

Is the presence of urinary indolyl-3-acryloylglycine associated with autism spectrum disorder?

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To test whether the presence of indolyl-3-acryloylglycine (IAG) is associated with autism, we analyzed urine from population-based, blinded cohorts. All children in York, UK with autism spectrum disorders (ASDs), diagnosed using ICD-10 research diagnostic criteria, were invited to participate. Fifty-six children on the autism spectrum (mean age 9y 8mo, SD 3y 8mo; 79% male) agreed to participate, as did 155 children without ASDs (mean age 10y, SD 3y 2mo; 54% male) in mainstream and special schools (56 of whom were age-, sex-, and school-matched to children with ASDs). IAG was found at similar levels in the urine of all children, whether IAG concentrations or IAG:creatinine ratios were compared. There was no significant difference between the ASD and the comparison group, and no difference between children at mainstream schools and those at special schools. There is no association between presence of IAG in urine and autism; therefore, it is unlikely to be of help either diagnostically or as a basis for recommending therapeutic intervention with dietary manipulation. The significance of the presence of IAG in urine has yet to be determined.

Although several studies have indicated a variety of metabolic abnormalities in autism, there are no consistent findings and few have led to any rational treatment or intervention. Despite the lack of evidence, there exist theories on autism that propose causal links and therapeutic interventions. One such theory is that of opioid excess. This proposes that autism may arise secondary to an opioid peptide excess (Panskepp 1979, Sher 1997). Abnormal peptide content has been described in the urine of children with autism (Shattock et al. 1990; Reichelt 1991, 1994). Part of this theory is based on the hypothesis that the bowel of some children with autism has an enteropathy due to gluten, which allows excess peptide absorption, particularly that derived from casein. There is no proven association between gluten enteropathy, coeliac disease, and autism with conventional diagnostic methods (McCarthy and Coleman 1979, Pavone et al. 1997, Fitzgerald 1999). A research group in Sunderland, UK (Shattock et al. 1990, 1991; Mills et al. 1998) hypothesized that not only are endogenous opioid peptides implicated in the aetiology of children with autism spectrum disorders (ASDs) but that an excess of indolyl-3-acryloylglycine (IAG) in the urine is a marker for autism and the presence of excess opioid peptides. This is supported by analysis of urine samples, which indicates that children with autistic spectrum disorders have high levels of IAG (Anderson et al. 2002). This last study does not report the urinary IAG levels of healthy comparison children, although a subsequent non-blinded study included 22 children with ASDs and 18 asymptomatic comparison children (Bull et al. 2003). Many children with autism in the UK are currently on gluten- or casein-free diets, based on analysis of urine samples for IAG and advice that this is due to excessive absorption from a bowel damaged by a diet containing gluten. A recent study (Hunter et al. 2003) exploring the presence of urinary opioid peptides in children with autism, using rigorous scientific methodology, failed to confirm the relationship and questioned the validity of the theory.

The aim of this study, using rigorous methodology on a population-based sample with a healthy comparison group, was to test whether there is an association between the presence of IAG in the urine and ASDs.

Method

York NHS Trust and Selby and York Primary Care Trust, UK serve a population of 270 000. Children and young people were approached to participate in the study if they had a diagnosis of childhood autism, atypical autism, or Asperger syndrome based on criteria from the International Classification of Diseases – Version 10 (ICD-10; World Health Organization 1993). Where there was uncertainty, the Autism Diagnostic Inventory – Revised (Lord et al. 1994) and the Autism Diagnostic Observation Schedule – Generic (Lord et al. 2001) were used, both of which are instruments that enhance and support ICD-10 diagnoses. This is the routine assessment and diagnosis protocol for the local multidisciplinary Autism Spectrum Disorders Forum. For comparison, healthy children without autism from special schools and mainstream schools were also recruited, and at least one age- and sex-matched child from a similar school was chosen for each child with ASDs.

After consent was obtained from head teachers and governors, a standard information leaflet was circulated to parents inviting them to participate in the study. Informed consent and assent was obtained from parents and children.

Full ethical committee approval was given for this study. Exclusion criteria included any known metabolic disorder, and, for the group without autism, any previous or current assessment for an ASD. A brief questionnaire was given to parents to elucidate concurrent dietary habits, use of medication or other remedies, and bowel habit history over the previous two weeks.

A sample collection method was used with standardized equipment; a leaflet for parents and children explained collection procedures. Urine samples were blinded with code numbers and delivered from one of several points of collection to the Department of Chemical Pathology at York Hospital within 24 hours and stored at -20°C . Each urine sample was divided into two parts. The first was used to perform quantitative analysis of IAG and the second for determination of the creatinine concentration using a standard method (Cook 1971) on an Hitachi 917 analyzer (Boehringer, Mannheim, Germany). IAG:creatinine ratios were calculated to control for variations caused by body mass, urine concentration, and other metabolic factors.

IAG was synthesized by a method adapted from Mills et al. (1998), which involved esterification of indole-3-acrylic acid with glycine methyl ester using dicyclohexylcarbodiimide and hydroxybenzotriazole in dichloromethane. In addition, a dideuterated analogue of IAG was synthesized by the same route according to a method by Tilley et al. (1994).

Analysis of urine samples for IAG was effected by using high performance liquid chromatography with tandem mass spectrometric detection (Imrie et al. 2004). Dideuterated IAG was used as an internal standard. The method was validated according to internationally recognized standards (Food and Drug Administration of the United States of America 2001) to ensure precision, accuracy, and specificity. All samples were analyzed within stability parameters established during the method validation. The analytical laboratory was blinded to sample identity. A independent statistician analyzed the statistics.

Results

POWER CALCULATIONS

Using unpublished data (MJ Mills, D Savery, PW Groundwater, personal communication 2002), we performed power calculations. The estimated effect size was $d=0.97$. At a power of 80% and significance level of 5% (two-tailed), we needed 18 children in each group to be able to show this difference in mean values. We set out to include larger numbers because the study we used for power calculations compared children with autism with adult laboratory technicians, so we were uncertain about the effect size.

One hundred and thirty-seven families with children on the autism spectrum were approached: 78 agreed to take part and 56 children produced samples of urine (providing a sample in a short time-scale was the biggest problem for families). One hundred and fifty-five healthy children were recruited. The age range for children with autism was 2 years 7 months to 20 years 11 months (mean 9y 8mo, SD 3y 8mo), and for children without ASD it was 3 years 6 months to 21 years 6 months (mean 10y, SD 3y 2mo). Forty-four (79%) of the ASD group were male compared with 84 (54%) of the comparison group. Of the 56 children with autism, 33 had childhood autism, 7 had atypical autism, and 16 had Asperger syndrome.

All children on the autism spectrum had IAG in their urine. However, contrary to expectation, all healthy children also had

IAG in their urine at similar levels. Mean IAG concentration for all children with ASD was 0.0511mmol/L ($n=56$). For children without autism the mean was 0.0508mmol/L ($n=155$). The mean for childhood autism was 0.0433 mmol/l ($n=33$), for atypical autism it was 0.0637mmol/L ($n=7$), and for Asperger syndrome it was 0.0444mmol/L ($n=16$). Because urine concentrations vary, an IAG:creatinine ratio was calculated. The distribution was skewed to the left, so the ratio was transformed by adding a constant of 0.001 and taking the natural logarithm to normalize the distribution. The transformed means, standard deviations, and standard errors are shown in Table I.

There was no significant difference between the ASD and comparison groups. A comparison of raw IAG concentrations also showed no significant difference. The correlation between age and the transformed IAG:creatinine ratio was -0.31 (95% confidence interval [CI] -0.43 to -0.17 , $p<0.001$), showing that there is a moderate negative correlation between age and IAG:creatinine ratio (i.e. levels tend to drop with age). We performed an analysis of covariance, controlling for age. The difference between the means of the two groups was 0.04 (95% CI 0.20 to -0.12 , $p=0.625$). Figure 1 shows the distribution of the transformed IAG:creatinine ratio (not controlling for age

Table I: Summary statistics for logged mean of indolyl-3-acryloylglycine:creatinine ratios

Group	Number	Mean	Standard deviation	Standard error
Comparison	155	-5.25	0.48	0.038
Autism	56	-5.29	0.62	0.083

Table II: Differences in (transformed) indolyl-3-acryloylglycine:creatinine ratios between comparison group and autism spectrum disorder subgroups

Group	Difference	Standard error	p	95% CI	
				Lower	Upper
Childhood autism	-0.061	0.100	0.539	-0.257	0.135
Atypical autism	0.217	0.201	0.280	-0.178	0.613
Asperger syndrome	-0.107	0.136	0.432	-0.376	0.161

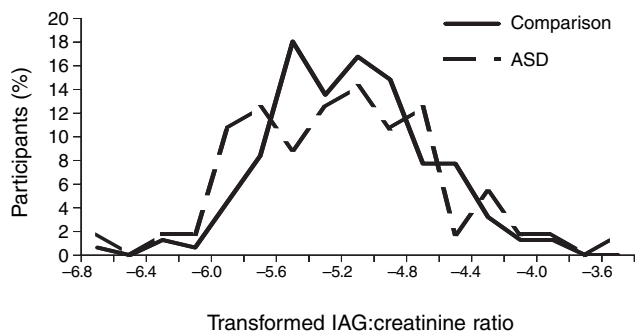


Figure 1: Frequency plot of autism spectrum disorder (ASD) and comparison groups. IAG, indolyl-3-acryloylglycine.

and sex). There was also no significant difference when paired age- and sex-matched children, controlled for school type, were compared with children with ASDs.

We further analyzed the group with autism by segregating it into childhood autism, Asperger syndrome, and atypical autism. Age was used as a covariate; the overall test of difference between the means was not significant ($F=0.76$, $df=-3$, 207 , $p=0.517$). Differences between each group and the comparison group, with 95% CIs and statistical significance, are shown in Table II.

Analysis of variance was used to investigate differences between mainstream and special schools ($F=0.42$, $df=1$, 205 , $p=0.514$), between children with ASDs and comparison children ($F=0.13$, $df=1$, 205 , $p=0.716$), and the interaction effect ($F=1.95$, $df=1$, 205 , $p=0.164$).

Discussion

IAG is known to exist in the urine of children with autism (see Anderson et al. 2002). We also found this, but that healthy children without ASD have similar amounts in their urine too. There was no significant difference between the groups and we found no trend. Our finding that IAG levels drop with age may account for the differences found between children with autism and healthy adults (MJ Mills, D Savery, PW Groundwater, personal communication 2002).

The origin of IAG in human urine is not known (Marklova 1999). Its structure is related to tryptophan and serotonin but the enzymatic production of α,β -dehydrotryptophan in humans has not yet been reported. However, the presence of L-tryptophan 2',3'-oxidase and related enzymes – capable of production of α,β -dehydrotryptophan (indole-3-acrylic acid) – has been described in some common bacteria which may populate the gut (see Roberts and Rosenfeld 1977, Zavala et al. 1983, Genet et al. 1995). Hence, the exogenous (gut flora) source of IAG cannot be excluded. Although gluten- and casein-free diets are recommended by some for autism, there is no randomized controlled evidence in the literature about the effectiveness of these diets. Research so far has also not shown a link between sensitivity to gluten and autism (McCarthy and Coleman 1979, Pavone et al. 1997). Food faddism is more common in children with ASDs (personal communication, Town J 2004), so the wisdom of restrictive diets needs to be carefully considered to avoid compromising healthy nutritional intake. Future research on specialized diets is needed and may be shown to have value, but we believe that because IAG does not appear to be a marker for autism it should not currently be used as an indicator for dietary restriction.

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