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Gut Permeability In Autism Spectrum Disorders

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Lay abstract

One hypothesis about the causation of autism is dependent on the theory of a ‘leaky gut’ that causes increased permeability to substances harmful to the brain. Some studies have found increased gut permeability in autism, others have not. Many studies have found increased reported symptoms of gastrointestinal dysfunction in autism. We measured gut permeability using a technique of sugar absorption from the gut and excretion in the urine. We did this in two groups of children, one with autism spectrum disorder (ASD) and one with special educational needs (SEN) from a variety of causes but no ASD. A few children (11 of 133) across both groups had some evidence of increased gut permeability, only 2 children at ‘pathological levels’, but there was no difference in the proportions in each group. The 2 children with definite increased gut permeability both had disorders of gut function which made the increased permeability explicable. We concluded that permeability is not increased in the majority of children aged 10-12 years with ASD compared with other groups of similar ages who have special educational needs.

Scientific Abstract

OBJECTIVE: To test whether gut permeability is increased in autism spectrum disorders (ASD) by evaluating gut permeability in a population-derived cohort of children with ASD compared with age- and IQ-matched controls without ASD but with special educational needs (SEN).

PATIENTS AND METHODS: 133 children aged 10-14 years, 103 with ASD and 30 with SEN, were given an oral test dose of mannitol and lactulose and urine collected for 6h. Gut permeability was assessed by measuring the urine lactulose/mannitol (L/M) recovery ratio by electrospray mass spectrometry-mass spectrometry. The ASD group was sub-categorised for comparison into those without (n=83) and with (n=20) regression.

RESULTS: There was no significant difference in L/M recovery ratio (mean (95% confidence interval)) between the groups with ASD: 0.015 (0.013-0.018), and SEN: 0.014 (0.009-0.019); nor in lactulose, mannitol, or creatinine recovery. No significant differences were observed in any parameter for the regressed versus non-regressed ASD groups. Results were consistent with previously published normal ranges. Eleven children (9/103=8.7% ASD and 2/30=6.7% SEN) had L/M recovery ratio >0.03 (the accepted normal range cut-off), of whom two (1 ASD and 1 SEN) had more definitely pathological L/M recovery ratios >0.04.

CONCLUSION: There is no statistically significant group difference in small intestine permeability in a population cohort-derived group of children with ASD compared with a control group with SEN. Of the two children (1 ASD and 1 SEN) with an L/M recovery ratio of >0.04, one had undiagnosed asymptomatic coeliac disease (ASD) and the other past extensive gastroschisis surgery (SEN).

Key Words: autism, autism spectrum disorders, gut permeability, lactulose/mannitol ratio

Introduction

One of the hypotheses implicated in the pathogenesis of autism has been the suggestion that autism “may be caused by endogenous overactivity of the child’s own brain opiate system” [Panksepp, 1979]. This was followed by data indicating abnormal peptides, “capable of modulating the function of major neurotransmitters”, in the urine of patients with autism [Reichelt et al., 1981]. These ideas and observations led to the “leaky gut” theory of autism in which “toxic” peptides with opioid activity derived from incomplete breakdown of foods, particularly casein and gluten, are absorbed, cross the blood-brain barrier and cause autism [Le Couteur, Trygstad, Evered, Gillberg, & Rutter, 1988; Panksepp, 1979; Reichelt et al., 1981; Shattock & Lowdon, 1991; Shattock & Whiteley, 2002]. The proposition is that, as a result of gastrointestinal inflammation, also causing gastrointestinal symptoms (GIS), the normal barrier to peptide absorption from the gut is compromised. Increased rates (22-70%) of GIS have been reported in ASD [Chandler et al., 2013; Erickson, Stigler, Corkins, Posey, Fitzgerald, & McDougle, 2005; Gorrindo, Williams, Lee, Walker, McGrew, & Levitt, 2012; Horvath & Perman, 2002; Smith, Farnworth, Wright, & Allgar, 2009; Valicenti-McDermott, McVicar, Rapin, Wershil, Cohen, & Shinnar, 2006; Wang, Tancredi, & Thomas, 2011], a variability that may depend on the sample (clinical or population-derived); the type, definition and number of symptoms; the method of investigation employed; and whether symptoms are current or life-time. The GIS most commonly reported in ASD are diarrhoea (loose frequent stools), constipation, and abdominal discomfort/pain. The American Academy of Pediatrics (AAP) consensus report [Buie et al., 2010a] found no conclusive evidence of a unique gastrointestinal pathophysiology specific to autism and related disorders.

Several reports have described increased gut permeability in children with autism [De Magistris et al., 2010; D'Eufemia et al., 1996; Horvath, Zielke, Collins, Rabsztyl, Medeiros, & Perman, 2000; Liu, Li, & Neu, 2005] and there has been considerable promotion of dietary manipulation, particularly of casein and gluten, in the treatment of autism [Knivsberg, Reichelt, Høien, & Nodland, 2002; Levy & Hyman, 2008; Millward, Ferriter, Calver, & Connell-Jones, 2004; Reichelt, Ekrem, & Scott, 1990]. However, it is important to emphasise that the fundamental basis of the “leaky gut” hypothesis does not rely on either the presence of inflammation or confirming neuropeptides in the urine, but on demonstrating impaired gut permeability in children with ASD; and the most recent consensus report of the AAP clearly highlights the limited evidence for abnormal gastrointestinal permeability in individuals with autism and recommends properly powered studies with appropriate controls to determine the role of abnormal permeability in ASD [Buie et al., 2010b].

We took advantage of a population-based epidemiological study of ASD [Baird et al., 2006] to perform formal gut permeability studies in as many participants as possible. Children with ASD were compared with children with other developmental disorders but without ASD. Contemporaneously, parents were asked to complete a questionnaire about current and past gastro-intestinal symptoms and evidence of coeliac disease was sought. Ethical permission was granted by the South Thames research ethics committee (MREC 00/1/50).

Patients and methods

Patients

The sampling methodology of the SNAP study has been described previously [Baird et al., 2006]. In brief, within a total population cohort of 56,946 children born between July 1st 1990 and December 31st 1991, all with a current clinical diagnosis of ASD (N=255) or

considered ‘at risk’ by virtue of having a Statement of Special Educational Needs¹ (SEN; N=1,515) were screened using the Social Communication Questionnaire (SCQ) [Rutter, Bailey, & Lord, 2003]. A stratified subsample (by coincidence also N=255) based on SCQ score received a comprehensive diagnostic assessment including standardized clinical observation (Autism Diagnostic Observation Schedule – Generic (ADOS-G)) [Lord et al., 2000], parent interview assessments of autistic symptoms (Autism Diagnostic Interview-Revised (ADI-R)) [Lord, Rutter, & Le Couteur, 1994], intelligence (intelligence quotient, IQ), and a medical examination. There were no exclusion criteria. ADI-R and ADOS-G information was used to derive a clinical consensus diagnosis of ASD (childhood autism and other ASDs: atypical autism, pervasive developmental disorder) with reference to ICD-10 research criteria. Severity of autism was measured in terms of number of ICD-10 symptoms (0-12). Regression was defined as a loss of 5 words used communicatively for 3 months before loss or, where the child had not reached the 5-word stage, as loss of words or babble with regression of social and play skills [Baird et al., 2008]. Cases not meeting criteria for a diagnosis of ASD were categorized as SEN; these children had special educational needs and a variety of other diagnoses including attention deficit hyperactivity disorder (ADHD), cerebral palsy, language disorders and intellectual disability [Baird et al., 2008].

Cognitive function was established using the Wechsler Intelligence Scale for Children-III-UK (WISC) [Wechsler, 1992], Raven’s Standard Progressive Matrices (SPM) [Raven, Court, & Raven, 1990a] or Coloured Progressive Matrices (CPM) [Raven, Court, & Raven, 1990b], depending on the child’s ability. For children for whom SPM or CPM but not WISC full-scale IQs were available, imputed full-scale IQs were obtained using the regression relationship of full-scale IQ to SPM/CPM within each diagnostic group.

¹A Statement of Special Educational Needs is a legal document issued by the local educational authority when children require significant additional support in school due to any learning and/or behavioural problems.

A blood sample was taken, with consent, from 173 of 255 of those in the ASD and SEN groups and a number of analyses performed. Where there was sufficient blood, a standard diagnostic assay of anti-endomysial IgA and anti-gliadin IgA was carried out, in a certified laboratory.

Gastro-Intestinal Symptoms (GIS)

Parental report on GIS was collected at the time of the diagnostic assessment using a questionnaire administered by researcher interview with the parents/carers of the ASD and SEN participants. A 20-item *GIS questionnaire* was constructed about current (last three months) and past (prior to the last three months) symptoms [Chandler et al., 2013]. The GIS included: *persistent vomiting* (at least once per day or more than five times in a week); *stool consistency* (formed, watery or mushy); *abdominal pain* (three or more episodes severe enough to interfere with activity); *abdominal pain associated with food, bowel movement or sleep*; *constipation* (decreased frequency of bowel movement to less than three times a week in the last three months); and associated symptoms such as *subjective difficulties with bowel movement and harder consistency of stools*; *stool withholding and soiling*; *diarrhoea* (loose/watery stools three or more times a day); *weight loss*; *mouth ulcer*; *presence of mucus or blood in the stools*. Sub-items of the current and past diarrhoea questions established chronicity with *persistent diarrhoea* being defined as lasting more than 14 days. 'Possible enterocolitis' was defined as the presence of 2 or more of 4 current GIS: *persistent diarrhoea*, *weight loss*, *abdominal pain* or *blood in stool* plus *past persistent diarrhoea* and excluding *current constipation*.

Parents of children with ASD and SEN were asked to record their child's typical *diet* for three days (including main meals, snacks and drinks over two weekdays and one weekend day), together with the number of different food items habitually eaten, and whether the diet was

supplemented in any way or limited by special diet or by faddiness (arbitrary and often unusual likes and dislikes about food) diets as reported by their parents.

Gut Permeability

Gut permeability was determined, following oral dosing with a mixture of lactulose and mannitol, as the urinary lactulose/mannitol (L/M) recovery ratio and lactulose recovery (percentage of oral dose administered), measured by electrospray mass spectrometry-mass spectrometry (MSMS). The differential sugar absorption test remains the primary objective test of gut permeability. It relies on the contrast between the almost complete absorption of the simple sugar alcohol, mannitol, and the virtually total exclusion, in normal individuals, of the disaccharide, lactulose, by the gut membrane. Neither mannitol nor lactulose is metabolised in the body, so that any absorbed is freely filtered by the kidney and appears in the urine. As a result, the permeability of the gut can be assessed, either by the lactulose recovery (percentage of oral dose administered) or by the ratio of the recovery of lactulose and mannitol, in an accurately timed urine collection. Accurate measurement of mannitol and particularly lactulose are crucial. In individuals with normal gut permeability the L/M recovery ratio is usually <0.03 [Wyatt, Vogelsang, Hubl, Waldhoer, & Lochs, 1993] and the lactulose recovery is usually $<0.5\%$ of the dose [D'Eufemia et al., 1996; Marsilio, D'Antiga, Zancani, Dussini, & Zacchello, 1998]. In severe active inflammatory bowel disease (IBD) the lactulose recovery may exceed 5% and the L/M recovery ratio may exceed 0.25 [Marsilio, D'Antiga, Zancani, Dussini, & Zacchello, 1998]. Although the true sensitivity of the test has not been formally assessed, even in mild IBD [Halme, Turunen, Tuominen, Forsstrom, & Turpeinen, 2000] and coeliac disease [Marsilio, D'Antiga, Zancani, Dussini, & Zacchello, 1998], the mean L/M recovery ratio is increased.

Participants were advised to have nothing to eat or drink after midnight. Immediately following early morning urine voiding, participants were given a mixture of mannitol (2g, 11.0 mmol) and lactulose (5g, 14.6 mmol) orally. They were advised to drink plenty of water.

In an attempt to ensure there was no food in the intestinal lumen that might interfere with the test, but acknowledging that it was unrealistic to expect families to travel the distance from home to hospital with water only, participants were advised to have no more than a slice of toast an hour later. All urine was then collected for approximately 6 hours, in a pre-weighed urine collection bottle containing 0.2g thiomersal, with a final voiding as close to 6 hours as possible. The urine collection bottle was re-weighed, to determine the volume of urine, before storage in 2 x 4 ml aliquots at -80°C until analysis. Lactulose and mannitol were measured in both the pre and 6 hours urine samples by MSMS by laboratory investigators who were blind to the diagnostic status of the participants.

Data Analysis

All analyses were undertaken in Stata 9 (Stata Corporation, 2005). Group differences were examined between children with ASD and those with SEN; within the ASD group, between those with and without regression, using the test command of stata. Sample weights were used in all analyses to allow all statistics such as proportions, means, and group differences to be presented as target population estimates, taking into account not only the differences in sampling proportions and the differential response to the SCQ screening questionnaire associated with a prior clinical ASD diagnosis, health district and child's sex, but also the differential take up of the gut permeability test. Standard errors of simple means, linear regressions, adjusted Wald test statistics and p-values were calculated using the linearization version of the robust parameter covariance matrix as implemented by the svy procedures of stata.

Results

Of the 255 participants, 158 met consensus ASD diagnosis. The other 97 had special educational needs (SEN) with a variety of other diagnoses and did not meet criteria for a diagnosis of ASD. All children were approached for consent to participate in the gut permeability test but some were unable to do so. The main reasons for failure to participate concerned individual consent and test procedure: reliable bladder emptying on demand, difficulty in getting some children to drink the mannitol/lactulose mixture and difficulty collecting 6 hours of urine, often while in transit. Gut permeability was measured in 103 with ASD and 30 with SEN (14 had a learning disability, 3 had a language disorder, 3 had a hearing impairment, 7 had hyperkinetic or conduct disorders, 2 had a chromosome disorder, and 1 had no clinical diagnosis). The participants' IQ ranged from 51 to 131 (SEN) and 28 to 136 (ASD). A comparison of those who participated in the gut permeability study with those who did not, showed that participants were younger ($t(249) = 7.34, p < .001$), had higher IQ ($t(251) = -3.63, p < .001$) and greater ASD severity ($t(253) = -4.23, p < .001$) than non-participants. Regression was reported in 20 of the ASD group; 83 had no history of regression. Group characteristics are summarised in Table 1.

Weighted mean scores for urine creatinine recovery, mannitol recovery, lactulose/mannitol (L/M) recovery ratio and lactulose recovery are presented in Table 2 and Figures 1-4. The recovery of urine creatinine was not significantly different in the ASD and SEN groups ($t(101) = 0.00, p = .98$). Similarly, there was no significant difference ($t(101) = 0.32, p = .76$) between the ASD group with regression and the ASD group without regression.

The recovery of urine mannitol, percentage of oral dose, was not significantly different in the ASD and SEN patient groups ($t(131) = 0.52, p = .60$). Similarly, there was no significant

difference ($t(101)=1.05$, $p=0.30$) between the ASD group with regression and the ASD group without regression.

As expected, there was a highly significant relationship between the recovery of creatinine and mannitol ($\beta=2.53$ (SE=0.77); $t(131)=3.28$, $p <.005$) but no significant association between the recovery of creatinine and lactulose ($\beta=0.02$ (SE=0.01); $t(131)=1.15$, $p =.25$). Critically, there was no correlation between the recovery of creatinine and the L/M recovery ratio ($\beta=-0.001$ (SE=0.0009); $t(131)=1.56$, $p = .12$), indicating that the latter was independent of the completeness of urine collection.

Analysis of the gut permeability indices demonstrated no significant differences between the ASD and SEN patient groups for either the L/M recovery ratio ($t(131)=0.56$, $p=.56$) or the lactulose recovery (percentage of oral dose) ($t(131)=1.26$, $p=.21$).

There was no significant difference ($p=.36$) for the L/M recovery ratio between the ASD group with regression and the ASD group without regression. In addition, there was no significant difference ($p=.46$) for the lactulose recovery between the ASD group with regression and the ASD group without regression.

Within the ASD group, there was no relationship between L/M recovery ratio and autism severity as measured either by number of ICD-10 symptoms ($t(101)=1.70$, $p = .93$) or ADOS-G severity scores ($t(101)= 1.10$, $p = .28$). Similarly, no relationship was found between lactulose recovery and ICD-10 symptoms ($t(101)=1.35$, $p = .18$) or between lactulose recovery and ADOS-G severity scores ($t(101)=0.94$, $p = .35$).

The GI symptom questionnaire was completed by 126 parents of the 133 children who had permeability measured. Mean unweighted current and past GI symptom counts are presented in Table 3, as are the rates of current and past vomiting, abdominal pain and diarrhoea. None of the children had GI symptoms that met the enterocolitis definition. Abdominal pain was the most frequently reported symptom currently and in the past. It was only moderately correlated with constipation (constipation measured by reported decreased frequency of bowel action $r = .42$, $p < .0001$; and when measured by decreased frequency, difficulty in passing stool or hardness of stool $r = .50$, $p < .0001$)

Diet

Sufficiently detailed parental information on food selectivity for analysis was available on only 46 participants who also completed the gut permeability test (12 SEN, 34 ASD) and who had also completed the GI symptom questionnaire. Those who completed the diet questionnaire did not differ from those who did not in terms of IQ, ICD-10 score, current or past reported GI symptoms (all $p > .32$). A 'limited' diet was defined as less than 10 food items in the diet; two parents reported diets with fewer than 5 food items (one with ASD and the other SEN without ASD). A limited diet was reported in 3/12 SEN cases (25%) and 14/34 (41%) ASD cases (no significant difference, Pearson χ^2 , $p = 0.318$). A faddy diet was reported in 4/12 (33%) SEN and 10/34 (29%) ASD cases. A limited or faddy diet did not account for any of the group differences found in relation to GI symptoms.

Adequate blood samples were collected from 63 of the children who had gut permeability measured; one child with ASD had positive endomysial antibodies and was subsequently diagnosed on retest and confirmatory mucosal biopsy as having coeliac disease.

No significant correlations were found between L/M recovery ratios and current or past GI symptom counts ($r = -0.07, p = .45$; $r = 0.07, p = .44$) or between lactulose recovery and current or past GI symptom counts ($r = -0.04, p = .68$; $r = 0.08, p = .38$). No significant associations were found between individual GI symptoms and either raised lactulose recovery ($>0.5\%$) or raised L/M recovery ratios (>0.03 , Fisher's Exact p all $>.1$). No significant associations were found between restricted or faddy diets and either raised L/M ratios or raised lactulose recovery.

Eleven participants (9 ASD, 2 SEN) had an L/M recovery ratio >0.03 , but only two of these, 1 ASD, 1 SEN, had a recovery ratio >0.04 . The child with ASD had no GI symptoms but was found to have undiagnosed coeliac disease. The child with SEN had current diarrhoea and past persistent vomiting, a history of gastroschisis and had had several surgical procedures including excision of part of the small bowel. Of the other 9, two had no gastrointestinal symptoms, six reported past diarrhoea (only one reported diarrhoea for more than 14 days) and one reported current diarrhoea of less than 14 days. Ten children (7 ASD, 2 SEN) had lactulose recovery between 0.5 and 1%, only 4 $>0.6\%$.

Five of 133 children (4 ASD, 1 SEN) had both a raised lactulose recovery ($>0.6\%$) and a raised L/M recovery ratio >0.03 , of whom two had a lactulose recovery $>0.6\%$ and L/M ratio >0.04 (1 ASD and 1 SEN), as reported above.

Discussion

In the present study we have compared children with ASD with an age and IQ-matched group of children with SEN but without ASD. We found no evidence of increased gut (small bowel) permeability in children with ASD compared with children with SEN. This remained the

case even when the ASD group was sub-categorised into those patients with and without evidence of regression. Using the “gold standard” measure of gut permeability, the mean L/M recovery ratios and 95% confidence intervals were equivalent in the ASD and SEN groups and well within the normal range. Gastrointestinal symptoms, abdominal pain, constipation, vomiting and diarrhoea were reported in a significant number of participants (also reported in Chandler et al) but there was no association found between the presence of gut symptoms and gut permeability measures.

Robertson and co-investigators [Robertson, Sigalet, Holst, Meddings, Wood, & Sharkey, 2008] reported that L/M recovery ratios were not significantly different in 14 patients with ASD: 0.032 (0.015), 7 siblings: 0.039 (0.016), and 8 control children: 0.031 (0.015). The mean values are all greater than 0.03, but this may reflect the use of 12h overnight urine collections. In contrast, Horvath and colleagues [Horvath, Zielke, Collins, Rabszryn, Medeiros, & Perman, 2000] described increased L/M recovery ratios in 19/25 children with autism and gastrointestinal symptoms, but there was no appropriate control group. D’Eufemia et al. [1996] reported no overall difference in mean lactulose recovery when comparing 21 patients with autism (but no evidence of gastrointestinal disorders) with 40 controls (mean lactulose recovery 0.38%). However, 9 of the 21 patients had increased lactulose recoveries (>0.5%; mean 1.64%), compared with none of the 40 controls, suggesting significantly increased gut permeability in a sub-group of children with ASD. De Magistris et al. [2010] reported that 33 out of 90 ASD patients had an L/M recovery ratio >0.03, mean L/M recovery 0.041, SD 0.08. SD at twice the mean indicates a skewed distribution and, therefore, might suggest at least a few patients with pathological gastrointestinal permeability problems. Interestingly, there was no correlation between L/M recovery ratio and faecal calprotectin, a marker of intestinal inflammation. Although the L/M ratio, mean (SD), was only 0.028 (0.05) in 138 first degree relatives, 31 also had a ratio

>0.03. In 8 siblings the mean (SD) was 0.051 (0.06), again the high SD suggesting that, in the single sibling with a ratio >0.03, there was severe gastrointestinal leakiness. De Magistris et al. [2010] also reported that mean lactulose recovery was 0.57% in 90 patients with ASD with a standard deviation (SD) of 0.8, indicating that a significant number had markedly increased gut permeability. Surprisingly, the recoveries were even higher and more variable in first degree relatives (n=138, mean (SD) = 0.60 (0.7)) and siblings (n=8, mean (SD) = 1.62 (1.6)), leading the authors to suggest the possibility of an inherited gut permeability abnormality in families with children with autism. In our study, in 11 of 133 participants tested (9 ASD, 2 SEN) L/M recovery ratios were marginally increased compared with the generally accepted upper limit and 10 had increased lactulose recovery. Five had both a raised L/M ratio and raised lactulose recovery. Significantly, two participants (1 ASD, 1 SEN) had a recovery ratio >0.04 (a more obviously pathological figure) and raised lactulose recovery and both had gastrointestinal pathology. The child with ASD had no GIS but had undiagnosed coeliac disease confirmed with biopsy; the child with SEN had past GIS and had had gastroschisis requiring bowel excision operations.

There are obvious disparities in the results and conclusions of the current study and those presented by Robertson and colleagues [Robertson, Sigalet, Holst, Meddings, Wood, & Sharkey, 2008], when compared to the previous studies reporting increased gut permeability in children with ASD [De Magistris et al., 2010; D'Eufemia et al., 1996; Horvath, Zielke, Collins, Rabsztyl, Medeiros, & Perman, 2000]. The protocols for the studies are very similar; the 12h overnight collection [Robertson, Sigalet, Holst, Meddings, Wood, & Sharkey, 2008] has already been highlighted, but all the studies used the maximum 2g of lactulose recommended [Bjarnason, MacPherson, & Hollander, 1995].

Analytical problems, particularly with respect to the measurement of lactulose in the presence of lactose, cannot be discounted as it is not stated in any of the studies that lactose was restricted before the studies were performed. We optimised the chromatography to separate lactose and lactulose and then tested our chromatographic system at the start and end of each analytical run to ensure the separation was maintained; the normal and spiked internal quality control samples both contained significant lactose. Obviously any method failing to separate lactose and lactulose will lead to artefactual increases in measured lactulose and this cannot be ruled out as an explanation of difference in results.

The possibility that severity of the disorder or regression might explain the differing results between studies is addressed by the lack of any evidence of increased gut permeability with severity of autism as measured by ICD-10 scores and autism versus other ASD diagnoses or in the sub-analysis of ASD with and without evidence of regression.

The possibility that there was transient permeability of the gut at a younger age cannot be ruled out.

Limitations of this study are the small number of children with SEN, the limited number with dietary information, the fact that a number of children and families did not manage to participate in the permeability test and the absence of a group of typically developing children. The latter would have enabled an additional control group for the measurement technique and further delineation of the normal range of the L/M ratio, particularly the upper limit of normal. The two definitely abnormal permeability results obtained were linked to clear gastrointestinal pathology (neither known to the laboratory). A further limitation is that the GIS questionnaire is not validated, GI symptoms were elicited by parent report and not by a gastroenterologist thus we cannot distinguish eg abdominal pain due to constipation or

some other cause. However Gorrindo et al's study shows high concordance between parent and gastroenterologist for the presence of GIS if not specificity of a particular diagnosis.

Strengths of the study are that this is a large sample for this kind of study with a wide range of IQ, that it is derived from a population cohort, and that the measurement technique was accurate particularly in ensuring lactose and lactulose separation. Obtaining 6 hour samples from children with autism is not easy and complete emptying of the bladder cannot be guaranteed. As reported, however, there was no correlation between the recovery of creatinine and the L/M recovery ratio, indicating that the latter was independent of the completeness of urine collection. We conclude that the data in the current study provide no evidence for differentially increased small bowel gut permeability in children with autism at the age of 10-12 years and, despite the reported gastrointestinal problems, provide no support for a persistent 'leaky' gut in children.

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Table 1: Group characteristics in terms of weighted mean scores, linearized standard errors (LSE) and 95% confidence intervals (CI) for age, IQ, number of ICD-10 symptoms and numbers of males and females

	<i>SEN controls</i> (N=30)	<i>ASD</i> (N=103)	<i>ASD, no regression</i> (N=83)	<i>ASD and regression</i> (N=20)
Age in years (LSE)	12.59 (0.31)	11.44 (0.11)	11.45 (0.13)	11.39 (0.15)
CI	11.98-13.20	11.22-11.65	11.20-11.70	11.09-11.69
IQ (LSE)	77.75 (3.38)	74.65 (2.68)	75.63 (3.19)	69.94 (2.73)
CI	71.06-84.44	69.35-79.95	69.29-81.96	64.52-75.36
ICD-10 total (LSE)	1.68 (0.31)	7.17 (0.34)	6.94 (0.34)	8.29 (1.23)
CI	1.07-2.23	6.50-7.84	6.28-7.61	5.85-10.74
Males, females	26, 4	89, 14	71, 12	18, 2

Table 2: Weighted mean scores (LSE) and 95% confidence intervals (CI) of urine creatinine recovery (mmol) (UCR), urine mannitol recovery (percentage of oral dose) (UMR), and the gut permeability indices urine lactulose/mannitol (L/M) recovery ratio and lactulose recovery (percentage of oral dose administered) (LR) across diagnostic groups

	<i>SEN controls</i> (N=30)	<i>ASD</i> (N=103)	<i>Adj Wald</i> <i>Test</i> <i>p</i>		<i>ASD, no</i> <i>regression</i> (N=83)	<i>ASD and</i> <i>regression</i> (N=20)	<i>Adj Wald</i> <i>Test</i> <i>p</i>
UCR	2.07 (0.15)	2.07 (0.11)	0.98		2.08 (0.12)	2.00 (0.25)	0.76
CI	1.78-2.37	1.85-2.28			1.84-2.32	1.50-2.49	
UMR	18.23 (1.43)	19.20 (1.20)	0.60		19.71 (1.31)	16.82 (2.40)	0.29
CI	15.40-21.06	16.83-21.58			17.10-22.31	12.06-21.58	
L/M	0.01 (0.003)	0.02 (0.001)	0.56		0.01 (0.001)	0.02 (0.01)	0.36
CI	0.01-0.02	0.01-0.02			0.01-0.02	0.01-0.03	
LR	0.22 (0.03)	0.27 (0.03)	0.21		0.27 (0.02)	0.29 (0.05)	0.67
CI	0.16-0.29	0.24-0.30			0.23-0.30	0.18-0.40	

Table 3: Mean (standard deviation) GI composite symptom counts, and proportions of endorsed individual GI symptoms for SEN controls and ASD cases

	<i>SEN controls</i> (<i>N=29^a</i>)	<i>ASD</i> (<i>N=97^a</i>)
Composite current GI symptoms		
Mean (SD)	0.18 (0.50)*	0.54 (0.89)*
Individual current symptoms	% (N)	% (N)
Persistent vomiting	3.5 (1)	2.1 (2)
Abdominal pain	3.5 (1)	16.7 (16)
Diarrhoea	5.9 (1)	2.9 (2)
Constipation (decrease in freq)^b	3.7 % (1)	9.9 % (9)
Constipation (any of 3 symptoms)^c	3.7 % (1)	11.3 % (11)
Composite past GI symptoms		
Mean (SD)	0.23 (0.54)*	0.61 (0.90)*
Individual past symptoms	% (N)	% (N)
Persistent vomiting	7.1 (2)	13.4 (13)
Abdominal pain	6.9 (2)	19.0 (18)
Diarrhoea	5.0 (1)	18.9 (14)

^a Total Ns for cell proportions vary slightly, depending on amount of missing data, largely due to ‘do not know’ responses on individual items of questionnaire.

^b Constipation defined as a decrease in frequency of passing a bowel motion (to less than 3 times a week)

^c Constipation defined as either a decrease in frequency of bowel movement, or a harder consistency of bowel movement, or difficulty in passing a bowel movement

* $p < .05$

Table 3: Mean (standard deviation) GI composite symptom counts, and proportions of endorsed individual GI symptoms for SEN controls and ASD cases

	<i>SEN controls</i> ($N=29^a$)	<i>ASD</i> ($N=97^a$)
Composite current GI symptoms		
Mean (SD)	0.18 (0.50)*	0.54 (0.89)*
Individual current symptoms	% (N)	% (N)
Persistent vomiting	3.5 (1)	2.1 (2)
Abdominal pain	3.5 (1)	16.7 (16)
Diarrhoea	5.9 (1)	2.9 (2)
Composite past GI symptoms		
Mean (SD)	0.23 (0.54)*	0.61 (0.90)*
Individual past symptoms	% (N)	% (N)
Persistent vomiting	7.1 (2)	13.4 (13)
Abdominal pain	6.9 (2)	19.0 (18)
Diarrhoea	5.0 (1)	18.9 (14)

^a Total Ns for cell proportions vary slightly, depending on amount of missing data, largely due to ‘do not know’ responses on individual items of questionnaire.

* $p < 0.05$

Figure Legends

Figure 1: Creatinine recovery (mmol) by diagnostic group as raw (unweighted) data; boxplots indicate median, lower and upper quartiles, and outliers

Figure 2: Mannitol recovery (% of dose) by diagnostic group as raw (unweighted) data; boxplots indicate median, lower and upper quartiles, and outliers

Figure 3: Lactulose/mannitol recovery ratios by diagnostic group as raw (unweighted) data, with cut-off indicating upper level of normal range; boxplots indicate median, lower and upper quartiles, and outliers

Figure 4: Lactulose recovery (% of dose) by diagnostic group as raw (unweighted) data, with cut-off indicating upper level of normal range; boxplots indicate median, lower and upper quartiles, and outliers

Appendix

Details of laboratory methodology:

Mixed lactulose (100, 500, and 1000 $\mu\text{mol/l}$) and mannitol (1, 5 and 10 mmol/l) standards were prepared in deionised water and stored, in aliquots, at -80°C . Spiked urine controls, lactulose (50 and 700 $\mu\text{mol/l}$) and mannitol (0.5 and 7.0 mmol/l) were prepared and stored, in aliquots, at -80°C . For analysis, standards, controls, and samples were thawed, vortex mixed, centrifuged at 1500 rpm and 4°C for 4 min, 10 μl diluted to 500 μl with deionised water, transferred to a 96 deep well polypropylene plate, sealed, and placed in a CTC autosampler maintained at 10°C . Chromatography was performed isocratically on an Agilent ZorbaxNH₂ carbohydrate analysis column (25 cm x 2.1 mm, packed by Hichrom, UK) with acetonitrile: water as mobile phase (200 $\mu\text{l}/\text{min}$). The ratio varied between 75 and 80% acetonitrile to optimise the separation of lactulose and lactose: inject to inject time was approximately 20 min. MSMS was performed in negative ion mode (-4200 V) on a SCIEX API4000 triple quadrupole mass spectrometer (Applied Biosystems, UK). Three experiments were performed (150 ms/scan) in multiple reaction monitoring (MRM) mode for disaccharides (m/z 340.9/160.9), hexitols (m/z 181.0/88.9), and hexoses (m/z 178.9/89.0) (data not included). Each MRM was optimised for maximum signal:noise ratio using aqueous lactulose, mannitol, and glucose standards, respectively. Results were calculated using Analyst 1.3.1 or 1.4.1. The lactulose response was linear over the range 0 to 1000 $\mu\text{mol/l}$ but the mannitol response over the range 0 to 10 mmol/l was best represented by a quadratic function. Measured mannitol concentrations $>10\text{ mmol/l}$ were re-diluted 1:200 with deionised water and re-analysed. Within-assay reproducibility ($n=5$), assessed using the spiked control samples, was 8.4% (53.5 $\mu\text{mol/l}$) and 9.3% (678 $\mu\text{mol/l}$) for lactulose and 6.1% (0.59 mmol/l) and 9.3% (7.28 mmol/l) for mannitol. Between-assay reproducibility ($n=21$) was 8.7% (53.5 $\mu\text{mol/l}$) and 13.8% (689 $\mu\text{mol/l}$) for lactulose and 5.5% (0.58 mmol/l) and 9.8%

(7.09 mmol/l). The possibility of ion suppression in patient samples was tested by analysing several samples at dilutions ranging from 1:5 to 1:200, but no suppression was detected. Urine creatinine was measured using a modified stable isotope dilution MSMS method³², to assess completeness of the urine collections and to correct for any interference in the pre-dose samples. Correction reduced the average L/M recovery ratio from 0.0156 to 0.0154 and the average lactulose recovery from 0.289 to 0.278%.