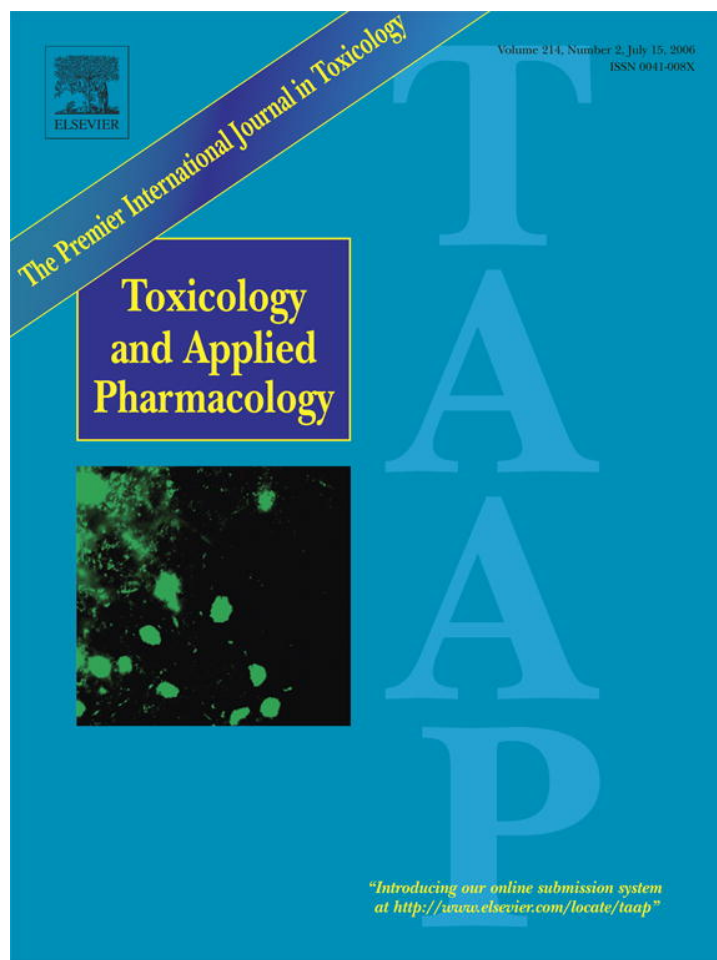


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the control group. These data demonstrate specific PRECOPRO elevation in autism and in autism with epilepsy. Levels in the two subjects with epilepsy alone were lower than with either autism or autism + epilepsy (mean PRECOPRO/URO ratio = 1.17, versus 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).

Pentacarboxy porphyrin is a further marker of heavy metal toxicity (Woods et al., 1993). Levels of this porphyrin, and its immediate precursors (hepta- and hexa-carboxy porphyrins), 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 pentacarboxy porphyrin ( $P < 0.001$ ) and hexacarboxy porphyrin ( $P < 0.002$ ), but without significant elevation of heptacarboxy porphyrin. Asperger and PDD-NOS did not differ significantly from control group in levels of any of these carboxy porphyrins. Autism with epilepsy was significantly higher than control for penta-carboxy porphyrin (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 heptacarboxy porphyrin (7CXP). Generally, in these disorders the same children with high values for hexa- had elevated values for heptacarboxy porphyrin. Of the other disorders surveyed, only mental retardation + epilepsy showed a significant increase in all three porphyrin intermediates while only pentacarboxy porphyrin was significantly increased in Rett's disorder (not presented).

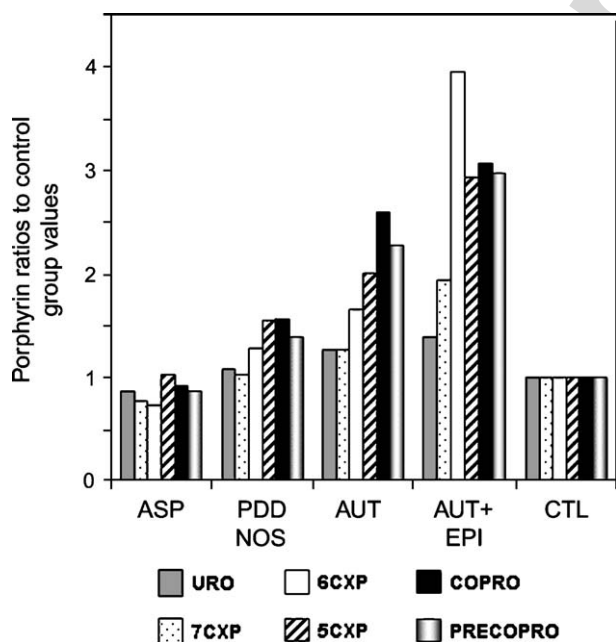


Fig. 5. Spectrum of mean (creatinine-normalized) porphyrin excess, expressed as a ratio of control group (CTL) values, for the different porphyrin subtypes uroporphyrin (URO), hepta-, hexa- and pentacarboxy porphyrin (7-, 6-, 5CXP), coproporphyrin (COPRO) and precoproporphyrin (PRECOPRO) in different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

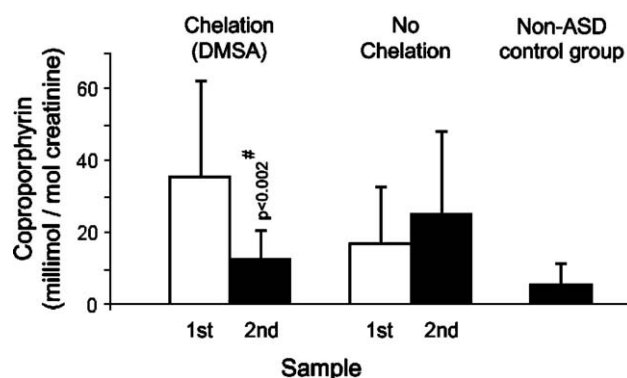


Fig. 6. Reduction in urinary coproporphyrin on chelation with DMSA. Parent-selected children with a diagnosis of autism or autism + epilepsy ( $n = 11$ , mean age 8.7 years at second sample) and evidence of porphyrin excess were treated (oral DMSA, Methods) and urine samples compared before and after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

#### 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 penta-carboxy porphyrin, 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 × 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 and Schneider-Yin, 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.

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 and

Schneider-Yin, 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 and Dissanayake, 2004), while others have argued that Asperger disorder can be distinguished from autistic spectrum disorders on the basis of cognitive testing (Ghaziuddin and Mountain-Kimchi, 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 and Snyder, 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 and Fowler, 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 and Miller, 1993; 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 and Cantrill, 1979; Muller and Snyder, 1977; Verma et al., 1987) and have been associated with neurologic disturbances, epilepsy and autism (Ruscito and Harrison, 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 and Weinberger, 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.

### Acknowledgments

We are indebted to Dr. Elisabeth Minder (Swiss Porphyrin Reference Laboratory SGK/IFCC, Stadtspital Triemli, Zürich, Switzerland) for providing her original results for reanalysis. We thank Julia Mizrahi and Martine Clair for their special help in collecting data. We also thank Elisabeth Minder, Boyd Haley, John O. Bishop, Marie-Paule Kieny, Ian Reid and David St. Clair for critical comments on the manuscript. The authors declare they have no competing financial interests.

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