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## Porphyrinuria in Childhood Autistic Disorder: Implications for Environmental Toxicity

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## **Abstract**

To address a possible environmental contribution to autism we carried out a retrospective study on urinary porphyrin levels, a biomarker of environmental toxicity, in 269 children with neurodevelopmental and related disorders referred to a Paris clinic (2002-2004), including 106 with autistic disorder. Urinary porphyrin levels determined by high-performance liquid chromatography were compared between diagnostic groups including internal and external control groups. Coproporphyrin levels were elevated in children with autistic disorder relative to control groups. Elevation was maintained on normalization for age or to a control heme pathway metabolite (uroporphyrin) in the same samples. The elevation was significant ( $p < 0.001$ ). Porphyrin levels were unchanged in Asperger's disorder, distinguishing it from autistic disorder. The atypical molecule precoproporphyrin, a specific indicator of heavy metal toxicity, was also elevated in autistic disorder ( $p < 0.001$ ) but not significantly in Asperger's. A subgroup with autistic disorder were treated with oral dimercaptosuccinic acid (DMSA) with a view to heavy metal removal. Following DMSA there was a significant ( $p = 0.002$ ) drop in urinary porphyrin excretion. These data implicate environmental toxicity in childhood autistic disorder.

*Keywords:* Autism, Asperger, Porphyrin, Mercury, Pervasive Developmental Disorder

## Introduction

Autism is a disorder of reciprocal social interaction, behavioral repertoire, and language and communication. Because the phenotype ranges from manifest disability to specific performance elevation, the term Autistic Spectrum Disorder (ASD) (Wing, 1996; Gillberg et al., 2000) is now commonly used to denote the DSM-IV (American Psychiatric Association, 1994) group of pervasive neurodevelopmental disorders encompassing autistic disorder, Asperger's disorder, Rett's disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS). A fraction of cases have a defined genetic cause, but the apparent increase in prevalence of ASD (California Department of Human Developmental Services, 2003; Smeeth et al., 2004; Barbaresi et al., 2005) as reviewed (Blaxill, 2004), is suggestive of an environmental contribution. Changes in awareness and diagnostic criteria may explain some of the rise (Croen et al., 2002; Rutter, 2005), but a true increase in prevalence has not been excluded (Rutter, 2005). Elevated ASD rates in urban versus rural areas (Deb et al., 1994; Palmer et al., 2006; Williams et al., 2006) are consistent with an environmental contribution. Several sporadic reports have suggested an association between heavy metal exposure and ASD (Cohen et al., 1982; Accardo et al., 1988; Shannon et al., 1996; Lidsky et al., 2005). Superficial similarity between mercury toxicity and ASD has prompted discussion of mercury exposure in the etiology of the disorders (Bernard et al., 2001), while ASD prevalence in Texas schools correlated with local environmental release of mercury (Palmer et al., 2006).

To address an environmental contribution to ASD several studies have explored the body burden of heavy metals. Because metal residues are immobilized in tissues, blood levels are a poor measure of exposure. Mobilization on treatment with chelating agents can release bound metals, affording a more reliable indication of exposure (Markowitz et al., 1991). Elevated excretion of heavy metals has been reported in ASD versus controls on chelation therapy (Lonsdale et al., 2002; Bradstreet, 2003), consistent with an abnormal heavy metal load. However, release of bound metals on chelation can be toxic (Markowitz et al., 1990), and chelation may be inappropriate for routine investigation of heavy metal burden. Hair samples provide an alternative, and though specific elevations have been seen in ASD (Fido et al., 2005) some children later becoming autistic appear to have a deficit in metal export into this tissue (Holmes et al., 2003; Hu et al., 2003), complicating analysis.

We therefore turned to an independent and non-invasive method to address environmental toxicity in ASD children. Porphyrins, derivatives of the heme synthesis pathway, afford an independent measure of adverse exposure (Brewster, 1988). Heme manufacture takes place most prominently in liver, kidney and erythroid cells. Synthesis proceeds in two steps from succinyl-CoA + glycine to uroporphyrinogen and in a further

series of steps via pentacarboxyporphyrinogen and coproporphyrinogen to heme (Fig. 1). Excess porphyrinogen metabolites are excreted in the urine as oxidized porphyrins, particularly uroporphyrin and coproporphyrin, reflecting the most abundant molecules in the rat kidney cortex (Woods et al., 1993b) and solubility: mid-pathway porphyrins are the most water-soluble and appear predominantly in urine, whereas hydrophobic protoporphyrin appears predominantly in bile and feces.

Excess urinary porphyrin excretion or porphyrinuria results from blockade of key enzymatic steps in conditions including genetic deficiencies in heme manufacture enzymes (Sarkany, 1999), hepatic, renal and erythroid disease (Gross et al., 2000), and also by toxic inhibition of heme synthesis enzymes. In both experimental animals and humans exposed to heavy metals porphyrins are exported at elevated levels into urine (Bowers et al., 1992; Woods, 1996). The most prominent targets for heavy metal inhibition are the uroporphyrin decarboxylase (UROD) (Woods et al., 1983) and coproporphyrinogen oxidase (CPOX) (Woods et al., 2005) reactions (Fig. 1), resulting in specific elevations of coproporphyrin and pentacarboxyporphyrin in urine. A causal relationship between heavy metal inhibition and porphyrinuria has been demonstrated: both in rats exposed to mercury (Pingree et al., 2001) and in humans exposed to lead (Rosen et al., 1993) heavy metal removal with chelating agents (dimercapto-propanesulfonic acid, DMPS, and ethylenediamine tetraacetic acid, EDTA, respectively) reduced urinary porphyrin levels towards control values. Although non-metal agents targeting the heme pathway can also elevate urinary porphyrin levels (Daniell et al., 1997), precoproporphyrin (also known as keto-isocoproporphyrin) is produced by *in vivo* conversion of pentacarboxyporphyrinogen under pressure of heavy metal interference (Woods et al., 2005; Heyer et al., 2006), providing a specific porphyrin marker of heavy metal (particularly mercury) toxicity.

To address the heavy metal burden of ASD children we carried out a retrospective study of levels of specific urinary porphyrins in a large group of French children with a primary diagnosis of autism or other neurodevelopmental disorders. No previous studies on porphyrin levels in ASD have been reported. Our analysis has focused on urinary markers of inhibition of the heme synthesis pathway including coproporphyrin; we also examined the specific marker of heavy metal toxicity, precoproporphyrin. We report significant elevation of these urinary porphyrins in autistic disorder.

## Methods

*Study subjects.* This study addressed 269 children presenting to the Clinique Dr. Skorupka, Paris (CS) between August 2002 and December 2004. Approximately 70% of these cases were first evaluated in specialist centres in France and subsequently referred to the clinic for further analysis. The remainder, principally the younger children, were first evaluated by psychiatric services selected by the parents before onward referral to the clinic. In all cases a second diagnostic assessment (CS and associated medical staff) was made on presentation; each child was seen by at least two independent clinicians. Definitive assessment was according to the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994) based on DSM-IV (American Psychiatric Association, 1994) and ICD-10 (WHO, 1992) adapted for use in France (CS). Diagnostic sub-groupings of Pervasive Developmental Disorders (also termed Autistic Spectrum Disorders (Wing, 1996; Gillberg et al., 2000) were: *Autism* (autistic disorder); *PDD-NOS* (pervasive developmental disorder not otherwise specified; atypical or sub-threshold symptomology); *Asperger* (Asperger's disorder); *Rett* (Rett's disorder). Other conditions were: *Attention Deficit* (attention deficit disorder without hyperactivity), *Hyperactivity* (disturbance of activity and attention, attention deficit disorder with hyperactivity), *Cerebral Palsy* (infantile cerebral palsy), *Mental Retardation*, in the subjects studied this was accompanied by epilepsy, and *Psychomotor Retardation*. One deviation from DSM-IV was separate inclusion of *Autism+Epilepsy*, the identical condition to Autism but with known co-morbidity of epilepsy, included to avoid an obvious confound (anti-epileptic medication). Other diagnoses (see text) also followed DSM-IV and ICD-10. All children were in the age range 1-16, and the children with autistic disorder in the range 2-15. The 269 study subjects analyzed over the period 2002-2004 are summarized in Table 1.

In no case was there prior evidence of heavy metal exposure or pica behavior that might be associated with heavy metal exposure. No child in the non-epilepsy groups received medication during the study period; subjects with epilepsy including the autism+epilepsy subgroup typically received anticonvulsant medication. The two individuals with Rett were confirmed by genomic MECP2 analysis; no child in the ASD group (n=191; Asperger, autism +/- epilepsy, PDD-NOS, Rett) harbored Fragile X.

An internal unselected control group was provided as follows: all children referred to the same clinic within the same timeframe with a diagnosis different from those above were included, with 6 exceptions. 3 children had multiple diagnoses and could not be included in any category, while 3 further children (Behcet Disorder, n=1; fetal distress, n=2) were unrepresentative in view of abnormal values of both uroporphyrin and coproporphyrin. The internal control group then comprised children presenting with abdominal pain (n=1), juvenile

arthritis with allergy (1), anorexia (1), bulimia (2), dyslexia (1), fibromyalgia (1), growth retardation (3), leukodystrophy (1) and one control sibling volunteer.

External control group data (COPRO and URO only) were obtained by extraction of values pertaining to the equivalent age-range from primary data kindly provided by Minder and colleagues regarding Swiss children (n=107), gender not specified, mean age 6.6 years, excluding individuals with overt porphyria and outliers (Minder et al., 1996).

*Analysis of urinary porphyrins.* Porphyrin analysis (RN, AL; Laboratoire Philippe Auguste) was blind of diagnosis. First matinal urines (10 ml) were stored in the dark (<2 d, ambient temperature; conditions where porphyrins are stable (Minder et al., 1996)) and then frozen (-20°C). Porphyrin analysis was by an HPLC spectrofluorometric technique (Bowers et al., 1992) After centrifugation (3000 g, 5 min) 1 ml supernatant was acidified (40 µl HCl 37% w/v), recentrifuged, and 50 µl injected (Econosphere column C18, 5 µm particle size, 250 x 46 mm; Alltech, Templemars, France). Elution was with a gradient (phase A: 50 mM KH<sub>2</sub>PO<sub>4</sub> pH 3.5 with CH<sub>3</sub>COOH; phase B: CH<sub>3</sub>OH), 1 ml/min, as follows: time 0, phase A:phase B, 50:50; time 3 min, 35:65; 8 min, 15:85; 18 min, 1:99; 28 min, 50:50. Fluorescence detection (excitation, λ=405 nm; emission, λ=618 nm) used dual on-line detection (UV, model 310; fluorescence, model 363; both from Varian, Les Ulis, France). Retention times for uroporphyrins (I and III), hepta-, hexa-, penta-carboxyporphyrin, coproporphyrins (I+III) and later mesoporphyrin IX were 7.93, 9.19, 11.25, 13.11, 15.07 and 19.58 min respectively; elution of the atypical metabolite precoproporphyrin was at 13.92. Subspecies I/III resolved poorly and were not separately tabulated. Detection was standardized against a mixed porphyrin reference sample (CMKIE, Porphyrin Products, Logan, Utah) and against purified coproporphyrin III (Sigma, France). Urinary creatinine (CRT) levels were determined using a spectrophotometric enzyme-linked assay as described by the supplier (Crea-Vitros Technical Bulletin; Ortho-Clinical Diagnostics, Johnson and Johnson, High Wycombe, UK); CRT reference standard was from the same source. Porphyrin levels were normalized to CRT. Detection protocols were independently validated according to ISO 9001 (2000 edition), COFRAC (Comité Français d'Accréditation), by AB Certification, Champlan, France. Sampling procedures, storage and analysis procedures (<2 days, -20°C, HPLC) for the external control group (Minder et al., 1996) were comparable to those used in the current study.

*Porphyrin reference values.* Literature mean values for urinary COPRO in Swiss children in the 2-16 age-range were 11.4 µmol/mol CRT (Minder et al., 1996), slightly in excess (34%) of our control group COPRO mean of 8.5 µmol/mol CRT. Male values are slightly higher

than for females but the extent of the difference (~17.5%) (Bloom et al., 1991) is not a significant confound, while grouping according to age (1-2, 3-6, 7-9, 10-16), despite local variations (Minder et al., 1996), excluded significant differences between the age groups in overall COPRO or uroporphyrin (URO) values normalized to CRT, although a trend towards age-dependent decline in both mean levels paralleled CRT elevation. Accordingly, primary results presented here were not adjusted for age.

*Re-normalization to age-changes in creatinine (CRT).* A second normalization was employed to account for possible artefacts introduced by changes in CRT. Urinary CRT roughly doubles between 3 and 17 years (Bloom et al., 1991; Remer et al., 2002; Skinner et al., 1996) in both genders. Averaged across all our data mean CRT level was not significantly different between males and females, but doubled over the 2-15 age-range, rising linearly from 640 mg/L (age 2-3) to 1316 at age 14-15 (5.66-11.65 mM). This established a normalization curve: factors employed (to age 10 equivalents) increased linearly from 0.63 at age 2 to 1.23 at age 15; age integral values at date of analysis were employed. Although there was no significant difference in COPRO values according to age either in the large external control group or in our entire group of data (all children including affected subjects and controls), there was a trend towards a small age-related fall in COPRO levels for both groups after compensation for changes in CRT. The decline approximated to a mean linear reduction of 2.9% per year; separate comparative analysis also compensated for this change.

*Internal ratios.* Levels of precoproporphyrin (PRECOPRO), an abnormal porphyrin seen in urine of rats or human subjects exposed to mercury (Woods et al., 1993b; Gonzalez-Ramirez et al., 1995; Pingree et al., 2001), and identified as keto-isocoproporphyrin (Woods, 1995; Woods et al., 2005; Heyer et al., 2006), are typically raised along with coproporphyrin (COPRO) on heavy metal toxicity while URO concentrations are far less (and not significantly) elevated (Woods et al., 1993a; Woods, 1996). Pentacarboxyporphyrin is also elevated in mercury toxicity (Woods et al., 1993a). Porphyrin ratios were either directly calculated or plotted (Excel, Microsoft Corp.); for plots of PRECOPRO/URO ratios two outliers (autistic disorder) with unusual levels of uroporphyrin were excluded due to further diagnosis of mononucleosis. Value-based ratios differed slightly but not significantly from those derived from regression plots constrained to pass through the origin. Age- or gender-bias were not significant confounds: reference data (Minder et al., 1996) grouped according to age (1-2, 3-6, 7-9, 10-16) or gender saw no significant between-group difference in COPRO/URO ratios (PRECOPRO levels were not determined in this study) or age-dependent trend; the mean COPRO/URO ratio from equivalent age-range samples in this study (n=107) was 6.1 +/- 5.3, somewhat lower than our mean value across all samples (n=269 including

affected subjects) of 10.7 +/- 9.3 but strictly equivalent to our internal control group value of 5.35 +/- 3.6. Statistical comparisons were performed to both internal (control group) and external (Minder et al., 1996) values.

*Chelation protocol.* A subgroup of parent-selected children with autism or autism+epilepsy, and all with evidence of frank porphyrinuria and with specific markers of heavy metal exposure, was treated under medical supervision (CS) to remove heavy metals with meso-2,3-dimercaptosuccinic acid (DMSA or succimer) (Aposhian et al., 1990). DMSA from Vitamin Research Products (Carson City, Nevada, USA) was given orally (10 mg / kg bodyweight every 8 h for 3 d), followed by an 11 d rest period. The two week cycle was repeated five times. Throughout the cycle per day recommended mineral replacement (Flora et al., 1990; Mercury detoxification consensus group, 2001) included zinc and a copper-free multimineral preparation; vitamin supplementation (vitamins C and E) (Flora et al., 2003) was also recommended. Urines were sampled outwith (>1 mo) the chelation period. Co-presenting children with the same diagnosis but not known to be receiving chelation treatment provided the control group. Criteria (in both the chelation and control groups) for retrospective analysis were (a) diagnosis of autism or autism plus epilepsy and (b) independent samples at least 6 months apart (the criterion excluded 3 children); giving two groups (chelation, n=11, 7m/4f, mean age at second test 8.7 yr, range 5-16, mean span between samples 18.6 mo; and control, n=10, 8m/2f, mean age 8.6 yr, range 4-16, mean span 13.8 mo). Porphyrin values were also normalized to urinary creatinine. Urinary and fecal metal levels during chelation were not measured.

*Statistical analysis.* Analyses (mean, standard deviation, linear regression) employed Excel (Microsoft Corp.) and Student's t test, two-tailed, unequal standard deviations, Satterthwaite approximation (GenStat; VSN International, Hemel Hempstead, Herts, UK). The minimum p value provided by this programme was  $p < 0.001$ .

*Ethical approval.* Urine samples analyzed retrospectively were obtained with informed consent of parents/guardians and where possible the patients. Approval for chelation treatment was also obtained with informed consent of the families. The present analysis was approved by the NHS Lothian Local Research Committee O4 (Scotland).

## Results

To address possible environmental toxicity in the etiology of autism we examined levels of urinary porphyrins, a robust marker of exposure, in a large (n=269) group of French children with neurodevelopmental and related disorders. Analysis was blind of diagnosis. 71% of these children had a diagnosis of autistic spectrum disorder (ASD); the majority of this subgroup (56%) had a diagnosis of childhood autistic disorder (autism). Other diagnostic groups in the study included Asperger, attention deficit, cerebral palsy, hyperactivity, PDD-NOS and the separate category of autistic disorder combined with epilepsy (Methods, Table 1). As expected, there was a male (M) excess both in the whole cohort (M/F=2.76) and in ASD (M/F=3.34), though in attention deficit there was a female (F) excess (M/F=0.29, n=9).

An internal control group (n=12) was provided by children referred to the same clinic within the same timeframe with a diagnosis unrelated to those listed (Methods). To ascertain the validity of internal control group values we reanalyzed primary data for 107 Swiss children (Methods). This validated internal control group values: there was no significant difference in porphyrin levels (or ratios) between the internal and external control groups. Asperger disorder provided further confirmation of internal control group data (below). Because of concerns regarding differences in measurement protocols, addressed further below, separate statistical comparisons were performed first against the internal control group, and then against the external (uroporphyrin and coproporphyrin values only).

*Porphyrin levels.* Urinary uroporphyrin (URO) and coproporphyrin (COPRO) mean values were compared between diagnostic categories and with the control groups. There was no significant change in URO levels in any disorder studied. In contrast, there was strong evidence of COPRO excess in two disorders (autism and the separate category of autism+epilepsy), where the means of COPRO levels exceeded the control group mean value plus twice the standard deviation (Fig. 2). The extent of the rise (mean increase 2.6-fold for COPRO) was comparable to the rise seen in arsenic (1.9-fold) and mercury (3.2-fold) exposure (Wang et al., 2002; Woods, 1996).

The increase was statistically significant ( $p<0.001$ ) for autism versus the internal control group, fulfilling the criteria for porphyrinuria (urinary porphyrin levels outwith the normal range), while the increase was marginal for autism+epilepsy ( $p<0.1$ ) in view of small group size. 3 other conditions showed even greater excess (epilepsy, mental retardation with epilepsy, and Rett disorder) but small sample sizes precluded assessment of significance. When compared to large group literature reference values for Swiss children (Methods) statistical significance was strengthened (autism and autism with epilepsy: both  $p<0.001$ ).

Unexpectedly, Asperger's disorder did not differ from either control group in urinary porphyrin levels. The biochemical distinction between Asperger's and either autism or autism with epilepsy group was of high statistical significance ( $p < 0.001$ ). Other disorders showing marginal porphyrin excess included allergy, attention deficit, cerebral palsy, PDD-NOS and psychomotor retardation, but were of low statistical significance ( $p > 0.05$ ) although cerebral palsy was of marginal significance ( $p < 0.1$ ) versus the internal control group.

Porphyrin levels are routinely normalized to urinary levels of the ubiquitous metabolite creatinine (CRT) to allow for individual differences in fluid consumption and excretion. Because CRT levels increase slowly with age in children, and to exclude this parameter as a source of variation, data were renormalized to a standard curve established with reference to internal and external data (Methods). The statistical significance of inter-group differences was maintained (autism versus control,  $p < 0.001$ ). Thus, age differences reflected in CRT levels do not explain the observed porphyrin excess. Although there was a small and non-significant trend for CRT-normalized COPRO values to fall with age (2.9% per year; Methods), specific adjustment to accommodate this trend did not affect the significance of between-group differences (not shown): this excluded inter-group mean age differences as a factor underlying the porphyrin excess in autistic disorder.

To eliminate CRT levels as a source of variation, we determined internal ratios of different porphyrin species within each sample. Specifically, we inspected the ratio of coproporphyrin (COPRO), a marker subservient to heavy metal toxicity, to uroporphyrin (URO), a precursor largely independent of environmental toxicity (Fig. 3). There was a slight elevation of URO levels in autism but the difference was not significant versus the control group. Both autism and autism+epilepsy presented high COPRO/URO ratios versus internal controls and Asperger; the differences in the ratios were significant (autism versus control,  $p < 0.001$ ; autism+epilepsy versus control,  $p = 0.008$ ; autism versus Asperger,  $p = 0.009$ ); Asperger was not significantly different from the control group. When compared to reference (external control group) values for the COPRO/URO ratio in Swiss children of the same age group (Minder et al., 1996), statistical significance was maintained (autism versus reference,  $p < 0.001$ ).

When compared against the external control group, COPRO/URO ratios in PDD-NOS (and cerebral palsy) were significant ( $p < 0.05$ ) but not when compared to the internal control group (marginal significance;  $p < 0.1$ ).

*Precoproporphyrin and pentacarboxyporphyrin, markers of heavy metal toxicity.* The data demonstrate significant (to the  $p < 0.001$  level) increase in coproporphyrin (COPRO) levels in autism. To address potential causes we inspected levels of precoproporphyrin (PRECOPRO), an atypical metabolite specifically linked to heavy metal exposure rather than chemical

toxicity or other disease processes. PRECOPRO levels were plotted against baseline uroporphyrin (URO) values for each sample and regression curves calculated (Fig. 4). The gradients in autism and autism+epilepsy were nearly twice as high as in the control or Asperger groups. Ratio values (rather than gradients) were compared: the differences were statistically significant (autism versus control group;  $p < 0.001$ ; autism+epilepsy versus control,  $p = 0.011$ ); Asperger did not differ significantly from the control group. These data demonstrate specific PRECOPRO elevation in autism and in autism with epilepsy. Levels in the two subjects with epilepsy alone were lower than with either autism or autism+epilepsy (mean PRECOPRO/URO ratio = 1.17, versus 1.2 in autism and 1.47 in autism+epilepsy; values from means, not shown) but above that seen in Asperger or control groups (0.69 and 0.6 by value; 0.56 and 0.62 by regression; Fig. 4).

Pentacarboxyporphyrin is a further marker of heavy metal toxicity (Woods et al., 1993a). Levels of this porphyrin, and its immediate precursors (hepta- and hexacarboxyporphyrins), were also elevated in urines of ASD children (particularly autism and autism+epilepsy) versus controls (Fig. 5).

Autism was significantly higher than control for both pentacarboxyporphyrin ( $p < 0.001$ ) and hexacarboxyporphyrin ( $p < 0.002$ ), but without significant elevation of heptacarboxyporphyrin. Asperger and PDD-NOS did not differ significantly from control group in levels of any of these carboxyporphyrins. Autism with epilepsy was significantly higher than control for penta-carboxyporphyrin (5CXP) ( $p < 0.02$ ) but not for hexa- (6CXP), despite the high mean excess (Fig. 5), due to the high variance (9.48) and small sample size ( $n = 9$ ). There was no significant difference for heptacarboxyporphyrin (7CXP). Generally, in these disorders the same children with high values for hexa- had elevated values for heptacarboxyporphyrin. Of the other disorders surveyed, only mental retardation+epilepsy showed a significant increase in all three porphyrin intermediates while only pentacarboxyporphyrin was significantly increased in Rett's disorder (not presented).

*Outcome of chelation.* 11 autistic (autism or autism with epilepsy) children were subjected to chelation therapy (DMSA, methods) with a view to heavy metal removal. Porphyrin values were compared prior to and following chelation, and compared with a control group ( $n = 10$ ) with the same diagnosis for which similarly spaced samples were also available (Fig. 6). A significant reduction in levels of urinary coproporphyrin (COPRO) was observed in the DMSA chelation group ( $p = 0.02$  despite small sample size) while an increase in COPRO values was recorded in the control group (not significant). There was also a marked reduction both in precoproporphyrin (PRECOPRO) levels and the PRECOPRO/URO ratio in the DMSA group (mean ratio falling from 1.63 to 0.71) but not in the group without chelation (not shown).

In depth statistical analysis confirmed this result. Because there was evidence of a dependency between the means and the variances of these measurements, data were then expressed as log values. Here there was no evidence of a difference between variances for the treatment and no-chelation groups; a standard t-test confirmed a significant difference in the log ratios of COPRO (sample 1:sample 2) and PRECOPRO (1:2) values for the DMSA versus the no treatment group ( $p < 0.002$ ,  $p < 0.01$  respectively).

## Discussion

We report porphyrinuria in the majority of a large group of French children with autistic disorder. Coproporphyrin (COPRO) excess was of high statistical significance ( $p < 0.001$ ) both versus an internal control group of unrelated disorders and versus a large external control group of Swiss children. Unexpectedly, porphyrin levels in Asperger's disorder were indistinguishable from the control group and provided a further reference point.

The mean extent of the COPRO rise in autistic disorder (2.6 fold) was comparable with the average elevation (3.2-fold) seen in a group of US dentists with significant Hg exposure (Woods, 1996) or on chronic arsenic exposure (1.9-fold) in Chinese villagers (Wang et al., 2002).

We report also that levels of two further markers of heavy metal exposure, precoproporphyrin (PRECOPRO) and pentacarboxyporphyrin, are elevated in autistic disorder. Though a majority displayed this excess, not all such children were porphyrinuric. The fraction of subjects with porphyrinuria was dependent on the specific parameter investigated, but in autistic disorder group (autism and autism+epilepsy,  $n=115$ ) 53% exceeded the internal control group Mean + 2 x Standard Deviation for PRECOPRO/uroporphyrin (URO) ratio.

Porphyrin excess in autism was also markedly and significantly reduced by treatment of children with a chelating agent, meso-dimercaptosuccinic acid (DMSA), that removes heavy metals, suggesting a causal relationship.

Because of the important implications of this study we have carefully considered possible confounds. Concerns have been expressed about different porphyrin results being obtained in different centres (Zuijderhoudt et al., 2003) but four normalizations firmly excluded technical artefacts associated with the detection protocol. First, by calibration of our high performance liquid chromatography (HPLC) equipment with purified standards, as was also done for the external control group (Minder et al., 1996); second, by comparison to an internal control group (unrelated disorders, and also to Asperger's disorder) where samples were processed on the same apparatus; third, by examining the ratio of coproporphyrin (COPRO, that elevates with heavy metal exposure) to uroporphyrin (URO, that remains largely unchanged on such exposure) determined simultaneously on the same HPLC run; fourth, by analysis, also on the same HPLC run, of the atypical molecule precoproporphyrin (PRECOPRO), a molecule seen only in heavy metal toxicity.

The fraction of subjects with porphyrinuria was dependent on the specific parameter investigated, but in autistic disorder group (autism and autism+epilepsy,  $n=115$ ) 53% exceeded the internal control group Mean + 2 x Standard Deviation for PRECOPRO/URO ratio.

One source of uncertainty concerns the control groups. First, the internal control group size was small (n=12) but, given the large numbers of study group subjects (257 total; 106 in the autistic disorder group), high statistical significance ( $p < 0.001$ ) was achieved in pairwise comparisons that explicitly take account of group sizes. Second, the internal control group was re-validated by careful comparison to an external control group of Swiss children (n=107) with reanalysis of corresponding primary data (Methods). There was no significant difference or trend between the internal and external control groups, confirming the reliability of the internal control group data. Third, values for the internal control group were unexpectedly confirmed by the finding that they were indistinguishable from the Asperger group (n=11); this sub-group therefore has provided a further internal reference point.

Despite the robustness of the control group data, both the internal and external control groups might under-estimate the true porphyrin excess in affected children. Both control groups comprised children referred for analysis, and may not be representative of the population. Plausibly, these subjects could have been differentially exposed to environmental agents. Indeed, porphyrin excess was seen in some control subjects (Minder et al., 1996) and could artificially diminish the extent of porphyrin excess in autistic disorder. The true extent of porphyrinuria might therefore be greater than that reported here. The overall evidence affirms that at least 53%, and possibly more, of children with autistic disorder excrete excess porphyrin in their urines.

Diagnostic accuracy is a further concern, given the complexity of applying international (English language) diagnostic instruments to French-speaking children and their families. Even in major international centres, ASD diagnostic accuracy hovers in the vicinity of 90% (Smeeth et al., 2004). Our study groups could therefore contain a small number of subjects who might more properly be classified as another disorder. However, autism and autism+epilepsy, with porphyrin excess, were clearly distinct from Asperger where no excess was seen, pointing to diagnostic accuracy.

The biochemical distinction between Asperger disorder and autism underscores the debate whether these are truly distinct disease entities. Some have questioned whether Asperger disorder merits a separate diagnostic categorization (Mayes et al., 2001; Macintosh et al., 2004) while others have argued that Asperger disorder can be distinguished from autistic spectrum disorders on the basis of cognitive testing (Ghaziuddin et al., 2004) and neuroimaging (Lotspeich et al., 2004). An etiology distinct from autism is consistent with the observed reduction in Asperger rates over the last 2 decades as a sub-proportion of ASD/PDD (MIND Institute, 2002). However, our results do not exclude historic exposure of Asperger subjects during an early window of developmental susceptibility.

Within other ASD categories, those diagnosed with pervasive developmental disorder (PDD-NOS) had only a mild (non-significant) increase in porphyrin levels, while 2 children

with Rett's disorder had extreme high values. This latter observation is of interest for Rett's is generally considered to be a genetic disorder of methyl DNA binding protein MECP2 (Amir et al., 1999). Nevertheless, precoproporphyrin levels were also elevated in Rett's disorder, pointing directly to heavy metal exposure, but because only two subjects were studied it is not known if this is representative of the disorder. We note that affected individuals range from classically symptomatic to asymptomatic (Naidu et al., 2003) and, although chromosome X-inactivation may explain much of the variability, environmental factors could exacerbate the condition.

We further report small elevations in urinary porphyrins in some non-ASD conditions. Most failed to achieve statistical significance, with the exception of epilepsy and mental retardation with epilepsy, while cerebral palsy only narrowly fell short of statistical significance (but was significant versus the external control group). Porphyrinuria was not generally significant in hyperactivity, attention deficit, or PDD-NOS, disorders that many consider to overlap with autism (though again significance was increased versus the external control group).

Porphyrin excess in autism+epilepsy was larger than in autism alone, raising the possibility that anti-epileptic medication might contribute to the elevation. However, two subjects with frank epilepsy alone, without a diagnosis of autism, displayed less precoproporphyrin than either autism or autism+epilepsy. Because levels were somewhat elevated above control groups, medication could contribute in part to the porphyrinuria seen in autism+epilepsy. Heavy metal exposure might also contribute to epilepsy (in the absence of autism): seizures are a sign of toxicity with heavy metals including mercury (Brenner et al., 1980; Bernard et al., 2001). However, medication is unlikely to contribute to porphyrinuria in autistic subjects where there is no evidence of seizure activity. First, all subjects (with the exception of children with epilepsy) were unmedicated. Second, precoproporphyrin elevation has not been reported in chemical toxicity, and fall in porphyrin levels on chelation therapy indicates heavy metal exposure rather than another cause.

Other variables include diet and disease. Children with neurodevelopmental disorders are often given restricted diets (Millward et al., 2004) and some may have GI involvement (White, 2003), notable because GI ulcerative conditions can be a rare cause of porphyrin excess (Sieg et al., 1991). However, precoproporphyrin excess points to heavy metal toxicity rather than another disorder, and the fall in porphyrin levels on chelation argues against a dietary or disease cause.

Our results accord with previous suggestions that heavy metal toxicity might contribute to the pathoetiology of autism (Bernard et al., 2001; Holmes et al., 2003) but do not identify the agent involved. The porphyrin spectrum provides an insight: specific excess of pentacarboxyporphyrin suggests interference with uroporphyrin decarboxylase (UROD)

and adjacent reactions (Fig. 1). In vitro, lead (Pb) does not block UROD while the same enzyme is potently inhibited by mercury (Hg) (Woods, 1995) and by certain other metals and metalloids (Woods et al., 1987; Garcia-Vargas et al., 1994).

Despite evidence for an association, one may not rigorously conclude that heavy metals are causally responsible for autism. Children exposed to heavy metals are likely to be co-exposed to other environmental toxins including polychlorinated biphenyls and dioxins that can also raise porphyrin levels (Marks et al., 1982; Hill, 1985; Daniell et al., 1997); chemical toxicants can synergize with heavy metals in the type and extent of damage (Stewart et al., 2003). Nevertheless, precoproporphyrin is a specific marker of metal toxicity (Woods et al., 1993b; Gonzalez-Ramirez et al., 1995; Woods, 1995; Pingree et al., 2001) and the porphyrin fall on chelation points to heavy metal exposure.

Excess urinary porphyrin, in addition to being a marker of toxicity, could play a contributory role in the behavioral manifestation of autistic disorder. Porphyrinuria is accompanied by elevated blood levels both of porphyrins and the precursor molecule 5-aminolevulinic acid ( $\delta$ ALA), (Costa et al., 1997; Opler et al., 2004) These metabolites target benzodiazepine receptors in the brain (Brennan et al., 1979; Muller et al., 1977; Verma et al., 1987) and have been associated with neurologic disturbances, epilepsy and autism (Ruscito et al., 2003; Gordon, 1999; Millward et al., 2001; Marion, 1995). Excess of these metabolites could contribute to the brain and behavior disturbances in some subjects with autism.

This then raises the question of whether heavy metal removal by chelation might alleviate the behavioral disturbances of autism. There has been an anecdotal report of benefit, particularly in younger children (Holmes, 2003) but this has not yet been confirmed. For the future, systematic evaluation of behavioral scores prior to and following chelation will be required. Chelation is also not without risk (Markowitz et al., 1990).

In conclusion, porphyrinuria, a reliable marker of environmental toxicity, is significantly over-represented in a large group of French children with autistic disorder. We stress that not all children with autistic disorder have porphyrinuria; nevertheless a majority of these children excrete excess porphyrins. The excess is not strictly confined to autistic disorder, and some subjects with other diagnoses also displayed somewhat elevated levels of urinary porphyrins. Because this is the first report addressing porphyrin levels in autism, our results will require independent replication. However, given evidence for increasing population exposure to heavy metals including mercury (Ozuah et al., 2003; UNEP Global Mercury Assessment Working Group, 2003), suggestions of increasing prevalence of autistic disorder (Blaxill, 2004), and a statistical association between mercury release and autism rates (Palmer et al., 2006), one may suspect that environmental toxicity, combined with genetic susceptibility (Holmes et al., 2003; Woods et al., 2005) contributes to ASD development, as discussed elsewhere (Lathe, 2006). Further investigations are warranted.

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**TABLE 1. Study subjects**

Condition/diagnosis	M	F	Total	Mean age (yr)	M/F	% total	% ASD group
Allergy	5	3	<b>8</b>	7.3	1.67	3	
Asperger	10	1	<b>11</b>	10	10	4.1	5.8
Attention deficit	2	7	<b>9</b>	9.4	0.29	2.3	
<b>Autism (autistic disorder)</b>	<b>79</b>	<b>27</b>	<b>106</b>	<b>6.4</b>	<b>2.9</b>	<b>39</b>	<b>55.5</b>
Autism + epilepsy	7	2	<b>9</b>	9.3	3.5	3.3	4.7
Cerebral palsy	6	6	<b>12</b>	8.3	1	4.4	
Epilepsy	2	0	<b>2</b>	10	na	0.7	
Hyperactivity	27	2	<b>29</b>	9.1	13.5	10.7	
MR + epilepsy	1	1	<b>2</b>	6	1	0.7	
PDD-NOS	51	12	<b>63</b>	6.6	4.3	23.4	33
Psychomotor retardation	1	3	<b>4</b>	7.3	0.33	1.5	
Rett	0	2	<b>2</b>	2.5	0	0.7	1
Control group	7	5	<b>12</b>	10.3	1.4	4.4	
<b>TOTAL</b>	<b>198</b>	<b>71</b>	<b>269</b>	<b>7.4</b>	<b>2.8</b>		

ASD = 71% of total sample (M/F = 3.34)

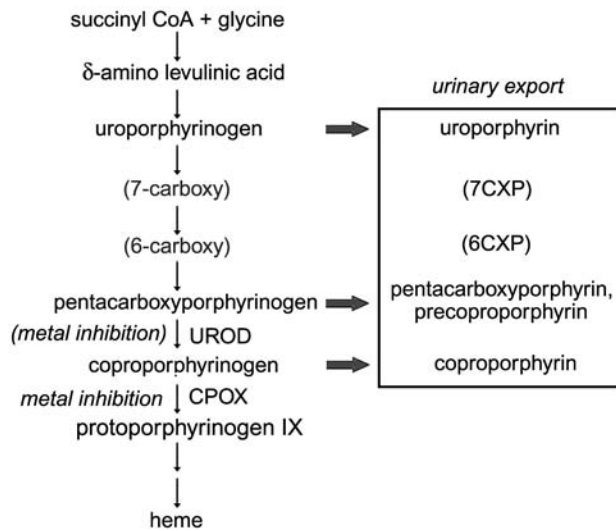


Fig. 1. Pathway of heme synthesis, major urinary metabolites, and inhibition by heavy metals. Porphyrinogens appear in urine as porphyrin derivatives (right): urinary penta-, precopro-, and coproporphyrin are indicators of inhibition of UROD (uroporphyrinogen decarboxylase) and/or CPOX (coproporphyrinogen oxidase); urinary uroporphyrin is not reported to alter with inhibition of these enzymatic steps. 7-carboxy, 6-carboxy; 7CXP, 6CXP; hepta- and hexa-carboxyporphyrinogens and -carboxyporphyrins respectively.

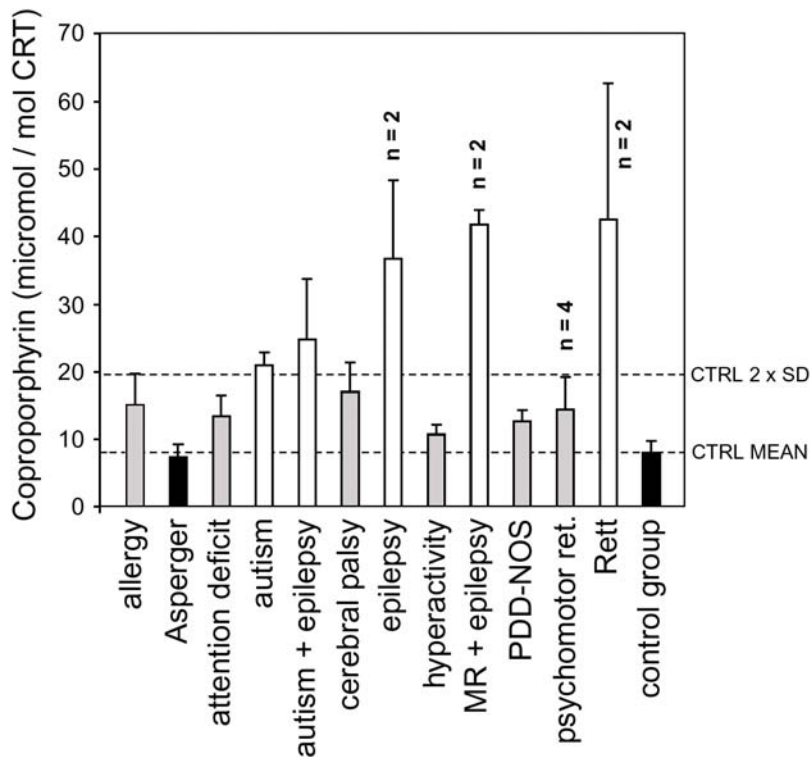


Fig. 2. Coproporphyrin levels in urines of children with neurodevelopmental and related disorders (Table 1 for details); the control group comprised children with unrelated conditions. Error bars are standard errors of the mean. Horizontal dashed lines indicate the control group (CTRL) mean and the mean plus 2 x standard deviation (SD). N values are indicated for groups with less than 8 subjects. MR, mental retardation; PDD-NOS, pervasive developmental disorder not otherwise specified.

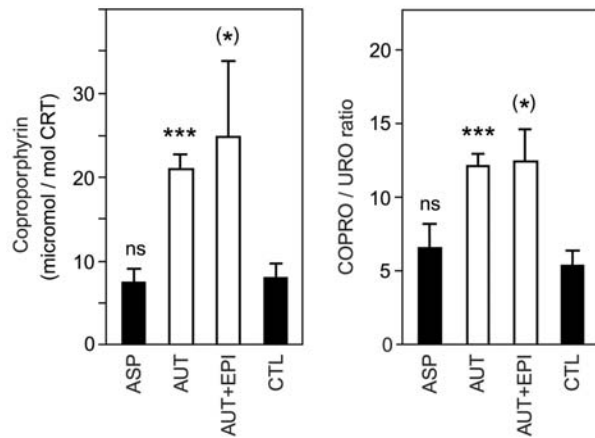


Fig. 3. Elevated urinary coproporphyrin (COPRO) levels in ASD expressed as absolute values normalized to creatinine (left) or as an internal ratio with uroporphyrin (URO) determined from the same HPLC trace (right). Values are means  $\pm$  SEM. ASP, Asperger disorder; AUT, autism; AUT+EPI, autism with epilepsy; CTL, internal control group. Statistical significances of differences versus the control group were \*\*\*,  $p < 0.001$ ; (\*),  $p < 0.1$ ; ns, not significant.

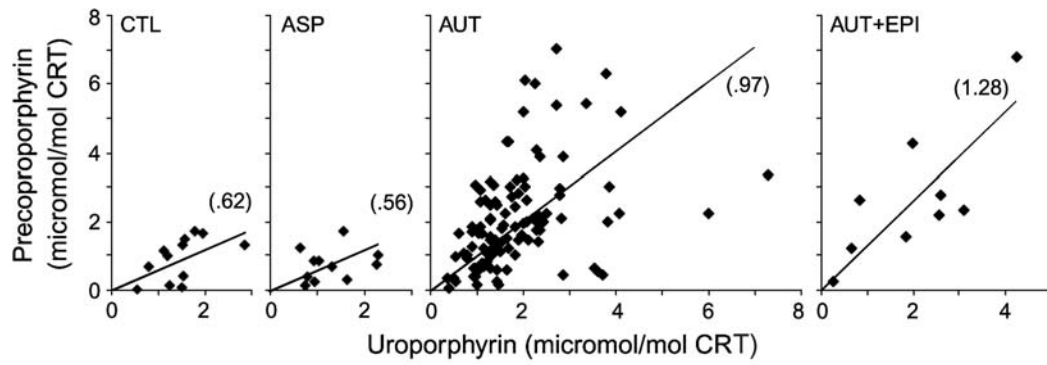


Fig. 4. Precoproporphyrin levels, a marker of heavy metal toxicity, plotted against baseline uroporphyrin values; the ratio is independent of age-related creatinine variation. Groups were CTL, control group; ASP, Asperger disorder; AUT, autism (with two exclusions, methods); AUT+EPI, autism with epilepsy. Bracketed figures give the linear regression (best-fit) gradient.

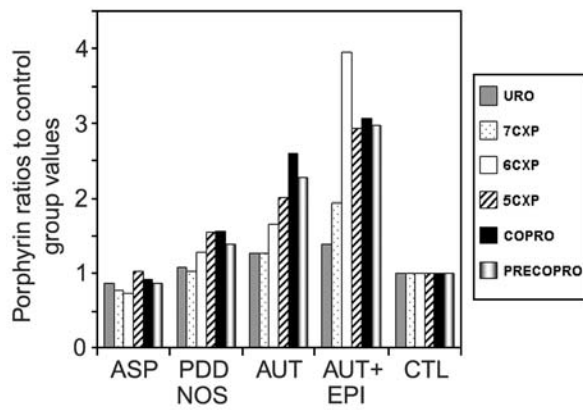


Fig. 5. Spectrum of mean (creatinine-normalized) porphyrin excess, expressed as a ratio of control group (CTL) values, for the different porphyrin subtypes uroporphyrin (URO), hepta-, hexa- and pentacarboxyporphyrin (7-, 6-, 5CXP), coproporphyrin (COPRO) and precoproporphyrin (PRECOPRO) in different conditions: ASP, Asperger disorder; PDD-NOS, pervasive developmental disorder not otherwise specified; AUT, autism; AUT+EPI, autism with epilepsy.

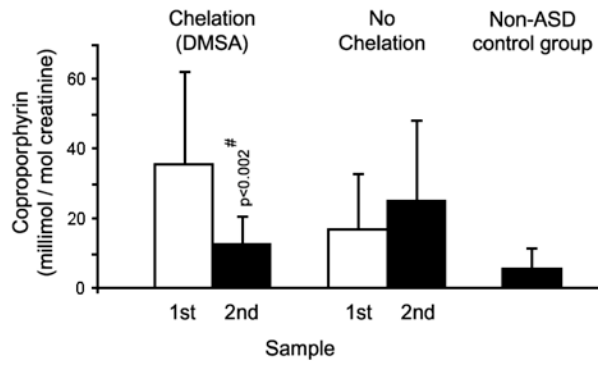


Fig. 6. Reduction in urinary coproporphyrin on chelation with DMSA. Parent-selected children with a diagnosis of autism or autism+epilepsy (n=11, mean age 8.7 yr at second sample), and evidence of porphyrin excess, were treated (oral DMSA, Methods) and urine samples compared before and after chelation (mean time between samples, 18.6 mo). The unselected control group (n=10, mean age 8.6 yr at second sample) represented all children with the same diagnosis and for which two independent urine samples >6 months apart were available (mean time between samples, 13.8 mo). The reference (non-ASD) box was the internal control group. Values are means +/- standard deviations to demonstrate the variance. The fall in coproporphyrin levels following DMSA treatment (#) was statistically significant.

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