



# Absence of urinary opioid peptides in children with autism

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## ABSTRACT

**Objective:** It has been claimed for a number of years that the urine of children with autism contains exogenously derived opioid peptides. This finding is said to reflect a disturbance in the integrity of the gut epithelium, act as a diagnostic marker for autism and predict treatment response to a diet excluding gluten and casein. The aim of the present study was to determine whether exogenous or endogenous peptides were present in the urine of children with autism or of control children.

**Design:** Case-control study

**Setting:** Cases were recruited from two tertiary referral centres specialising in autistic spectrum disorders, while controls were recruited from mainstream primary and secondary schools in the same geographical area.

**Participants:** 65 boys with autism, mean age 7.4 years (range 5–11) and 158 control boys, mean age 7.8 years (range 4.2–11).

**Investigations:** Urine samples were examined by high pressure liquid chromatography (HPLC) and matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) for the presence of a number of putative opioid peptides.

**Outcomes:** There were no significant differences between the HPLC urinary profiles of the children affected by autism and the typically developing controls. In those cases where HPLC showed peaks in the locations at which opioid peptides might be expected to be found, MALDI-TOF established that these peaks did not, in fact, represent opioid peptides.

**Conclusions:** Given the lack of evidence for any opioid peptiduria in children with autism, opioid peptides can neither serve as a biomedical marker for autism nor be employed to predict or monitor response to a casein- and gluten-free diet.

Autism is a severe lifelong developmental disorder, defined on the basis of behavioural characteristics which result from impairments in social communication and reciprocal social interaction, repetitive and restrictive behaviours, and imaginary thought.<sup>1</sup> It is one of the most common serious developmental disabilities of childhood.<sup>2</sup> An underlying aetiology for their autism is identified for a minority of children, but this proportion rises in the learning disabled population.<sup>3</sup> For the majority of children for whom no underlying aetiology is identified, there are no evidence-based biomedical investigations to guide intervention. This has been identified as a key area for research.<sup>4</sup>

This can be a frustrating state of affairs for parents who are contending with a diagnosis of autism for their child. Most psycho-educational interventions are considered helpful, although they too lack an evidence base.<sup>5</sup> In addition, many parents continue

## What is already known on this topic

- Claims that autistic children suffer from a condition known as leaky gut, resulting in exogenously derived opioid peptides being present in their urine, have resulted in large numbers of parents seeking urinalysis in commercial laboratories.
- Proponents of the leaky gut theory advocate the use of opioid peptiduria as a biomedical marker of the disorder and a pointer towards dietary intervention.

## What this study adds

- This study finds no evidence of opioid peptiduria in male children with autism or Asperger syndrome, nor evidence for any other differences in urinary profiles between children with autism and Asperger syndrome and a matched control group.
- There is therefore no evidence that opioid peptiduria performs as a biomedical marker of autism, nor that it can be usefully employed to predict or monitor response to a casein and gluten exclusion diet. These findings are counter to the putative mechanism of the leaky gut syndrome.

to pursue experimental biomedical treatments. These include adopting a casein- and gluten-free exclusion diet for their affected children, although a recent Cochrane Review established that this intervention lacked an evidence base.<sup>6</sup>

The "leaky gut" hypothesis proposes that there may be gluten sensitivity and a specific enterocolitis in children with autism, resulting in an exogenously derived opioid peptiduria. These opioid peptides are assumed to cross the blood-brain barrier and result in autistic symptomatology.<sup>7</sup> A number of researchers have proposed that casein and gluten exclusion diets exert their effect by reducing circulating exogenously derived opioid peptides.<sup>8–11</sup> These investigators have also presented evidence that prior to commencing this exclusion diet, some children with autism have opioid peptiduria and that this can guide whether they are likely to respond to this type of dietary intervention.<sup>12</sup>

There are many problems with this hypothesis, including major weaknesses in terms of biological and clinical plausibility.<sup>13</sup> Children with autism do

not have an increased rate of coeliac disease.<sup>14</sup> Neither are they more likely to present to their general practitioner with chronic inflammation of the gastro-intestinal tract, food intolerance or recurrent gastro-intestinal symptoms.<sup>15</sup> Furthermore, the early techniques for establishing the presence of opioid peptiduria employed either immunological approaches<sup>16</sup> using antibodies raised against gluten or casein derivatives which are subject to difficulties of cross-reactivity and non-specific binding, or high pressure liquid chromatography (HPLC). Thus they do not provide information concerning sequence length or the identity of the peptide(s). Other researchers employing HPLC have been unable to replicate the findings of urinary peptides.<sup>17</sup> A study with a small number of subjects was also unable to identify exogenously derived peptides employing mass spectrometry.<sup>18</sup>

The aim of the present study was to determine whether exogenous or endogenous peptides were present in the urine of children with autism in physiologically significant quantities. We aimed to determine if differences existed in the amount and nature of such peptides when comparing urine samples from children with autism and age- and sex-matched control children.

## METHODS

### Subjects

Cases consisted of 68 male children with autism aged 4–11 years recruited from two tertiary diagnostic centres specialising in the assessment of children with neurodevelopmental disorders. Informed consent and assent was obtained from parents and from children where possible. In addition to relevant local ethics committee approval, the National Autistic Society research ethics committee gave approval for the study.

Diagnosis in all cases was based on ICD-10 classification for autism. A total of 61 subjects received a diagnosis of autism, and seven a diagnosis of Asperger syndrome. Unclear cases or those that met partial criteria, for example atypical autism, were excluded. Diagnosis was achieved through a multi-disciplinary, multi-agency assessment as described in the National Autism Plan for Children,<sup>19</sup> and cases were only selected for this study when there was complete agreement between all team members on the basis of interview, history and observations. Diagnoses were further validated by formal Autism Diagnostic Interview-Revised (ADI-R) interviews<sup>20</sup> in 41 cases (60%) with 100% concordance between clinical and ADI-R diagnosis. IQ was assessed in all cases: 16 of the autistic group (and by definition, all seven of the Asperger group) had IQs >70.

Children with progressive neurological disorders and unstable epilepsy were excluded, as were those taking regular medications including stimulants and anticonvulsants. Three cases were on gluten-free diets and three cases were on gluten- and casein-free diets.

A total of 202 male controls aged between 4 and 12 years were recruited from mainstream infant and primary schools, based on the same ethics committee approval as given for the cases with autism. After permission was obtained from head teachers, a standard information leaflet was circulated to parents inviting them to participate in the study. Informed consent and assent was obtained from parents and, where possible, from control children. The Strengths and Difficulties Questionnaire (SDQ) was completed by the parents of the controls in order to screen out children with possible neuropsychiatric disorders.<sup>21</sup> Fifteen of the control group scored in the borderline range on the SDQ with a further 18 scoring in the abnormal range. Seven parents elected not to complete the SDQ. All these individuals were excluded from further analysis leaving 162 controls.

### Urine collection and sampling

For all cases and controls, first morning urine was collected on dry ice and immediately transferred to a  $-70^{\circ}\text{C}$  freezer. Samples were bar coded to allow analysis to be carried out blind to diagnostic status. Urine samples for creatinine (see technical appendix 1 in supplementary data) and peptide measurement were successfully captured and analysed for 158 of the controls and 65 of the cases (58 children with autism and seven children with Asperger syndrome).

### HPLC analysis

All HPLC analysis was carried out on an Amersham Biosciences AKTA Basic system. All chromatography was conducted using a reversed phase C18 column (ACE 5 C18 250×4.6 mm; cat. no. ACE-121-2546) with a Phenomenex pre-column guard (AJO-6073) (see technical appendix 2 in supplementary data). UV absorbance measurements (215 nm) were automatically taken every 0.8 s over the first 118 min of the run, generating an ASCII file listing approximately 8800 data points.

Where HPLC analysis of a urine sample indicated the presence of high and well-defined peaks exhibiting the correct retention time for one or more of the suspect opioid peptides cited in previous studies,<sup>8–12</sup> the sample was classified as a candidate for further analysis. Samples from all groups showed such peaks, but only those from the autism and Asperger syndrome group were reanalysed, as being the most likely to present opioids given the assumptions of the leaky-gut hypothesis. Twenty five such samples were re-fractionated by HPLC, and individual fractions were taken for further analysis by matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) to determine if any peaks exhibiting the expected  $m/z$  ratio were observed.

### Power calculations

Power calculations were carried out using NQuery Advisor software. The calculations show that the creatinine adjusted spectra generate statistics which, given the number of children in the study, would have high statistical power to detect differences between the control and autistic spectrum disorder groups (see column 7 in table 1). Comparisons between the control group and the autism group had similar properties. The low number of replicates in the Asperger syndrome group means that all comparisons to that group alone would have low power (not shown).

### Prior validation of biochemical techniques

Before embarking on the analysis described in this study, we carried out extensive validation of every aspect of our laboratory methodology and assay technique. This included HPLC and MALDI-TOF analysis of synthetic opioid peptides (see technical appendix 3 in supplementary data), calculation of the lower limit of detection of peptides in urine for both techniques (see technical appendix 4 in supplementary data), measurement of peptide stability in urine and then calculation of the coefficient of variation for system reproducibility to guide subsequent statistical analysis (see technical appendix 5 in supplementary data).

## RESULTS

### Statistical analysis of total peak area

The total peak area (ie, the area under the curve) for each urine sample was calculated and the data were examined to determine if a statistically significant difference in means existed between the groups (table 1). Data were compared either in raw uncorrected form or normalised to the equivalent of 200 nM creatinine per sample to account for differences in urinary creatinine content between samples. Comparisons were made

**Table 1** Comparison of mean total peak area from the complete (0–78 min) and truncated spectra (40–78 min) between the control and autistic groups, using uncorrected and creatinine corrected data

	Mean values (SE)						
	1	2	3	4	5	6	7
	Control (n = 158)	Total cases (n = 65)	Autism (n = 58)	Asperger syndrome (n = 7)	p Value, control/cases	p Value, all groups	Size of difference detectable
Total peak area							
A. Complete spectrum*	1209 (18)	1229 (28)	1219 (30)	1311 (86)	0.55	0.51	± 7.9%
B. Truncated spectrum*	506.8 (8.0)	518.1 (12.4)	516.9 (13.2)	528.6 (37.9)	0.44	0.71	± 8.2%
C. Creatinine adjusted complete spectrum†	2.685 (0.003)	2.690 (0.005)	2.691 (0.005)	2.683 (0.014)	0.34	0.56	± 0.6%
D. Creatinine adjusted truncated spectrum†	2.558 (0.003)	2.567 (0.005)	2.569 (0.006)	2.545 (0.016)	0.18	0.16	± 0.7%
E. Creatinine content*	28.58 (0.62)	27.7 (0.96)	27.09 (1.02)	32.55 (2.93)	0.44	0.16	± 11.3%

\*Using square root transformed data; †using repeated log transformed data.

A comparison of the creatinine concentration in the urine is also shown. The final column presents the minimum difference between the control and autistic spectrum disorder groups detectable at 8% statistical power given the observed variability, as a percentage of the observed mean of the control group. SE, standard error.

between the control (n = 158) and complete autism and Asperger syndrome dataset (n = 65) (ie, two groups in total), and also between the control and the two diagnostic subgroups (ie, three groups in total). In each case, analysis was carried out using one-way ANOVA, and p values were controlled to allow for multiple comparisons using both Fisher's and Dunnett's methods, where the individual and family error rates respectively were 5%, and the normal group was selected as the baseline comparison for the Dunnett procedure.

Although the cases as a group exhibited a larger average total peak area than the control group for both the full and truncated spectra (the latter being the region where suspect opioid peptides would be expected to elute if present), the relatively large variation between children within each group ensured that these effects were not statistically significant (table 1, rows A and B, columns 1, 2 and 5). When the peak area data were corrected for differences in creatinine concentration, the effect was to increase the apparent difference in average total peak areas between the two groups (rows C and D, columns 1 and 2). However, no statistical significance was apparent when comparing the means of the two groups (column 5).

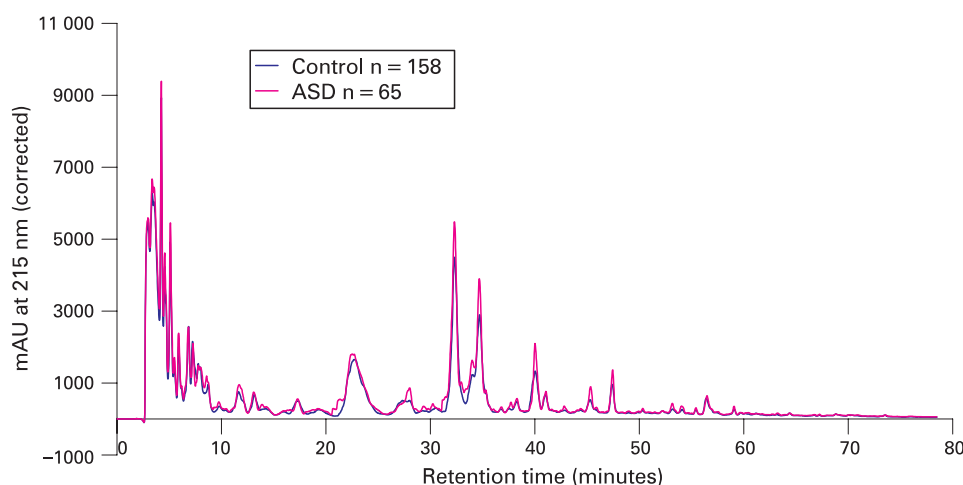
When repeating these analyses for the autism subgroups, there was no statistically significant evidence for differences in the urinary mean peak areas associated with different subgroups (columns 3, 4 and 6), either with or without creatinine correction. In all cases, the differences in mean total peak areas are small compared with the differences seen between different children in the same groups.

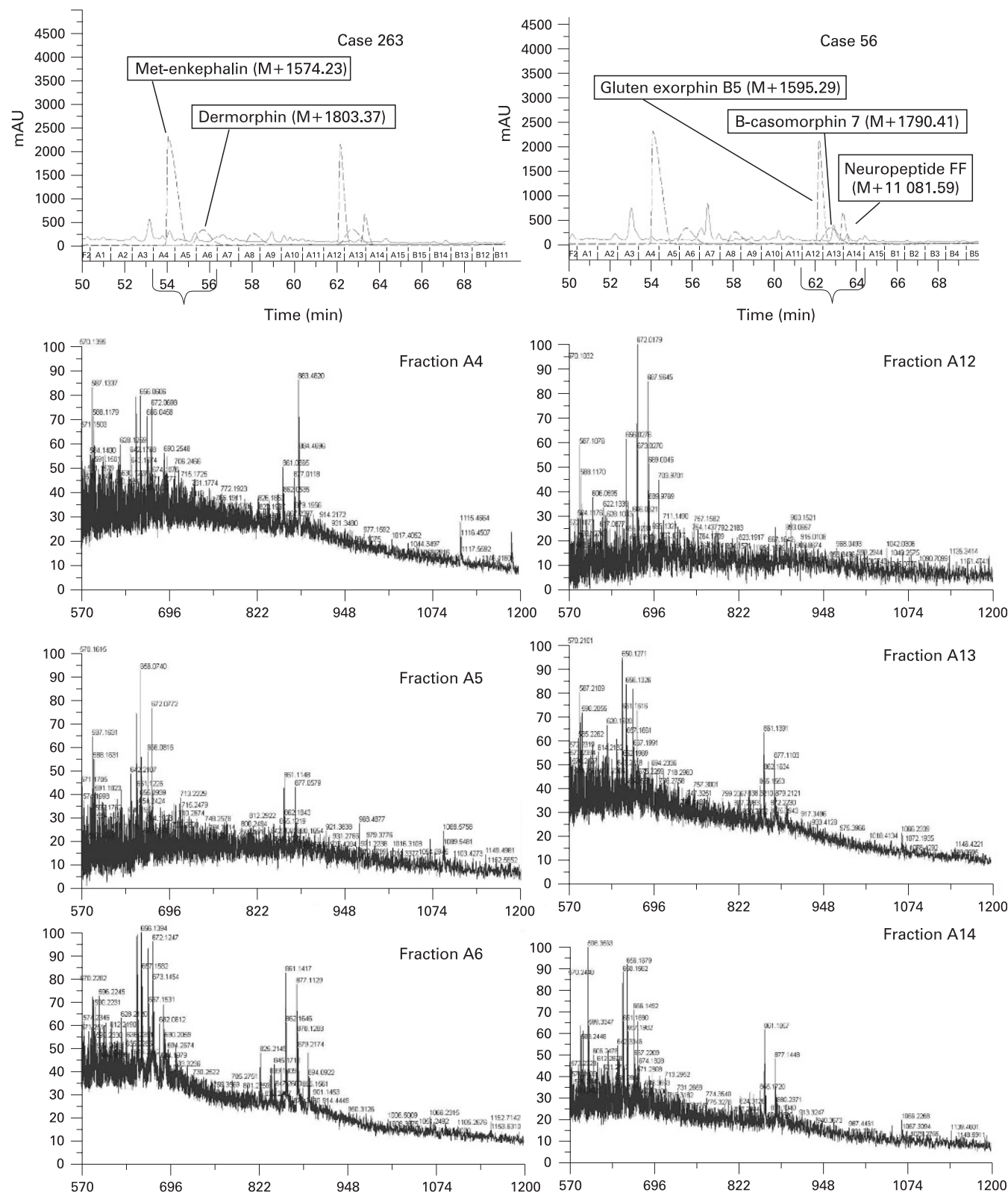
### Visual comparison of average urinary traces of case and control urines: whole spectra

The average HPLC spectra for case and control urine samples were generated by calculating the average absorbance reading at every 0.8 s data point throughout the run for the entire dataset (fig 1). The overall amount of material under the curve was slightly larger for cases compared to the control group for most peaks throughout both traces and not just in the region 40–70 min. The averaged autism/Asperger trace and control group trace were very similar in overall profile, with no peaks being case or control specific and any differences in peak height being small (10–15%). Average HPLC spectra for the autism and IQ subgroups (see technical appendix 6 in supplementary data) show no obvious peaks in any groups.

### MALDI-TOF MS for opioid peptides

By HPLC analysis alone, 25 urine samples from the autism/Asperger group were selected as showing peaks in approximately the correct locations to be opioid peptides. For all 25, MALDI-TOF MS analysis of the relevant fraction found no instances of ions of m/z corresponding to opioid peptides. These results indicate that the peaks observed on the HPLC trace were not opioid peptides. Two examples of this are shown in figure 2. HPLC data suggested that met-enkephalin and dermorphin may have been present in sample 263; however, this was not substantiated by subsequent MALDI-TOF MS of the relevant fractions. Similarly with autism sample 59, the HPLC data

**Figure 1** Creatinine-corrected (equivalent of 200 nM loaded) average urinary trace from case and control samples over the full 78-min run.



**Figure 2** HPLC traces of cases 263 (top left) and 56 (top right) showing peak intensity (absorbance at 215 nm) plotted against retention time. HPLC trace is shown plotted with continuous lines, overlaid with peaks demonstrating the location of pure opioid peptides shown with dotted lines. The fractions marked below the x axis were taken for further analysis by MALDI-TOF MS, and the traces generated by each individual fraction are shown plotted underneath. The opioid peptides predicted to be present by virtue of the correct retention time are shown marked on the HPLC chart, together with the  $m/z$  ratio which should be present on the MALDI trace if the peptide was indeed present in the fraction.

suggested the presence of  $\beta$ -casomorphin 7 and neuropeptide FF. Again MALDI-TOF MS showed they were not opioid peptides. These negative observations were reported for all samples examined by a combination of HPLC and MALDI-TOF MS.

## DISCUSSION

This study finds no evidence of opioid peptiduria in male children with autism or Asperger syndrome, nor is there any evidence for any differences in urinary profiles between children with autism and Asperger syndrome and a matched control group. There is therefore no evidence that opioid peptiduria performs as a biomedical marker of autism, nor that it can be usefully employed to predict or monitor response to a casein and gluten exclusion diet. These findings are counter to the putative mechanism of the leaky gut syndrome.

There are a number of strengths in this present study which support these conclusions. Firstly, we report the findings from urine analysis of children with well-defined autism and Asperger syndrome across a broad range of cognitive abilities.

Secondly, while the initial stage of our study employed the same HPLC technique as described by the main research groups reporting opioid peptiduria, we had the advantage of well-defined synthetic opioid peptides with which we were able to conduct extensive bench-side laboratory tests. These confirm that the conditions under which we conducted our experiments were rigorous and appropriate and that methodological errors cannot explain our failure to replicate their apparent demonstration of opioid peptiduria.

Thirdly, the present study employed mass spectrometry as an additional means of analysing urinary derived material. This technique provides an exact molecular weight for any molecules and is more sensitive to peptides than HPLC. HPLC only gives information regarding retention time, but thousands of highly dissimilar molecules may share a common retention time. HPLC allows no conclusions to be drawn regarding mass or structure and therefore cannot be used to separate a complex mixture of analytes with any degree of accuracy at these levels. The importance of being able to compare HPLC and MALDI-TOF analyses was demonstrated, for example, in those cases where HPLC showed peaks in the locations at which opioid peptides might be expected to be found. In all such cases, MALDI-TOF established that these peaks did not, in fact, represent opioid peptides. It is worth revisiting assumptions described in earlier work by Ek *et al* that illustrate the importance of this point: "The known peptides were recognised by their points of elution and by chromatography with peak augmentation, using commercially bought synthetic peptides. It is, of course, conceivable that a chromatographically similar peptide may have appeared and run with the known peaks... However, it is reasonable to think that these peaks are caseomorphin 1-8-like peptides".<sup>22</sup> We have shown that such interpretations are not, in fact, reasonable and have led to false assumptions about the presence of exogenously derived opioid peptides.

One possible weakness of this study is the fact that cases were recruited from tertiary centres and hence may have been more clinically severe or may have differed systematically from children presenting to community services. We would argue that our decision in this particular study to deliberately only include full-blown cases of autism or Asperger syndrome serves to strengthen the findings.

Another possible weakness is our lack of a control group matched for the very low IQ children with autism. This would have been a significant weakness if peptides had been identified in either group and shown an association with IQ. However,

peptides were not found in either case. Although it may be argued that peptides should be lower or absent in the three children on gluten-free diets and three children on gluten- and casein-free diets, this does not explain their absence in the other 62 cases.

The hypothesis of the leaky gut has long been controversial, with proponents citing links between autism and gut abnormalities, and advocating the use of opioid peptiduria as a biomedical marker of the disorder and a pointer towards intervention. However, there is now a large body of work which refutes the theory and cannot replicate the findings. We suggest that this present study demonstrates that children with autism should not be subjected to investigation of urinary opioid peptides or their parents to the expense of the assays which are still widely advertised on the internet by commercial laboratories around the world. There is still a need to scientifically establish for parents whether a casein- and gluten-free diet is an effective intervention for autism, but we have shown that there is no evidence that exogenously derived opioid peptides are the mechanism by which such diets may exert their effect.

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**Competing interests:** JM has previously acted as an expert witness for the litigants in the MMR litigation case conducted by Alexander Harris against three MMR vaccine manufacturers, which involved urinary analysis of both litigants and non-litigant controls using MALDI-TOF mass spectrometry. He has an autistic child who was not part of this legal case.

**Ethics approval:** Obtained from the local ethics committee and the National Autistic Society research ethics committee.

**Patient consent:** Obtained.

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## Images in paediatrics

### Recurrent papular urticaria in a 6-year-old girl

#### CASE REPORT

A 6-year-old female was referred for multiple pruritic lesions on her legs. No other members of the family had similar skin manifestations. The family reported having fleas at home previously, but the house had since been fumigated. Examination revealed numerous 4–8 mm excoriated, erythematous papules in clusters on the legs (fig 1).

The family was reassured that these lesions were probably secondary to flea bites. However, the patient continued to have intermittent recrudescences. The family became frustrated and sceptical because no one else at home showed signs of insect bites.

At the sixth-month follow-up, the patient's mother brought in some debris from the patient's bed. The material was examined microscopically and identified to be a cat flea (*Ctenocephalides felis*) larva (fig 2). The family cat was treated for fleas and at follow up the patient was found to be clear.

Children under 2 years typically do not have sufficient exposure to insect antigens to mount a robust hypersensitivity response.<sup>2</sup> Conversely, children over 10 years typically develop tolerance to the antigen and produce transient wheals instead of long-lasting papules.<sup>2–4</sup> This age-based susceptibility explains the common but counterintuitive scenario illustrated in this case wherein only a single child is affected despite the whole family being exposed to the insect.

Management of papular urticaria – prevention and eradication – is usually implemented without confirmatory evidence of



**Figure 2** Photomicrograph of the cat flea larva (original magnification 10×).

the culprit.<sup>1–3</sup> Unfortunately, preventative measures such as home fumigation and treatment of pets are costly and may seem excessive when families are sceptical of the diagnosis.<sup>3–4</sup>

Identifying the culprit helps motivate families to invest in prevention and can uncover failures in eradications. By verifying the persistence of the cat flea in the patient's home despite fumigation, we pinpointed the cat as the source of re-infestation and potentially averted a frustrating cycle of recurring eruptions for the patient.

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**Figure 1** Clusters of excoriated erythematous papules and healing macules on the patient's lower extremities.