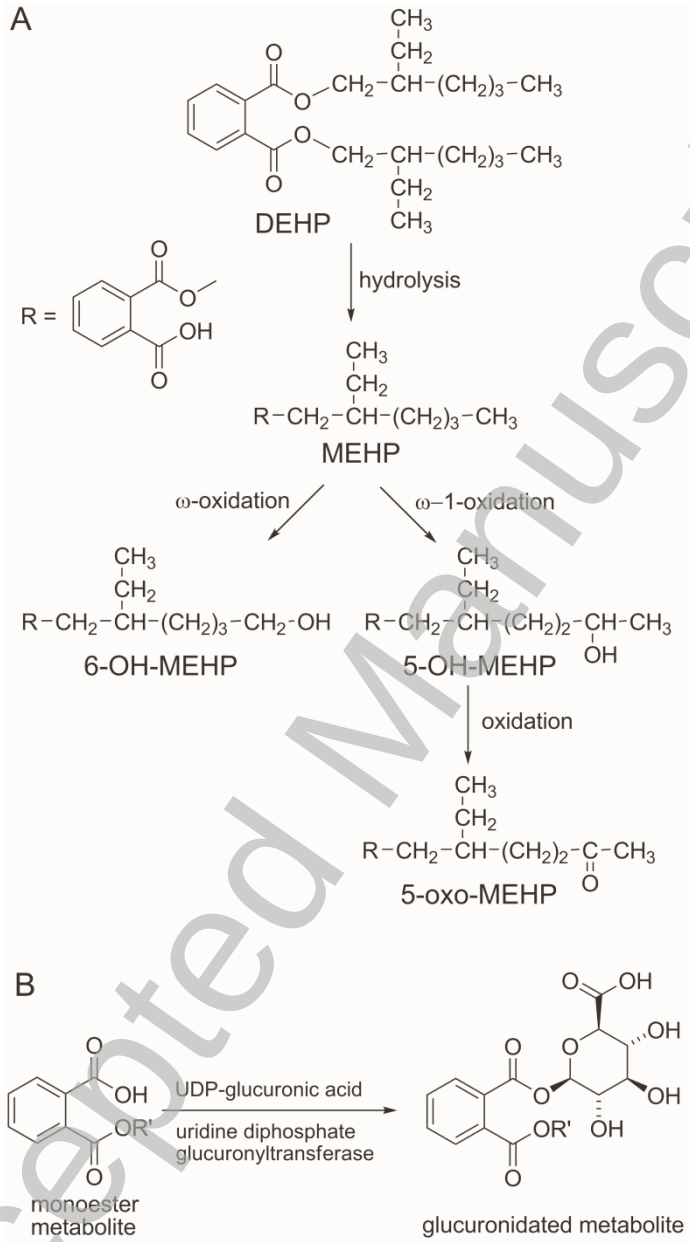
Figure 1. Metabolism of DEHP

(A) Hydrolytic/oxidative pathway leading to MEHP and secondary metabolites formation.

(B) Formation of glucuronic conjugate of DEHP metabolites.



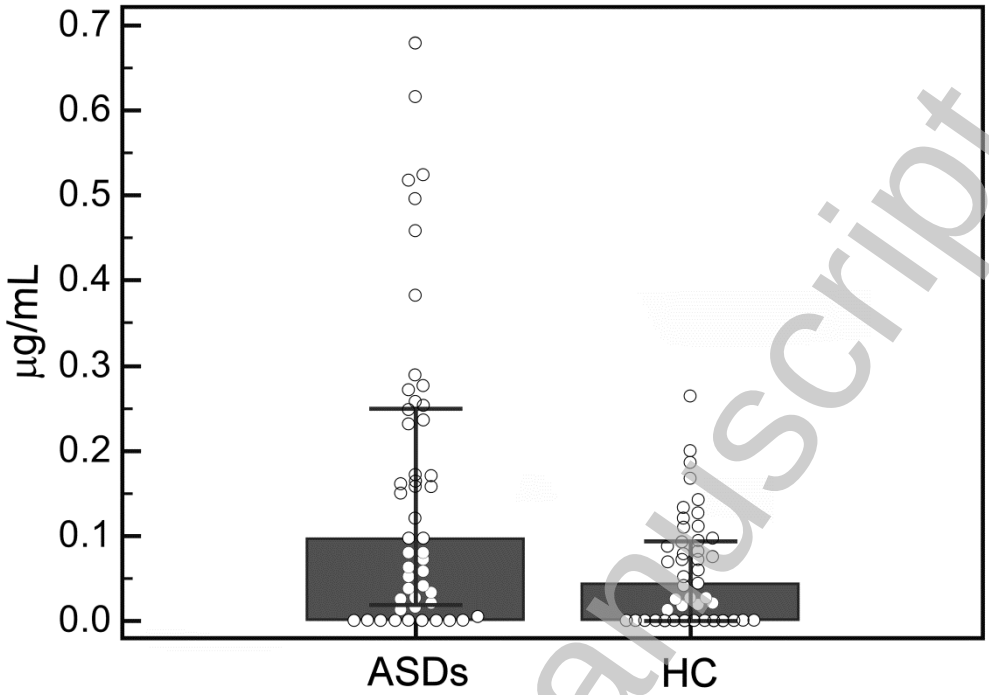


Figure 2. Differences between groups: scatterplots for 5-oxo-MEHP values in ASDs' urine samples (ASDs, n = 48, and Healthy Controls (HC, n = 45)
Data are medians and inter-quartile range.

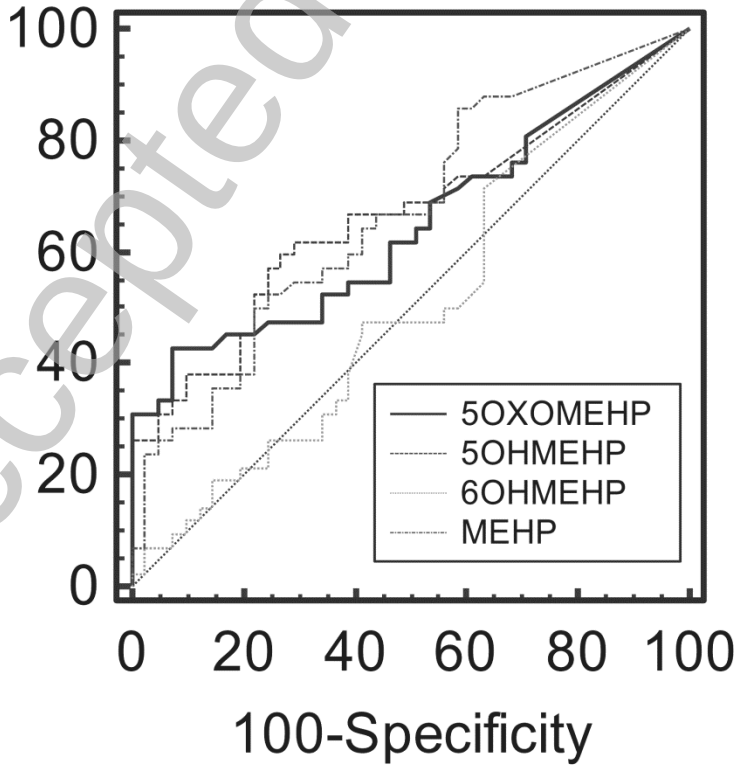


Figure 3. ROC curve analysis (ASDs patients vs. HC) for DEHP metabolites