Correlation of urinary excretion of cysteine - sulphate metabolites and *trans*-indolyl-3-acrloylglycine (IAcrGly) in ten children diagnosed with pervasive developmental disorders.

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Abstract.

Background.

A number of organic parameters are under investigation in childhood pervasive developmental disorders (PDD). Aberrant cysteine - sulphate metabolism characterised by perturbed levels of urinary cysteine / sulphate / sulphite, and elevated levels of urinary *trans*-indolyl-3-acryloylglycine (IAcrGly) represent two such areas of research. These factors share common mechanisms within the opioid-excess hypothesis of autism, but have not been systematically investigated together. We report the results of an exploratory study correlating quantitative urinary cysteine - sulphate-related metabolites and IAcrGly levels (normalised for creatinine) for 10 children diagnosed with PDD.

Methods.

Analysis of urine samples by colormetric and High-Performance Liquid-Chromatography (HPLC) assays were conducted for total urinary protein (mcg/ml), cysteine (nmol/ml), free sulphate (nmol/ml), total sulphate (nmol/ml), sulphite (nmol/ml), thiosulphate (nmol/ml), thiocyanate (nmol/ml) and IAcrGly, as a function of urinary creatinine (area under the urinary curve, mV / mmol/L). Urinary sulphate / glucuronide (S/G) ratios were also measured following paracetamol challenge.

Results.

A significant positive correlation (Spearman's, $p<0.05$) was found between levels of urinary cysteine and urinary IAcrGly (normalised for creatinine) (0.685, $p=0.029$). Chronological age (in months) showed no significant correlation with any parameter.

Conclusions.

A significant correlation between urinary cysteine and IAcrGly levels provides preliminary support for a possible link between the immune system and tryptophan and cysteine pathways in PDD.

Introduction.

Consistent physiological findings specific to the multitude of conditions included under the description "*pervasive developmental disorder*" (PDD) are at present a rarity. The lack of universally accepted aetiological or pathological biomarkers contributes to the continued dominance of psychological and behavioural models of the PDD condition, which are primarily based on the overt presentation of the triad of impairments [APA, 1994; WHO, 1992]. Biological and genetic studies have only just begun to categorise differences in sub-groups within the PDD spectrum that may subsequently yield objective biomarkers for the condition. The problem has occurred to a large extent because of the heterogeneous nature of the condition. Promising strands of research have begun to emerge from various biochemical and physiological investigations, in particular the impact of gastrointestinal factors in PDD [Wakefield *et al*, 1998; Furlano *et al*, 2001] supporting a metabolic basis to the condition [Shattock & Whiteley, 2002].

Aberrant cysteine - sulphate metabolism is a key finding in PDD, as exemplified by findings of low plasma sulphate levels and elevated quantities of urinary sulphate and precursor metabolites [O'Reilly & Waring, 1993; Alberti *et al*, 1994; Waring & Klovrza, 2000]. The consequences of problems with cysteine - sulphate metabolism are numerous, including inadequate deactivation of phenolic neurotransmitter amines such as dopamine, insufficient release of digestive enzymes and abnormalities associated with the connective tissue that make up the membranes of the gastrointestinal tract.

Excessive permeability of the gastrointestinal membrane, whether through decreased levels of free sulphate or as a consequence of other factors has been reported in patients with PDD [D'Eufemia *et al*, 1996]. Several studies have also indicated the presence of a number of elevated compounds in the urine of people with PDD [Reichelt *et al*, 2003; Dhossche *et al*, 2002]. Some of these compounds are congruent with the opioid-excess hypothesis of PDD [Shattock & Whiteley, 2002] including the increased transport of incompletely hydrolysed peptide species derived from exogenous sources across the gastrointestinal wall.

Excretion of the compound *trans*-indolyl-3-acryloylglycine (IAcrGly) in urine fractions from people with PDD [Mills *et al*, 1998; Anderson *et al*, 2002a; Whiteley & Shattock, 2003; Bull *et al*, 2003] presents an alternative explanation for abnormal permeability of the gastrointestinal membranes principally derived from the aberrant metabolism of tryptophan to indole-3-acrylic acid (IAcrA). Interest has focussed on the role of this compound, a precursor to IAcrGly, in promoting excessive permeability of biological membranes [Hooper, 2000]. Preliminary investigation has shown that use of a gluten-free diet decreases levels of urinary IAcrGly as well as ameliorates some of the symptoms of PDD [Whiteley *et al*, 1999], although the exact mechanism has yet to be fully elucidated.

Both areas of study have been incorporated into a coherent metabolic hypothesis of PDD [Shattock & Whiteley, 2002], although as yet there has been no attempt to correlate the findings of perturbed levels of cysteine - sulphate metabolites and increased urinary IAcrGly output. We provide preliminary data showing a correlation between quantitative levels of urinary cysteine - sulphate metabolites and urinary IAcrGly in ten patients diagnosed with PDD.

Methods.

Subjects.

Ten children (9 males, 1 female) resident in the UK with a formal diagnosis of PDD using DSM-IV / ICD-10 criteria (4 diagnosed with autism, 1 diagnosed with Asperger syndrome, 5 diagnosed with ASD equivocal to PDD-NOS [Wing, 1996]) (median age = 89.5 months, inter-quartile ranges 66-140 months) were studied. Participants were selected on the basis of completion of urinary IAcrGly screening as part of an on-going research programme [Whiteley & Shattock, 2003] and accompanying urinary cysteine / sulphate screening [Waring *et al*, 1997]. Formal diagnosis was defined as: parental report of specific PDD diagnosis given, time and place of receipt of diagnosis, and details of the diagnosing clinician (and institution where available). Diagnoses and level of intellectual ability were not independently verified.

Urinary cysteine / sulphate metabolites assay

Standard colormeteric methods were used to estimate cysteine / sulphate metabolites as reported by Waring & Klovrza [2000].

Urinary Sulphate / Glucuronide assay

The method of analysis for paracetamol sulphate/glucuronide (S/G) ratio has been previously described [Alberti *et al*, 1999]. Participants were given one tablet of paracetamol (500mg) after a light breakfast. All urine passed for an 8-hour period was collected and totals measured. Analyses by HPLC of urine fractions were conducted using the method described by Howie *et al* (1977). Results are expressed as the S/G ratio.

Urinary IAcrGly assay.

Sample collection, preparation and the generic reverse-phase HPLC method used for IAcrGly analysis have been previously described [Mills *et* al, 1998; Whiteley *et al*, 1999; Bull *et al*, 2003]. Confirmation of the peak IAcrGly is made by comparison with synthetic standards. UV detection is made at 215nm. Urinary creatinine levels were determined by spectrophotometric assay (Olympus). Results are expressed as area under urinary curve (mV) / creatinine (mmol/L). The research was carried out in accordance with the Declaration of Helsinki. All statistical analysis of data were done with SPSS for Windows© (v10.1 SPSS Inc. Chicago, IL, 2000) at $p<0.05$.

Results.

Individual patient results are shown in Table 1. Correlation analysis (Spearman's rho 2-tailed test) showed a significant positive correlation between urinary cysteine and quantitative levels of IAcrGly (normalised for creatinine) (0.685, p=0.029). No significant correlation was found between levels of IAcrGly and any other parameter: total urinary protein (0.109, p=0.763), free sulphate (-0.152, p=0.676), total sulphate (0.176, p=0.627), sulphite (-0.437, p=0.207), thiosulphate (-0.413, p=0.235), thiocyanate (0.594, p=0.070), urinary sulphate / glucuronide (S/G) ratio (-0.097, p=0.789).

Other factors that did show significant correlations were: urinary cysteine and total urinary protein (0.711, p=0.021), total sulphate and free sulphate (0.636, p=0.048) and urinary sulphite and creatinine levels (0.649, p=0.042). Chronological age (in months) was not significantly correlated with any factor.

Discussion.

Our preliminary results suggest that levels of urinary cysteine significantly correlated with quantitative levels of IAcrGly (adjusted for creatinine concentrations). Cysteine is an important intermediary in the production of inorganic sulphate and related anions. Conversion of cysteine to cysteine sulphinic acid is governed by the polymorphic enzyme cysteine dioxygenase (CDO). Waring *et al* (1997) reported initial evidence that nearly half of children with autism studied *in-vivo* showed impairment with the S-oxidation of cysteine via this route. Possible factors affecting this reaction by inhibition of CDO have focused on the role of specific classes of cytokines, including tumour necrosis factor alpha (TNF-α) [Wilkinson & Waring, 2002]. Cysteine produced by antigen presenting cells plays a key role, along with tryptophan in the activation and proliferation of T-cells [Edinger & Thompson, 2002; Angelini *et al*, 2002].

The precise origin of urinary *trans*-indolyl-3-acryloylglycine (IAcrGly) is still comparatively unknown. Two possible routes have been suggested thus far. The first reports IAcrGly to be derived from catabolism of tryptophan via intestinal micro-organisms followed by conjugation of the acidic precursor with glycine [Hooper, 2000]. The second has focused on inhibition of the rate-limiting enzyme tryptophan hydroxylase (TPH) and enzyme co-factors such as tetrahydrobiopterin (BH4) and iron by environmental agents [Anderson *et al*, 2002b]. Only very small amounts of IAcrGly were found in the urine of piglets reared in an aseptic environment [Marklova, 1999] suggesting an exogenous source of both IAcrGly and IAcrA.

The heterogeneous nature of PDD implicitly implies that numerous biological factors will feature in the aetiology and pathology of the condition. This, combined with the lack of common universal biomarkers, makes the task of finding relevant common ground between

these variables more difficult. Suspicion as to the role of the immune system and the serotonergic pathway in PDD provides some of the biggest clues as to the nature of any link. Both sulphate and tryptophan metabolism are known to impact on these factors.

Aside from the direct link between tryptophan and production of 5-hydroxy tryptophan (5-HT), investigations into the effect of immune activation on tryptophan metabolism provide some possible connections. Saito *et al* (1992) showed that the effect of administration of interferon-gamma (INF-γ) on tryptophan metabolism resulted in increased levels of kynurenic and quinolinic compounds through induction of the indoleamine-2,3-dioxygenase enzyme (IDO). Both these compounds are known to have neurotoxic and pro-convulsant properties, and subsequent metabolites (kynurenate and xanthurenate) may also inhibit the TPH enzyme. Tryptophan catabolism has also been implicated in the modulation of the immune system through induced immune cell apoptosis or depletion of tryptophan suppressing proliferation of immune cells [Moffett & Namboodiri, 2003]. Tryptophan levels are crucial for the proliferation of T-cells thereby modulating the immune response [Mellor & Munn, 1999; Widner *et al*, 2000]. Any changes in cysteine and/or tryptophan levels would therefore be expected to have significant effects on the functioning of the immune system, something known to be compromised in many children with PDD [Warren *et al*, 1986; Jyonouchi *et al*, 2001]. T-cells also play an important role in orchestrating intestinal mucosal shape and integrity [MacDonald *et al*, 1999] providing another role for this important amino acid and a further link to a major feature of autism, increased gut permeability.

Preliminary studies examining the use of a gluten-free diet have shown it to be effective in ameliorating some of the behavioural symptoms of PDD [Whiteley *et al*, 1999]. This was also accompanied by a non-significant reduction of levels of IAcrGly compared to non-dietary intervention PDD participants. The precise role of IAcrGly in this abatement of symptoms is still unclear. Jyonouchi *et al* (2002) reported that peripheral blood mononuclear cells (PBMCs) from children with PDD showed significantly higher levels of cytokine response (INF-γ, TNF-α) to gliadin, casein and soy compared to controls. It is feasible that elevated levels of cytokines (TNF-α) produced as a consequence of immuno-reactivity to dietary proteins could contribute to the inhibition of CDO.

The successful conversion of cysteine to both sulphate and taurine via the CDO enzyme are also important compounds for a range of biochemical processes. These include detoxification of phenolic amines such as 5-HT and dopamine. Dopamine has been reported to show both high affinity for TPH and also non-competitive inhibition [Naoi *et al*, 1994]. A further role in the maintenance of mucin structure of connective tissue on the gastrointestinal wall demonstrates the feasibility of aberrant cysteine – sulphate metabolism in the pathology of PDD within the opioid-excess hypothesis.

It is imperative that further work be conducted to ascertain the significance of these preliminary findings to PDD. The combination of variables such as small sample size, lack of objective verification of diagnoses, and lack of detailed clinical history of participants are all potential sources of bias in this study. Further research is necessary to elucidate the relationship between these parameters and other potential biomarkers, for example, measures of gastrointestinal permeability, levels of PBMC cytokines and levels of circulating neurotransmitter metabolites.

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Table 1: Individual patient results for biochemical tests

Patient	Diagnosis	Age (months)	protein	cysteine	free sulphate	total sulphate	sulfite	thiosulphite	thiocyanate	S/G	creatinine	IAcrGly / creatinine
1	Autism	104	194.8	52.6	3972.1	6347.1	0	379.5	3.3	0.75	5.5	347
2	ASD	65	98.6	43.5	4205.6	8763.4	78.2	98.4	0	1.2	14.4	296.6
3	ASD	194	882.5	54.8	4053.8	8067.2	0	0	0	0.8	7.8	403.2
4	ASD	152	37.8	5.5	4319.2	5407.1	521.8	90.3	0	1	12.1	193.9
5	Asperger	137	37.8	42.9	6572.8	11116.2	0	0	28.1	1.9	6.6	376.4
6	ASD	67	155.7	391.5	10604.4	16048.1	0	27.3	89.9	0.8	1.7	8110
7	ASD	65	45	36.8	3507.3	6103.1	0	28.2	0	1.9	1.3	2645
8	Autism	74	62.1	52.1	4586.1	10296.3	528.1	256.8	59.9	0.74	9	917
9	Autism	75	64.9	21.8	5119.8	5607.5	0	193.5	0	0.68	15.3	173.5
10	Autism	109	87.1	34.6	9940.1	21401.9	576.1	229.5	0	3.9	15.9	77.94

Results are expressed in the following units: total urinary protein (mcg/ml), cysteine (nmol/ml), free sulphate (nmol/ml), total sulphate (nmol/ml), sulphite (nmol/ml), thiosulphate (nmol/ml), thiocyanate (nmol/ml), urinary sulphate / glucuronide ratios (S/G), creatinine (mmol/L), IAcrGly, as a function of urinary creatinine (area under the urinary curve, mV / mmol/L).