Dietary issues in children with Autistic Spectrum Disorder (ASD):

Comparing patterns of urine and blood opioid peptides in groups of children with and without ASD.

"The face was drawn but the curtains were real"

A major project submitted in partial fulfilment for the award of the degree B.Nutrition & Dietetics University of Wollongong

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Table of Contents:

Acknowledgments:

For assistance, guidance, friendship and tolerance, I would like to thank:

Abstract:

Background: Some current beliefs with regard to dietary issues in children with ASD submit that these children are more prone to gastrointestinal challenges as compared to children without ASD. Implementation of gluten-and casein-free diets have been consistent in clinical improvements seen in children suffering from ASD and gastrointestinal problems. This study was conducted to see whether children with ASD are more prone to gastrointestinal problems as compared to a population of children without ASD and to compare the patterns in two specific opioid peptides present in their urines and bloods, which are purported to affect the behaviour in children with ASD.

Method: 24 hr urine specimens were collected from subpopulations of children with and without ASD. Creatinine was measured by standard clinical method based on the Jaffe reaction, prior to urine being analysed by reverse phase column HPLC. The reference peptides used were β-casomorphine bovine and gluten exorphine A5. 4mls of blood were collected from populations of children with and without ASD. Coeliac diagnostic screening was ordered to assess the presence of IgG and IgA and antigliadin autoantibodies in order to assess the prevalence of any gastrointestinal pathology between groups. A 4 day diet record was kept over the time the urine and blood specimens were collected, in order to validate consumption of food versus the excretion of wastes, of particular interest being the opioid peptides previously mentioned.

Results: The creatinine results suggested that there was non-compliance by the subjects. The trace patterns for the HPLC showed hyperpeptiduria, particularly in the region where gluten is eluted, for subjects with ASD and some subjects in the control group, with little significance where casein is eluted. The blood results showed normal results in a population for prevalence of HLA DQ2 genes, which indicate a genetic propensity toward coeliac disease.

Discussion: It is not possible to ascertain whether an opioid peptide effect exists in this population, as in general, everyone excretes peptides and the excretory pattern is highly individual. A relationship between wheat and milk in diet and excretion of peptides could not be found. A comparison of HPLC patterns shows similarity between those subjects with ASD and those subjects with positive HLA DQ2 screening tests, which may indicate that gluten may contribute to gastrointestinal sensitivities. It is essential that food intolerance issues are explored further, as most gluten containing foods contain salicylates, and some contain amines and glutamate as well as the salicylates, which are all known to cause reactions in food chemical sensitive individuals. The clinical improvement previously reported with dietary interventions such as gluten-and casein-free diets may be attributed to reducing the load of naturally occurring chemicals in the diet, hence behaviour and gastrointestinal problems decreasing.

Introduction:

Autism Spectrum Disorders (ASD) encompasses Asperger's Disorder/Asperger's Syndrome, Autistic Disorder, Atypical Autism and other pervasive developmental disabilities (including Rett's Disorder, Childhood Disintegrative Disorder and PDDNOS (Pervasive Developmental Disorder – not otherwise specified)) (NCBDDD 2003). The two most common forms of ASD are Asperger's Disorder and Autistic Disorder, being differentiated by the capacity of intellectual functioning and language abilities (Warren 2003). ASD occurs in approximately 1 in 100 persons, is not racially, culturally or socio-economically specific and is four times more common in males (Warren 2003).

ASD's often present unexpectedly following a normal developmental period from birth to between approximately 12-18 months of age (Erickson 1992). At this time developmental regression becomes apparent in the child's language ability and social interaction skills (Kessler 1988). After an initial period of dramatic regression, generally some improvement is seen however the child usually does not regain expected normal developmental progress (Kessler 1988). This may suggest that the onset of ASD's are either programmed events, such as genetic factors, or the effect may be due to an introduction of some new environmental factor that creates an adverse pathophysiological reaction. It may be a combination of both. Previous research has hypothesised that proteins specifically from gluten and casein have entered the blood due to what is known as the "leaky gut syndrome" and crossed the blood-brain barrier which causes the neurological affects seen in ASD's (Knivsberg et al. 2000).

ASD as a metabolic disorder.

Some theories (Unit 2002) subscribe to autistic spectrum disorder being purely a metabolic disorder, whereby ingested proteins, such as gluten and/or casein (but not limited to) are not metabolised correctly. It has been purported by others (Knivsberg et al. 2000; Wakefield et al. 2002) that people with ASD have greater permeability of their intestinal mucosa and as a result larger proteins particles leak into the blood from the gut. These proteins tend to be collected in the kidneys and excreted in the urine (Unit 2002), hence the ability to explore this hypothesis via HPLC methods described previously (Reichelt 1998; Ek 1999). It has been theorised that the proteins that have permeated through the gut wall into the bloodstream, may mass at the end of synapses in the brain, causing nerve impulses to be interrupted and hence the behaviour and/or cognitive issues arising as symptoms of ASD (Nelson et al. 2001). Abnormal peptide content has been described in the urine of most participants with autism by two research groups (Shattock 1990; Reichelt 1994) whom proposed that children with autism developed an enteropathy from gluten sensitivity which then allowed opioid peptides derived particularly from casein to enter the circulatory system and exert an effect on the opioid receptors in the brain (Hunter 2003). It has been argued that metabolic abnormalities in individuals with ASD could provide valuable information to the

underlying cause of ASDs, which may be genetic and the metabolic disorders being symptoms of the disease, rather than the cause (Johnston 2000).

Opiate effect of peptides

It has been suggested that the dopaminergic system within the central nervous system has a pivotal role in the study of autism (Shattock 1990). It is generally accepted that there are two types of receptors for the neuropeptide, dopamine, D_1, which activates the release of andenyl cyclase and D_2, which does not result in the release of adenyl cyclase (Shattock 1990). The distribution and structure of these dopamine receptors differ throughout the CNS and specific opioid peptides have a greater affinity for D_2 receptor sites, casein being thought to be one opioid peptide that can profoundly modify synaptic transmission. As the receptor sites are modified, the symptoms and developmental abnormalities seen in autism are thought to become prevalent (Shattock 1990).

It has been suggested that dietary gluten and casein have particularly morphine-like (opioid) effects (Shattock 1997) and may contribute to the causation of autism (Hunter 2003). Gluten (from wheat and certain other cereals) and casein (from milk and other dairy produce) may act either directly as neuroregulators (by modifying the structure of the D_2 receptors) or act as ligands to the enzymes which would catabolise these naturally occurring compounds (Shattock 1997). It follows that should the proteins in the body be limited via modulating the ingestion of gluten and casein (which have a particularly opioid effect (Hunter 2003)) then behavioural improvements may be seen.

Gastrointestinal issues

The occurrence of gastrointestinal problems in children with ASD has been discussed in a number of articles. Wakefield et al (2000) highlighted evidence that physiological symptoms of ASD, specifically gastrointestinal symptoms such as diahorrea, constipation, bloating, oesophageal reflux and pain were predominant in their subpopulation of children with ASD. Similarly studies involving a cohort of 62 children with ASD aged between 3-8 years, showed that a higher frequency of gastrointestinal symptoms existed (Levy 2001). A nested case-control study performed in the UK contrasted these results with exactly the same prevalence of gastrointestinal symptoms being observed in the cases and controls (Black 2002), however, these results are subject largely to general practitioner interpretation and bias as the criteria used for assessment was taken from an anonymised general practice research database (Black 2002). In a cohort of 349 children aged between 1 month and 12 years, children with ASD were assessed for constipation, diahorrea, gastro-oesophageal reflux (GOR), and delayed gastric emptying (Field 2003). The prevalence of such were all shown to be significantly associated with those children with ASD (Field 2003). However, the study omitted from differentiating between food allergy and food intolerance factors in their assessment for simplicity sake and no control group was assessed in comparison (Field 2003).

Gluten and casein-free diets

Since the early eighties gluten-and/or casein-free diets have been purported to offer relief of symptoms for gastrointestinal problems which may encompass food allergy, food intolerance, irritable bowel syndrome, coeliac disease and any or all of them combined (Knivsberg et al. 2000). Implementation of these diets have been consistent with clinical improvements seen in children suffering from ASD (Wakefield et al. 2002), however, as a clear mechanistic explanation has been absent the results have been difficult to replicate (Page 2000). Much of the literature states that primary caregivers often seek "alternative" or fringe intervention methods as conventional treatment therapies rarely recommend dietary intervention (Cornish 2002). This is due to research on dietary intervention effectiveness being scarce (Cornish 2002) and such diets are seen as a controversial treatment in the wider medical community. Current treatments usually include a range of behavioural modification strategies such as positive behaviour support (ABC), visual supports applied singularly or in combination with prescription drugs (Warren 2003). Interaction by the primary caregivers with associations and other support networks usually invokes discussion on dietary modification to combat some of the gastrointestinal symptoms, however, little solid proof or relevant information can be easily accessed by the caregivers about the treatments (Cornish 2002).

Materials and Methods

SUBJECTS

A population of 15 subjects, 6 children without ASD (5 males and 1 female) and 9 children (7 males and 2 females) with ASD, consented to this study involving the collection of urines and 16 subjects, 6 children without ASD (5 males and 1 female) and 10 children (8 males and 2 females) with ASD, consented to this study involving the collection of bloods, validated with a 4-day diet record. The mean age of the subjects were 5.75 (+/- 2). All children with ASD were diagnosed according to the DSMIV. The reference group was compromised of age and sex-matched healthy children of some of the RPA Allergy Clinic's staff members.

URINE

Collection of samples

A 24-hour urine collection was prescribed for the analysis. The first urine of the day was discarded after the child woke, and the subsequent urine was collected over the following 24-hours. The first urine the following morning was collected prior to the collection ceasing. A 3 litre vacutainer was supplied to each participant, which contained 1 teaspoon of ascorbic acid for preservation of the urine. Instructions that were given to participants are located in Appendix 1. Participants were requested to record the total volume output of urine over the 24-hour period, the date the urine was collected and to freeze the urine until delivery to the clinic was possible.

Sample preparation

After thawing, the urine was decantered into three specimen jars. One of the jars were reserved to assess creatinine levels, which was measured by the standard clinical chemical method, based on the Jaffe reaction as described by Popper et al, Seeligand and Wust and modified by Bartels. by the Department of Biochemistry, Royal Prince Alfred Hospital, Sydney. Two vials each containing 1ml of sample urine were filtered and stored separately for direct insertion into the HPLC. The remaining two specimen jars were labelled according to their unique identifier, total output volume and date of collection and stored for future considerations.

Standards

Standards for thymol, ascorbic acid, and the two opioid peptides β-casomorphin (H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH) and gluten exorphin A5 (H-Gly-Tyr-Tyr-Pro-Thr-OH) were prepared and run. The β-casomorphin and gluten exorphin A5 were run as concentrate and dilutions (1:10).

Buffers

Two buffers were prepared according to Reichelt et al 1999 method and applied utilising the same gradient. Buffer A consisted of 0.1% by volume of trifluroacetic acid (TFA) and Buffer B 0.1% TFA in 95% by volume acetonitrile with 4.9% water.

HPLC Analysis

A high-performance liquid chromatography system (Hewlett Packard 1050 series), with automatic sample application and 250mm x 4.6mm C18 reversed phase columns (Alltech, Apollo C18 series) were used. The columns were run at room temperature with an automatic gradient control and a flow rate of 0.5ml/min. Standard injection was 0.1ml and absorbance was set at 215nm and 280nm to identify peptide bonds and aromatic compounds respectively with a slope sensitivity of 0.2. Total run time was set at 100 minutes per sample, and each sample was run in duplicate. Details of the gradient used are contained in Table 1.

Table 1: Gradient used in HPLC for urine analysis

	BUFFER A	BUFFER B	MINS
1	99%	1%	0-15
2	Linear decrease to 60%	Linear increase to 40%	15-75
3	Linear decrease to 40%	Linear increase to 60%	75-80
4	40%	60%	80-89%
5	Linear increase to 99%	Linear decrease to 1%	89-95
6	Reequilibration		95-100

An automatic calculation of the area under the concentration curve was output by the enhanced integrator for both 215nm and 280nm in the peptide elution area, with ascorbic acid (preservative used) appearing at 4 mins.

Collection of samples

Subjects were screened for coeliac disease by assessing for antibodies and genetic markers of coeliac disease. 5ml of blood was collected via venapuncture by a trained blood-collecting nurse at the RPAH Medical Centre Pathology suite. Children were given a local anaesthetic patch (EMLA) applied superficially prior to the blood collection to minimise discomfort. Immunoglobulin A, Antigliadin IgA, Antigliadin IgG and Antigliadin EMA (IgA & IgG) were measured by standard clinical chemical methods. A further screening test for HLA DQ2 gene which indicate a propensity to coeliac disease was administered to the samples. All testing was performed by the Department of Clinical Immunology, Royal Prince Alfred Hospital, Sydney.

Results

CREATININE

Please see methodology described in Appendix 2.

Table 2: Mmol/day creatinine excretion utilised to standardise urine

SAMPLE ID	SAMPLE VOLUME (ML)	CREATINE (MMOL/L)	ADJUSTED VOLUME
1064	800	6.3	5.04
1065	400	9.5	3.8
1056	400	4.5	1.8
2004	500	7.8	3.9
2005	1600*	9.8	15.68
2009	300	2.5	0.75
2023	2400	3.3	7.92
3037	2350	2.2	5.17
3040	800	3.2	2.56
4074	900	6.8	6.12
4061	300	11.3	3.39
4064	1250	8.6	10.75
062	600	4.7	2.82
4063	625	7.1	4.44
4067	735*	10.5	7.72

*Volumes not recorded so mean volume within subpopulation taken for calculation.

URINES

Gluten was eluted at 44.362 minutes and β-casomorphine (bovine) was eluted at 63.384 minutes in the standards run with the urines. Figure 1 shows the urinary peptide trace pattern of a subject without ASD, Figure 2 shows the urinary trace pattern of a subject with ASD and Figure 3 shows the urinary trace pattern of a subject with a positive result for HLA DQ2 coeliac screening.

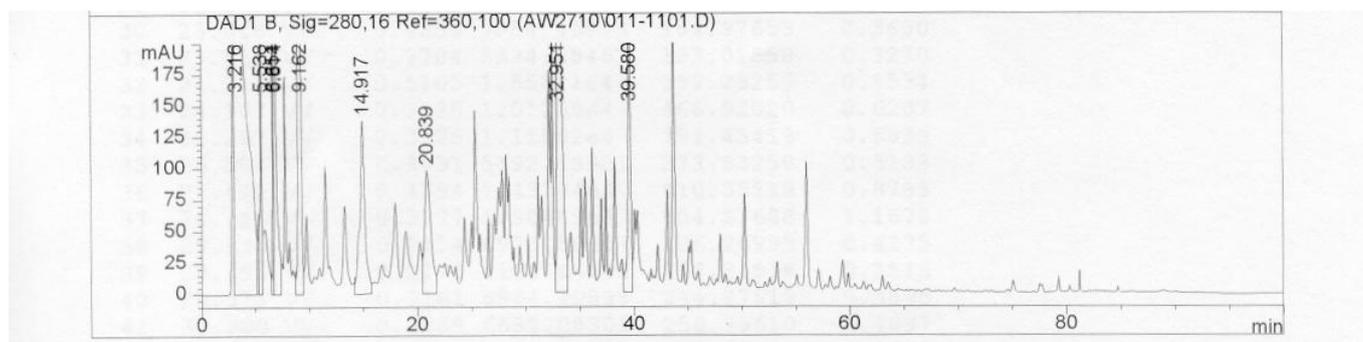*Figure 1: HPLC trace pattern for a subject without ASD*

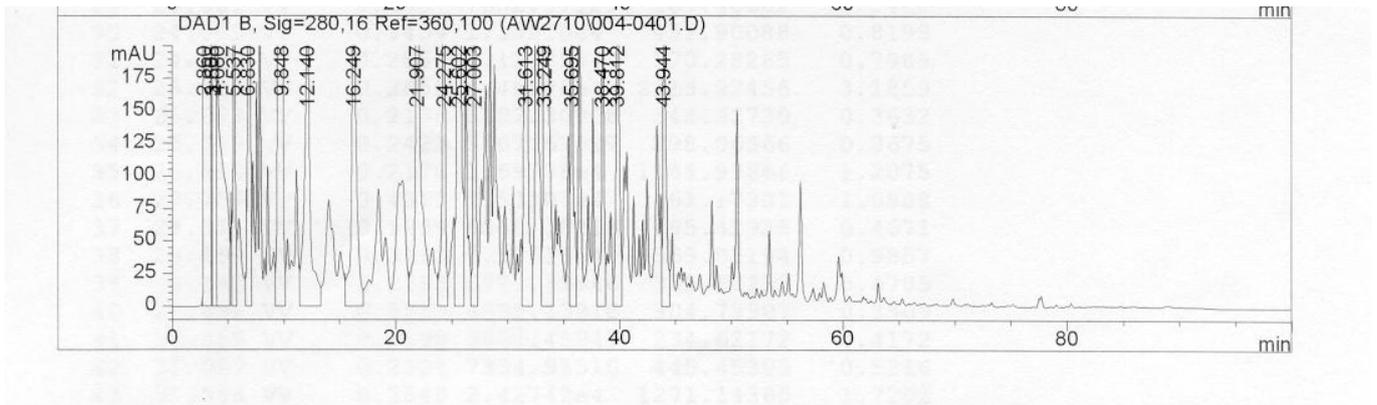*Figure 2: HPLC trace pattern for a subject with ASD*

Figure 3: HPLC trace pattern for a subject without ASD and positive result from HLA DQ2 screening test.
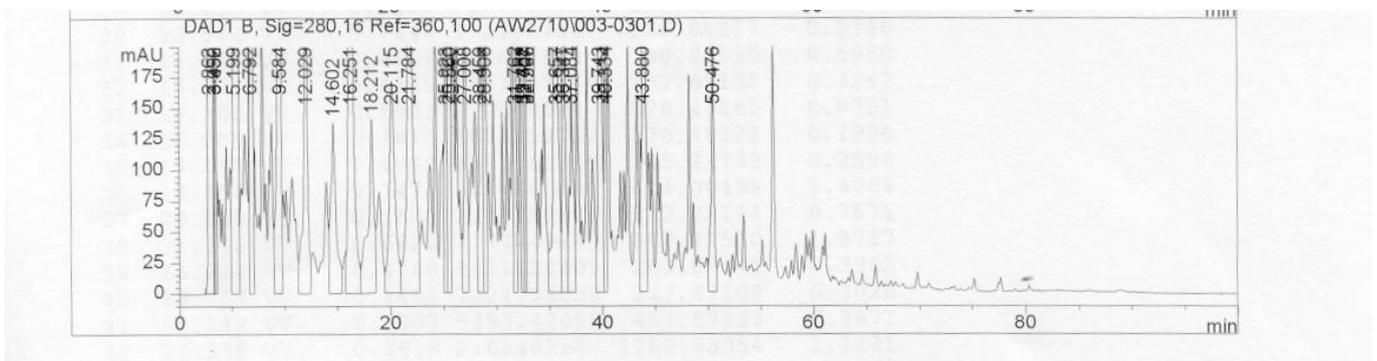

Table 3 is contained in Appendix 3 and outlines the serves of wheat and milk for each subject and the area under the curve for the peptides of interest (+/- 1.00min). Trace patterns and elution times for all peptides are contained in Appendix 4.

Hyperpeptiduria is evident in Figure 2 and Figure 3. There is no significant association with amount of serves of wheat and/or milk in the diet and the average area under the curve for the peptides of interest.

BLOODS

All subjects in both subpopulations scored normal for age for IgA, Antigliadin IgA, and Anti EMA. One subject (#1065) in the subpopulation of children with ASD had high Antigliadin IgG scores, with the remainder scoring normal for age. See Table 4 in Appendix 5.

Figure 4: Average area under the curve for peptide of interest (gluten exorphin A5 and β-casomorphine (bovine).
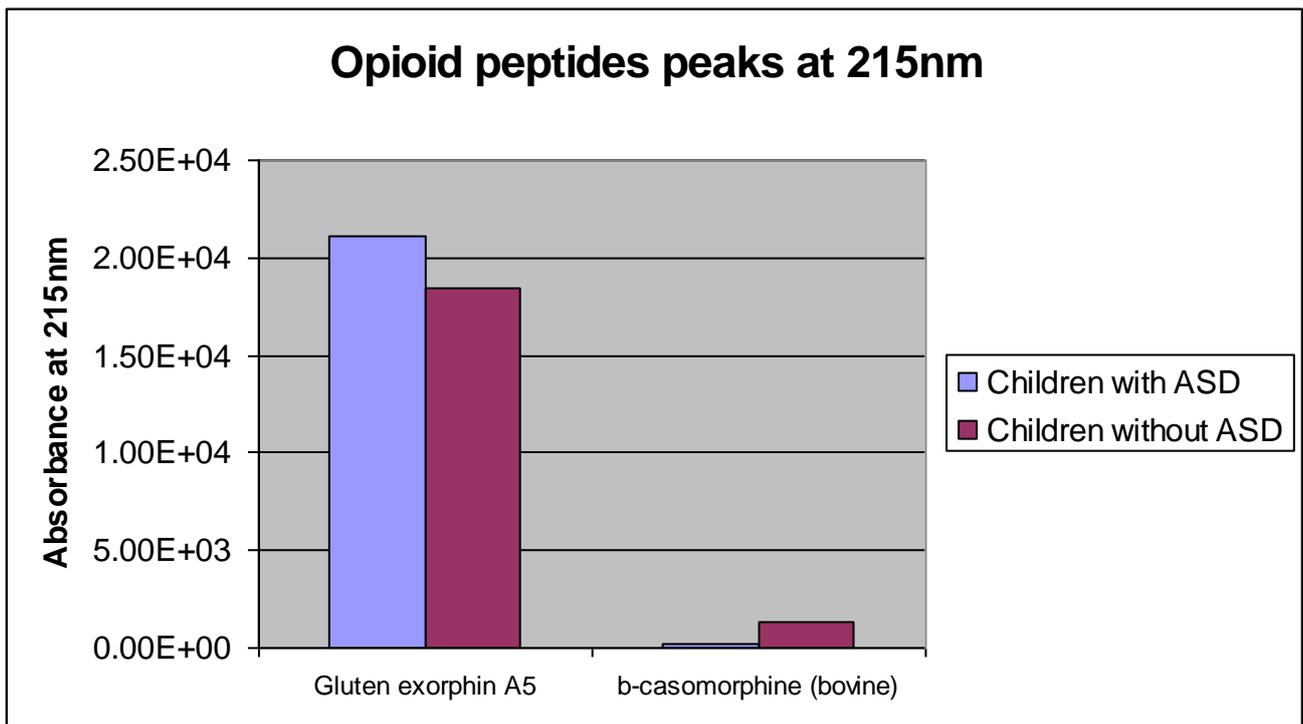
Discussion

Opioid peptide excess

Our observations of the HPLC trace patterns indicate it is not possible to ascertain whether an opioid peptide excess exists. It is apparent that everyone excretes peptides, but the concentration of the peptide excretion is individual. Similar results have been published (Hunter 2003) whereby it is impossible to detect the presence of opioid peptides or their breakdown products in the urine of the subjects. The standards used in this study were gluten exorphin A5 and β-casomorphine (bovine), which were excreted at specific times. These peptide excretion times were not replicated in any of the samples, which indicates that either these peptides were not represented in the foods consumed by the subjects or that the subjects excreted them in a highly individual manner. Microheterogeneity of subsyndromes of autism have been discussed previously (Reichelt 1994), where the amino acids, urea and ammonia were eluted over a range of times indicating that different peptidases are different in different individuals (Reichelt 1994). It therefore follows that peptide excretion is highly individual and that an excess or even existence of a specific opioid peptide is more complicated to elucidate than the methods described and replicated here suggest.

Method

The vast array of excretory products in an individuals urine is highly dependent on foods consumed and the manner in which they are metabolised, that is, exogenous and endogenous peptides. The method replicated in this study was one described by Reichelt et al 1999 and utilised chromatography methods to determine the amount of various peptides in an individuals urine. It is our suggestion that due to the highly individual nature of the content of urine, HPLC methods of assessing this content are inaccurate and provide little more than a tool to compare patterns of urine excretion. No definitive evidence can be determined by HPLC peaks in our subjects for the reference opioid peptides being present in excess. This study and others highlights the complexity of low molecular weight compound/peptide content of urine (Hunter 2003) and methods to validate food diaries versus peptide content of urine are yet to be described accurately. It is only possible to find a relative intake of, for example, wheat or milk as compared to other subjects in the study and relate this back to the pattern of peptide excretion in the subjects urine. Measurement for creatinine has been described and the clinical method for determining the amount of creatinine in the individuals urine is well documented. However, the results can only determine compliance of the subject within the study and/or how concentrate the urine is, and/or whether there is possibly a collection issue in the excretory system. The creatinine levels for these subjects is only useful in determining that no abnormality as far as urine collection is occurring.

Food intake versus excretory patterns

The purpose of this study was to measure dietary intake of wheat (gluten) and milk (casein) against excretory patterns of peptides in the wheat and milk family, in the subjects urine as it has been purported that gluten and casein contribute to the aetiology of autism in some individuals (Reichelt 1994; Mehl-Madrona 2000; Hunter 2003). Hyperpeptiduria has been reported in a number of studies (Reichelt 1994; Shattock 1997) however, validation involving specific dietary intake during the course of the study, has not been previously reported. Reference has only previously been made to clinical improvements being seen when subjects are on a gluten-and casein-free diet. It was not possible to define a relationship between the subjects milk and wheat intake and their gluten and/or casein peptide excretion in this study, thereby suggesting that the benefits of any dietary intervention are anecdotal and subjective.

Comparison of HPLC trace patterns

A comparison of HPLC trace patterns show similarity between those subject with ASD and those subjects with positive HLA DQ2 screening test for coeliac disease. This may contribute to explaining why gluten-free (and casein-free) diets have been shown to have a clinically positive effect. Coeliac disease is an enteropathy that primarily affects the proximal small intestine, causing small bowel injury (Anderson 2002). It has been shown that HLA-DQ2 molecules are present in almost 100% of coeliac subjects, although not all subjects with positive HLA-DQ2 have coeliac disease. Many clinical associations with coeliac disease and other disorders have been established or suggested and include Down's syndrome. It is suggested that this disorder may raise the suspicion of coeliac disease (Cook 2002). As Down's syndrome is considered a purely genetic disorder, and it has been suggested that ASD's are a genetic disorder, it may be possible that ASD's and coeliac disease may also be associated as is suggested for Down's syndrome. It has been reported that up to 15% of Down's syndrome subjects also have coeliac disease (Cook 2002).

When comparing the trace patterns, it is observed that many peptide peaks exist within the first 45-50 minutes approximately for all subjects, however, the area under the curve, ie the concentration of the substance, is greatest in those subject with positive HLA DQ2, followed by those subjects with ASD and the subjects without ASD and negative HLA DQ2 have the least concentration of peptides in their urine. This suggests that the enteropathy for coeliac disease may exist in ASD patients albeit in a less amplified manner. Other evidence exists that ASD patients have malabsorption issues (Reichelt 1994; Shattock 1997) and that there may exist some association between coeliac patients and ASD subjects, based on biopsies, serum IgA analysis and gluten challenges after an elimination diet (Reichelt 1994). The exact mechanism of the adversarial response to gluten type products and specific peptides cannot be elucidated as the method is not refined enough.

Dietary intervention

From our observations of the HPLC trace patterns, it is obvious why gluten-and casein-free diets have shown clinical improvements for subjects with ASD. Management of coeliac disease involves a strict adherence to a gluten-free diet and histological and symptomatic improvements are seen when gluten has been removed completely from the diet (Cook 2002). It follows that removing gluten from the diet of an ASD subject is also going to be beneficial as it is evident that proteins are being metabolised in a similar manner to those subjects that have a genetic propensity to coeliac disease, although to a lesser degree. The positive effect of a gluten-and casein-free diet has been extensively reported throughout the literature (Reichelt 1990; Ek 1999; Knivsberg et al. 2000; Cornish 2002) however the improvements seen and reported correlate to the incidence of coeliac disease in the normal population.

Conclusion

It has been shown in this study that a pattern of urinary peptide excretion exists between subjects, however, a greater concentration of urinary peptides are found in subjects with positive HLA DQ2 yet they do not have a diagnosed ASD. This suggests that hyperpeptiduria can exist without ASD existing, and hyperpeptiduria does not necessarily need to be present for a subject to have ASD. We would then subscribe to the theory that autism is probably a genetic disposition (Reichelt 1994) and some subjects with ASD have dietary intolerances that complicate their condition.

Further studies are required to validate the findings of this study, however, it may be beneficial for them to be directed toward an investigation of food intolerance and/or evidence of an association between coeliac disease and ASD, as found in Down syndrome. Should an association be found, advice with regard to implementation of gluten-free diets, including allowances within the Australian government's Carers Benefit being directed toward education on special dietary needs, may be strongly argued for.

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