Alterations of the Intestinal Barrier in Patients With Autism Spectrum Disorders and in Their First-degree Relatives

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ABSTRACT

Objectives: Intestinal permeability (IPT) was investigated in patients with autism as well as in their first-degree relatives to investigate leaky gut hypothesis. Faecal calprotectin (FC) was also measured in patients with autism, either with or without gastrointestinal symptoms, and in their first-degree relatives.

Patients and Methods: IPT results, assessed by means of the lactulose/mannitol test, were compared with adult and child controls and with FC values.

Results: A high percentage of abnormal IPT values were found among patients with autism (36.7%) and their relatives (21.2%) compared with normal subjects (4.8%). Patients with autism on a reported gluten-casein–free diet had significantly lower IPT values compared with those who were on an unrestricted diet and controls. Gastrointestinal symptoms were present in 46.7% of children with autism: constipation (45.5%), diarrhoea (34.1%), and others (alternating diarrhoea/constipation, abdominal pain, etc: 15.9%). FC was elevated in 24.4% of patients with autism and in 11.6% of their relatives; it was not, however, correlated with abnormal IPT values.

Conclusions: The results obtained support the leaky gut hypothesis and indicate that measuring IPT could help to identify a subgroup of patients with autism who could benefit from a gluten-free diet. The IPT alterations found in first-degree relatives suggest the presence of an intestinal (tight-junction linked) hereditary factor in the families of subjects with autism.

Key Words: autism, calprotectin, first-degree relatives, gastrointestinal symptoms, intestinal permeability

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Pertinent articles and information related to the subject of autism and gastrointestinal abnormalities.
The presence of a genetic predisposing factor for the leaky gut condition could be inferred through the study of IPT in first-degree relatives of subjects with ASD, just as it has already been done in inflammatory bowel disease (IBD) and other GI conditions (18–20).

GI inflammation can be investigated by measuring faecal calprotectin (FC), a protein produced by intestinal granulocytes (22). Besides being far less invasive than endoscopy, it has proved to be a useful tool in the study of GI inflammation. Its evaluation, together with that of IPT, should help to identify definite subgroups of patients with ASD.

The aim of the present research is to verify whether the GI barrier is actually impaired and whether gut inflammation is present in a large cohort of patients with ASD and in their first-degree relatives, using noninvasive tools.

PATIENTS AND METHODS

To carry out the present research we investigated 90 children with ASD and 146 of their first-degree relatives; data were compared with 64 children and 146 adult normal subjects as control groups. All of the subjects were given an IPT test (lactulose [LA]/mannitol [MA]), and all of the patients and their first-degree relatives underwent an FC determination. Blood tests for coeliac disease markers—serum anti-tissue transglutaminase antibodies (anti-tTG), anti-gliadin antibodies (AGA IgG and AGA IgA), anti-endomysium antibodies (EMA), and human leucocyte antigen (HLA) DQ2-DQ8 (DQ2/8)—were performed in all of the patients with ASD as well as in all of the first-degree relatives and controls who showed high values of IPT and/or FC. Informed consent was obtained from the parents of each patient and control subject before starting any procedure.

Subjects

Ninety subjects with ASD were recruited from either the outpatient or inpatient services of the Child and Adolescent Psychiatry Unit at the Second University of Naples, Italy. The sample included 81 boys and 9 girls (mean age ± SD 7.4 ± 5.1 years). There were 4 couples of twins. All of the children were administered the Autism Diagnostic Interview-Revised version (23), the Childhood Autism Rating Scale (24), and the Autism Diagnostic Observation Schedule-Generic (25) to document the diagnosis of autism. In addition, 2 expert clinicians (A.P. and R.M.) observed all of the children to confirm that they met the Diagnostic and Statistical Manual of Mental Disorders-IV criteria for AD.

One hundred forty-six first-degree relatives were included (mean age ± SD 40.2 ± 8.7; female = 72, male = 74; siblings = 8, female = 4, male = 4, mean age ± SD 12.4 ± 5.0). A total of 85 families took part in the study, including 2 children whose (foster) parents did not take part in the study. An expert clinical review was conducted of their social functioning and participation history to exclude the presence of symptoms related to pervasive developmental disorders.

One hundred forty-six healthy subjects (age range 19–66 years, mean age ± SD 31.8 ± 12.3; female = 98, male = 48) were recruited from among the staff members of the department and their families, together with 64 healthy children (mean age ± SD 7.1 ± 3.1; female = 30, male = 34). None of these subjects claimed any recent GI symptoms, nor were they affected by any major GI diseases.

A carefully detailed GI anamnesis was collected for each subject, with special regard to type of special diet if any (eg, gluten-free or gluten-casein–free diet [GCFD]), reported food intolerances, and recently determined aspartase aminotransferase and alanine aminotransferase values to assess the general state of health of the liver.

Inclusion criteria for control subjects and first-degree relatives were as follows. All of the subjects were requested not to smoke, drink alcohol, or take any kind of nonsteroidal anti-inflammatory drugs (or other anti-inflammatory drugs) for at least 3 days before the test. The exclusion criteria were pregnancy and/or the concomitant condition of known coeliac disease and/or other major diseases of the intestinal tract, such as IBD and hepatic disorders, as well as known and serologically proven food intolerances. In all of the subjects with ASD, serum anti-tTG, AGA IgG and AGA IgA, EMA, and HLA DQ2-DQ8 were determined. The above parameters were also investigated in all of the first-degree relatives and controls who showed high values of IPT and/or FC.

Informed consent was obtained from each selected subject and from the parents of children. The study was approved by the ethics committee of our department and was carried out in accordance with the Helsinki Declaration of 1975.

Intestinal Permeability

IPT is regarded as a valuable and noninvasive test for monitoring mucosal damage of the small intestine. The procedure is based on the simultaneous oral administration of 2 sugar probes of different molecular sizes and absorption routes and the estimation of urinary recovery of each molecule. In the present study, an LA/MA test was administered, as previously described (26,27). Briefly, an oral isomolar load of the 2 probes (MA 2 g plus LA 5 g) is orally administered to fasting subjects and urine samples are collected for the following 5 hours. The MA and LA detection in the urine samples was performed by high-performance anion exchange chromatography with pulsed amperometric detection (26). IPT is expressed as the ratio (LA/MA) of the recovered percentage of LA versus MA. The cutoff value for the normal range, as previously described (26,27), was set at LA/MA <0.030.

Faecal Calprotectin

FC was determined from a stool sample from each subject to investigate intestinal inflammation. FC was detected by means of enzyme-linked immunosorbent assay (ELISA) (Calprest, Eurospital, Italy). Briefly, this method is based on the use of a polyclonal antibody against calprotectin in an ELISA system, with the addition of a final coloured product. Normal values were estimated to be <100 μg/g stool (adult and children) on the basis of previous reports (28,29) and our own laboratory experience.

Anti-gliadin Antibodies

The determination of specific IgA and IgG antibodies against α-gliadin in serum was achieved by means of an ELISA (Eurospital, Italy). Normal values were set as <8 U/mL and <50 U/mL, respectively.

Anti-tissue Transglutaminase Antibodies

The determination of specific IgA antibodies against tissue transglutaminase in serum was achieved by means of an ELISA (Eurospital, Italy). Normal values were <8 U/mL.

Anti-endomysium Antibodies

The detection of class IgA EMA was achieved by indirect immunofluorescence on sections of human umbilical cord (Eurospital, Italy).
Human Leucocyte Antigen

The Eurospital Eu-DQ kit was used for the determination of HLA II, DQ2, and DQ8 haplotypes in human whole blood, respectively coded by alleles DQA1*05-DQ81*02 and DQB1*0302. It requires the preliminary DNA extraction and purification from whole blood, subsequent DNA amplification in PCR, and detection of amplificates on agarose gel.

Statistical Analyses

Statistical significance was assessed at a level of 0.05. Variables were summarised either as mean ± SD and/or as frequency and percentage. The Student *t*-test, the Mann-Whitney test, and analysis of variance (ANOVA) with Bonferroni correction were used to evaluate the differences among means. The dependence between pairs of parameters was evaluated as a simple linear correlation (*r*) with the Spearman test. Data handling and analysis were performed through Graph Pad Prism 5 (GraphPad Software Inc, La Jolla, CA).

RESULTS

Among patients with ASD, 33 of 90 (36.7%) showed abnormal IPT compared with 31 of 146 (21.2%) first-degree relatives, 7 of 146 (4.8%) adult controls, and none of the child controls. The percentage of abnormal values of patients with ASD and that of their relatives were significantly different from those of the respective control groups (Fisher exact test, *P* < 0.0001) (Table 1).

IPT mean value results were significantly different among the 4 investigated groups: ASD, 0.041 ± 0.08; relatives, 0.028 ± 0.050; adult controls, 0.013 ± 0.01; and child controls, 0.023 ± 0.01 as shown in Table 1 and Figure 1. The 1-way ANOVA with Bartlett correction gave the results *P* < 0.0001. The difference between mean values from relatives and adult controls was significant, with *P* = 0.019 (Mann-Whitney test).

IPT mean values of the children on GCFD showed significantly different results from those of the control children (*P* = 0.039, Student *t*-test) and the children on an unrestricted diet (*P* = 0.014, Student *t*-test).

To examine any possible correlation between abnormal IPT values and the presence of GI symptoms, as well as other specific characteristics of the patients (eg, sex, age, HLA DQ2/DQ8) children with ASD were divided into 2 groups, abnormal or normal IPT. As shown in Table 2, there was no correlation between the presence of GI symptoms and abnormality of IPT values. GI symptoms were referred by the parents and were present in 42 of 90 children (46.7%), namely constipation in 20 of 44 (45.5%), diarrhoea in 15 of 44 (34.1%), and others (eg, alternating diarrhoea/constipation, abdominal pain) in 7 of 44 (15.9%). Sex and age distribution were comparable between the 2 subgroups and HLA DQ2/DQ8 were present, as is normally found in the general population.

An ANOVA conducted to compare individual IPT values with various scores from the Autism Diagnostic Interview-Revised, Autism Diagnostic Observation Schedule, and Childhood Autism

![FIGURE 1. IPT is measured in the 4 investigated subgroups and expressed as LA/MA values (mean ± SD): patients with autism spectrum disorders (N = 90), relatives (N = 146), adult controls (N = 146), child controls (N = 64). One-way ANOVA with Bartlett correction was *P* < 0.0001. The difference between mean values from relatives to adult controls was significant, with *P* = 0.019 (Mann-Whitney test).](https://www.jpgn.org/)

**TABLE 1. Demographic characteristics and permeability data of the investigated subjects**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>LA % recovery</th>
<th>MA % recovery</th>
<th>LA/MA</th>
<th>Abnormal LA/MA (&gt;0.030)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult controls (N = 146)</td>
<td>48/98</td>
<td>31.8 ± 12.3</td>
<td>0.20 ± 0.2</td>
<td>14.9 ± 13.5</td>
<td>0.013 ± 0.01</td>
</tr>
<tr>
<td>Child controls (N = 64)</td>
<td>30/34</td>
<td>7.1 ± 3.1</td>
<td>0.22 ± 0.1</td>
<td>11.1 ± 8.2</td>
<td>0.023 ± 0.01</td>
</tr>
<tr>
<td>ASD patients (N = 90)</td>
<td>81/9</td>
<td>7.4 ± 5.1</td>
<td>0.57 ± 0.8</td>
<td>19.4 ± 14.0</td>
<td>0.041 ± 0.08</td>
</tr>
<tr>
<td>Relatives (N = 138)</td>
<td>70/68</td>
<td>40.2 ± 8.7</td>
<td>0.60 ± 0.7</td>
<td>26.4 ± 17.5</td>
<td>0.028 ± 0.05</td>
</tr>
<tr>
<td>Siblings (N = 8)</td>
<td>4/4</td>
<td>12.4 ± 5.0</td>
<td>1.62 ± 1.6</td>
<td>29.3 ± 3.4</td>
<td>0.051 ± 0.06</td>
</tr>
</tbody>
</table>

LA and MA % recovery are reported (mean ± SD), as well as LA/MA values (mean ± SD). The final column reports the number and % of subjects with abnormal LA/MA values. LA = lactulose; MA = mannitol.

* *P* < 0.0001 vs children and adult controls, respectively (Fisher exact test).

\( ^{1} P < 0.0001 \) 1-way ANOVA with Bartlett correction.

\( ^{2} P = 0.019 \) vs adult controls (Mann-Whitney test).
FIGURE 2. Small intestine barrier function is more deregulated in the children with autism spectrum disorders with regular eating habits than in those who are on a gluten-casein–free diet (GCFD) \( (P = 0.034, \text{Mann-Whitney test}) \). Intestinal permeability, expressed as LA/MA values (mean ± SD), in the 2 groups and child controls is reported. The differences between the 2 groups (GCFD and free diet) vs controls are both significant \( (P = 0.039 \text{ and } P = 0.014, \text{respectively; Student } t \text{ test}) \).

Rating Scale questionnaires showed no significant correlations \( (F = 1.708; F = 0.595; F = 1.464) \).

The values of FC were abnormal in 22 of 90 (24.6%; range 102.5–387.4 μg/g) of patients with ASD and 17 of 146 (11.7%; range 108.7–375.7 μg/g) of relatives; that is, these subjects showed FC values higher than normal (ie, FC >100 μg/g). In Table 3 the means of the pathological values in the 2 groups are given to show how their entity could account for a condition of mild inflammation \( (30) \). The upper limit of the normality range (ie, FC >100 μg/g) is commonly used in the diagnostic procedure and is in agreement with our personal diagnostic laboratory experience. Because of budget restrictions, FC was investigated only in those controls (adult or child) who showed abnormal IPT values; none of them had FC values above the normal range.

There was no correlation between the IPT and FC values; the linear correlation analysis gave the results \( r = 0.09 \) (ASD) and \( r = 0.23 \) (relatives) (Spearman test).

Serological parameters to exclude coeliac disease are reported in Table 4. The investigated patients with ASD were negative for tTG, EMA, and AGA IgA, whereas 2 of them showed high AGA IgG values. These 2 high values (both >100 U/mL) were associated with high FC values (200 and 248 μg/g) and normal IPT \( (0.012 \text{ and } 0.008) \). Familial and personal anamneses were both free from any indication of food intolerances.

**DISCUSSION**

In the present study, evidence is provided that supports the view that a genetically determined abnormal IPT is present in ASD \( (4,14) \), hence defining a subgroup among patients with ASD, which could tentatively be named "barrier function deficit." We do not know yet how and whether this aspect is related to the development of ASD; a considerable amount of work is still required.

As a matter of fact, IPT was abnormal in a large percentage of subjects with ASD; subjects with ASD were reported to benefit from a gluten-free diet \( (1) \); gluten itself augments IPT \( (31–35) \). We can hypothesise that subjects with ASD are gluten sensitive, as well as other recently described conditions \( (36–38) \), and hence their intestinal barrier function will ameliorate with a gluten-free diet. The well-recognised intestinal mucosal effects of gliadin—the major component of gluten—would justify a treatment with gluten-free diet in ASD.

A recovered barrier function would eventually prevent digestion products of natural food from entering the blood through the leaky mucosa and inducing antigenic responses as well as reducing the interference with the central nervous system. Some recent studies have found a close relation between dietary change and the onset of symptoms in patients with ASD \( (6,39–42) \). It was

**TABLE 2. IPT values of patients with ASD \( (N = 90) \)**

<table>
<thead>
<tr>
<th>Sex: M/F ratio</th>
<th>Abnormal IPT (33 patients)</th>
<th>Normal IPT (57 patients)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic HLA predisposition: DQ2 and/or DQ8 yes/no (%)</td>
<td>29/4</td>
<td>52/5</td>
</tr>
<tr>
<td>Mean age ± SD at recruitment</td>
<td>8/25 (32.0)</td>
<td>15/38 (39.5)</td>
</tr>
<tr>
<td>Presence of referred GI symptoms (%)</td>
<td>6.3 ± 3.7</td>
<td>8.9 ± 6.0</td>
</tr>
<tr>
<td>Presence of referred GI symptoms (%)</td>
<td>15/33 (45.5)</td>
<td>27/57 (47.4)</td>
</tr>
</tbody>
</table>

Some specific characteristics are compared to show that the 2 subgroups were almost overlapping. ASD = autism spectrum disorders; GI = gastrointestinal; HLA = human leucocyte antigen; IPT = intestinal permeability; SD = standard deviation.
proposed that some forms of ASD may arise from toxic effects of intestinal products on the developing brain. On the contrary, it is well known that the gut–brain axis is central to certain encephalopathies of extracranial origin, of which hepatic encephalopathy is a prime example (15,43). Some GI pathology, such as coeliac disease, characterised by abnormal permeability, may also include disturbed behavioural symptoms (9). Shattuck and Whiteley (15) formulated the hypothesis that ASD may be caused by an inappropriate central activity of dietary-derived opioid peptides (exorphins) from the gut. Such peptides include gliadomorphin and casomorphin from the substrates wheat gliadin and bovine casein, respectively. Under normal circumstances, these abundant dietary opioids are digested by brush border peptidases, such as dipeptidyl peptidase IV. An increased IPT may facilitate the absorption of dietary peptides.

Another possibility is that 1 or more enzymatic defects affecting the digestion of such proteins are present; they could produce “aberrant” peptides, which would cross a weak barrier—a weakened intestinal barrier still is a necessary condition—thus reaching peripheral areas of the body causing damage (4). On the contrary, the intestinal lesions that increase IPT may also arise in pathological conditions such as viral infections and immune deficiency states. We found that the abnormal IPT was not, however, related to any of the scored developmental characteristics; that is, the barrier function deficit subgroup of patients with ASD does not match any of the main behavioural subgroups.

The detection by D’Eufemia et al (14) of aberrant IPT in asymptomatic children with ASD indicates that reliance upon symptoms substantially underestimates the percentage of individuals with ASD with possible GI pathology. Similarly, we found that half of the patients complained about GI symptoms, but no correlation between them and abnormal IPT values was found (Table 2). Again, a possible subgroup stratification evolves in which “GI symptomatic” does not always overlap to “barrier function deficit.” In many cases, however, the perception remains that intestinal symptoms are expected in children with developmental disorders, reflecting the effect rather than a primary GI dysfunction. A recent article (21) does not confirm any IPT increase in a small sample of patients with ASD and their siblings. The results of the present study confirm the IPT increase in a much larger sample of patients with ASD in comparison with the above studies.

Many studies were conducted in the first-degree relatives of patients with IBD in the attempt to prove the existence of a genetic defect (18–20) in the GI barrier. The obtained results presented here, showing increased IPT values in >20% of healthy relatives of children with ASD, suggest the existence of a genetic GI factor, possibly at the tight junction level, which seems involved in the pathology of ASD. The finding that abnormal IPT clustered in 5.6% of the families further reinforces this.

Abnormal IPT was found in asymptomatic subjects, including relatives and adult controls, suggesting that a weakened intestinal barrier function could be present and underestimated in a part of the general population. The identification of increased IPT in a subject is not a diagnostic endpoint, but does indicate the need for additional detailed investigation.

Coeliac disease was excluded in all of the investigated subjects, based on the clinical and serological markers, thus confirming the previous findings (44). Although ASD is a totally different condition with respect to coeliac disease, a recent case report showed a strong correlation between the 2 clinical conditions, and resolution of behavioural and GI symptoms using a gluten-free diet was reported (45). The diagnostic question about the 2 children with ASD with elevated AGA IgA remains open: they are not coeliac (negative tTG and EMA, absence of DQ2/DQ8 alleles) or wheat allergic; the answer will most probably be provided by the endoscopist.

In the present study, the increased IPT is mainly due to the 2- to 3-fold increase in passive LA absorption. The MA, being a monosaccharide and relatively small, is absorbed via a transcellular route (reflecting absorptive capacity), and LA, a disaccharide, is absorbed via an inter- or paracellular route (reflecting barrier function). An increase in LA urinary recovery compared with that of MA indicates a fall in the intestinal barrier function (16,46). This is a common finding in coeliac disease, which is accompanied by a flattening of the intestinal mucosa. In other conditions, such as type 1 diabetes mellitus (27) the increased IPT was associated with a partial disruption of narrow junctions within microscopically apparently normal mucosa.

IPT was reported to be often high in the presence of intestinal inflammation, as in Crohn disease and coeliac disease (18–20,47). GI inflammation can be inferred by measuring the FC (22), which is a useful, specific, and noninvasive tool. In the present study, bowel inflammation—based on FC determination only—was present in 24.6% of patients with ASD. FC evaluation was made once, on a single sample of stools obtained the day before IPT test administration. Even though the possibility of day-to-day FC variations exists (48), our findings are in keeping with previous reports (49) and do not confirm those by Tibble et al (50). The loss of intestinal barrier function is not associated with gut inflammation, as testified by the lack of correlation between LA/MA and calprotectin. This lack of correlation, in our opinion, is a significant finding indicating that each parameter is a sign of different pathways of intestinal damage.

FC is a protein typically produced by granulocytes, and its quantity in stools is directly correlated to the degree of inflammation. In IBD, increased permeability is the consequence of inflammation; in ASD, IPT is most likely a primary defect independent of inflammation related to functional changes that can be secondary to specific genetic predisposition. IPT alteration in ASD is not directly correlated with inflammation: A massive intervention of immune system cells is secondary to a prolonged and/or disruptive breakdown of the intestinal barrier. The mean pathological value of FC found in these patients (159.7 ± 74.0 μg/g; Table 3), although the results should be confirmed by repeating the measurement (48), indicates a mild degree of inflammation of the bowel (29,30), which is in keeping with previous findings.

**TABLE 4. Serum parameters (mean ± SD) to evaluate presence of concomitant coeliac disease and/or hepatic involvement**

<table>
<thead>
<tr>
<th></th>
<th>AGA IgA, U/mL</th>
<th>AGA IgG, U/mL</th>
<th>tTG, U/mL</th>
<th>EMA</th>
<th>AST, U/L</th>
<th>ALT, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with ASD (N = 90)</td>
<td>1.6 ± 2.5</td>
<td>13.8 ± 24.2*</td>
<td>1.04 ± 0.91</td>
<td>100% neg</td>
<td>22.3 ± 5.0</td>
<td>26.1 ± 6.3</td>
</tr>
<tr>
<td>Relatives (N = 33)</td>
<td>0.8 ± 0.4</td>
<td>8.4 ± 5.9</td>
<td>3.0 ± 3.7</td>
<td>100% neg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AGA = anti-gliadin antibodies; ALT = alanine aminotransferase; AST = aspartate aminotransferase; EMA = anti-endomysium antibodies; tTG = anti-transglutaminase antibodies.

* Mean ± SD value from 88 subjects; the only 2 extremely high values (>>100) were excluded from the presently reported mean data.
To summarise, our results show that IPT is abnormal in a subgroup of children affected by ASD. This result may be extremely important in that IPT determination could be used as a biomarker for a subgroup of children who can benefit from treatments targeting specifically the leaky gut. The presence of a genetic factor influencing the intestinal barrier is suggested through the finding that a large number of first-degree relatives showed IPT impairment as well. The influence of environment (ie, “toxic” gluten) (35,51) on the intestinal barrier is also suggested. Different IPT values were obtained in children with ASD based on their diet. The latter data require controlled studies to be confirmed.

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