Can urinary peptides explain the pathophysiology of Autistic Spectrum Disorder (ASD)?

The University of Sydney

A major project submitted in partial fulfillment
for the award of the degree Master of Science
(Nutrition and Dietetics)
University of Sydney

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ACKNOWLEDGEMENT

I would like to begin by acknowledging and thank all the parents and children who took part in this project. This work would not have been possible without all of your generous 'samples'.

I also wish to give my special thanks and undying gratitude to **Dr. Velencia Soutter** and **Dr. Rob Loblay**, who gave me invaluable assistance, knowledge, guidance and massive amount of supports in this project.

Dr. Anne Swain, also deserve special mention for her active involvement and continuous input and feedback in preparation of this work. Your patience, tolerance and understanding are immeasurable.

Mr. Zia Ahmad and **Ms. Jenny Phuyal**, from Human Nutrition Unit (HNU) of University of Sydney, who generously lending their expertise and '24-hour' support to me without any hesitation.

Mr Tim Watkins, for his technical advice and **Ms. Katherine Jukic**, for being such a great company and sharing her knowledge and experience to me at all time.

All staff at the Allergy unit, RPAH for their kind hospitality, making us feels so comfortable and helping us out wherever they could

Ada, **Liz**, **Tomoko** and **Katinka** for their friendship, great accompaniment and all the work they put together into this project.

Last but not least, to all others, who have provided direct or indirect assistance to the project, are also gratefully acknowledged. The preparation of this report would not have been possible without the support and valuable contributions of these individuals.

ABSTRACT

Introduction: Evidence is growing that the "opioid excess" theory may have a role in the origins of autism. This theory suggests that peptides, derived from gluten and casein poses excessive opioid activity in the brain can cause autistic behaviour. Research has reported abnormal levels of peptides in the urine of person with autism. Diets to exclude these opioids, commonly gluten and casein free diet has appeared to improve symptoms in some but not all autistic individuals

Aim: To validate the 'Opioid Excess' Theory

Method: 24 hour urine & 4-day food records specimens were collected from 2 cohorts of 20 children (ASD & non-ASD) aged between 3 and 10 years. All urines was analysed to detect the presence of seven peptides: Gluten exorphin A4, Gluten exorphin A5, Gluten exorphin B5, Beta-casomorphin (bovine), Dermorphin, Deltorphin II and Indolyl-acrylolglycine (IAG) in both groups.

Results: It was not possible to differentiate ASD and non-ASD group based on the urine profiles of the UV chromatogram. There were no peptides specific to autism as the excretion pattern in ASD group is no different from the control group. There were no significant difference found between presence of IAG in urine of the ASD and non-ASD group

Conclusion: The present study demonstrated that it is not possible to ascertain that the 'Opioid excess theory' exist in children with ASD due to the small sample size involved in this study. However, some preliminary finding indicates it is not possible to identify peptides specific to autism. All individual excrete peptides in their urines and the excretory pattern is extremely individual. It is not possible to adapt chromatographic methods to be a diagnostic tool for ASD or form a basis for recommending therapeutic intervention with dietary modification. It is also not possible ascertain whether IAG could be potential biomarker for ASD. Further research is required to identify the effect of dietary intervention. Increase the number of participants would assist by enhancing these findings through providing more conclusive outcome.

Can urinary peptides explain the pathophysiology of Autistic Spectrum Disorders (ASD)?

INTRODUCTION

Autism Spectrum Disorder (ASD) is a lifelong pervasive developmental disorder that present in a variety of ways. Individuals with ASD have marked impairment of social interaction and of both verbal and non-verbal communication. Affected people tend to have extreme difficulty learning from experience and modifying their behaviour to accommodate varying situation. Other spectrums of diagnoses which fall under the umbrella of ASD include Autistic Disorder, Rett's disorder, Childhood Disintegrative Disorder, Asperger's Disorder (ASP), and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS). These diagnoses come under the classification of Pervasive Developmental Disorder in the Diagnostic Statistical Manual IV (DSM-IV) [1].

There are indications that the prevalence of the disease is rising [2,3]. In the United States, Autism was once considered relatively rare condition until the early 1990s, after which its prevalence has increased to 40-50 per 10000 children by 1997 [4]. Autism was also identified four times more commonly in males than females [3,4]. Recent preliminary studies conducted in Australia discover that the annual incidence of all ASD was 5.1, 8.0 and 4.3 per 10000 children in New South Wales (NSW), Western Australia (WA) and Victoria (VIC) respectively [5,6]. This finding is comparable to other study conducted in United Kingdom, where an incidence of 8.9 per 1000 was found for all ASD [7].

Recent developments have generated debate over the possibility that autism is a systemic, treatable illness precipitated by genetic vulnerability plus an early environmental trigger [8,9]. Due to the complex aetiology of the disorder, its management was largely empirical. To date, majority of the interventions in this area of disorder are based primarily on behavioural and educational approaches aimed at treating symptoms. However these did not address the fundamental causation of the specific deficit observed in autism.

‘Opioid Peptide Excess’ hypothesis

‘Opioid peptide excess’ theory was first raised by Panksepp in 1979. He proposed that people with autism may have elevated levels of opioids which occur naturally in the brain [10]. This finding was further extended by other groups where they have found elevated levels of opioid peptides presence in the urine of people with autism [11-14]. They suggested that these compounds were exogenous (exorphins) derived from partially digested food proteins as the quantities of these compounds are much too large to be of CNS origin. They hypothesised that the intestinal mucosa is abnormally permeable in autism [15] and peptides form from incomplete breakdown of gluten and casein are able to enter the blood through the leaky mucosa and thus exhibit direct opioid activity or form ligands for the peptidase enzymes which break down endogenous endorphins and enkephalins [14,16]. It was furthermore hypothesised it is the presence of this intense opioid activity that explain the behavioural and developmental problem of people with autism [12].

Urinary Analysis

Urinary investigation using high performance liquid chromatography (HPLC) revealed the presence of distinct urinary profiles common peaks in the urines of people with autism[12,13,17] except for one study [18]. Opioid peptides derived from dairy and cereal produces had been identified in the urine of children with autism using a techniques combining HPLC and electrospray mass spectrometry. These peptides were mainly bovine beta-casomorphin, alpha gliadin, glutemorphin A4, glutemorphin A5, gliadinopmorphin, dermophin, deltorphin I, deltorphin II, and morphine-modulating neuropeptide, as well as breakdown of these parent peptides [13,14]. While beta-casomorphin, alpha-gliadin and glutemorphin are derived from food products, the source of the other peptides was unknown.

There were also evidences indicating the presence of elevated levels of indolyl-acryololyglycine (IAG) in the urine of children with autism [16,19,20] a compound previously found in the urine of people with metabolic disorders. The source of IAG in

autistic subjects is not clearly understood but is not thought to be an opioid peptide directly derived from dietary sources [20]. The appearance of IAG has been proposed to be the best marker for infantile autism [20]. Recent report, however, found no significant difference from urine samples taken from ASD and non ASD group [21].

Dietary Intervention

Based on analysis of urine samples, dietary intervention involving the exclusion of foods containing gluten and/or casein has been proposed to be effective in ameliorating some of the behavioural symptoms of autism [13,22,23]. The results from a gluten and casein free dietary intervention have shown significant improvements in the behavioural and cognitive functioning of participant, with regression reported following the suspension of the diet. Similar changes in the pathological urine patterns and levels of peptides have also been demonstrated in children with autism on dietary intervention [23]. However, laboratory tests often failed to detect normalization of urinary peptides even when clinical improvement was indicated [24]. Improvement in behavioural and gastrointestinal symptoms has been link to food intolerance with no correlation found between gluten and casein intake and excretion of gluten exorphin and casomorphin peptides [25].

It is apparent that a need exist to establish the relative correctness of the hypothesis. If the opioid excess theory is correct, it should be feasible to detect and/or quantify specific substances to this disorder through HPLC analysis. Furthermore, if majority of autistic patients has been related to gluten/casein intolerance, a diet free of gluten and/or casein should show changes in the pathological urine pattern and reduce the symptoms associated with autism. The scope of this study is to (i) reproduce the findings based on the method adapted from Shanahan (2002) [13] and Reichelt and Coworkers (1998) [26]; (ii) confirm the presence of opioid peptides in the urine of children with autism; (iii) determine the effect of gluten and/or casein free diet in the urinary profile of children with autism.

Materials and Methods

Subjects

A total of 20 children (17 males and 3 females) age ranging from 3-10 years old were involved in this study. As this was a follow-up study based on preliminary findings from previous studies, subjects were recruited from previous studies or new patients who attended the Allergy clinic this year. Subjects were categorized into the following two groups:

a) Autism Spectrum Disorder (ASD) diagnosed according to the criteria of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (n = 10)
b) Healthy individual without diagnosis - Control (n = 10)

The first group consist of 10 ASD children (10 male and 1 female) with mean age of 7.9 years. They were either patients' of the Royal Prince Alfred (RPAH) Allergy Unit, or members of Autism Association who have not consulted a Paediatrician or dietitian at the Allergy Unit. Subjects were not under any medication or special dietary intervention at the time of urine testing. Subjects who were on special dietary intake were noted in their Four Day Food Dairy to prevent misinterpretation of results.

The control group were comprised of 7 males and 3 females of age and sex-matched healthy school children with mean age of 8.3 years. They were not diagnosed with any Autism Spectrum Disorder or involved on any specific dietary/drug interventions at time of urine testing. They were either children recruited from the childcare centre and primary schools in the CSAHS region or children of some staff member. Unaffected siblings of the ASD children were also included in this group.

This study was conducted with ethics approval from Sydney South West Area Health Services (SSWAHS) Ethics Review Committee. Parents were informed and provided consent on behalf of their children

Four Day Food Dairy

A Four Day Food Dairy was completed by the participant's parents who are involved in recording the child's dietary intake for 4 consecutive days including 3 weekdays and 1 weekend day (i.e. from Wednesday till Saturday). Participants' parents were required to initiate the food record three days prior to the 24 hour urine collection in order to allow clearance of the urines if they were on any dietary intervention.

Urinary Analysis

Sample collection and preparation

When specimen jars containing urine were returned to the clinic, the volume of each sample was determined and the pH and presence of protein were measured using a multistik. All specimens were labelled and one of the specimen jars were reserved to measures creatinine level based on the Jaffe reaction at the Department of Biochemisry, RPAH. The remaining specimens were kept frozen until analysis.

Urine samples were subjected to preliminary clean up using Solid Phase Extraction as described by Shanahan (2000) [13]. Frozen urine samples were thawed overnight and approximately 10mL of urine samples were centrifuged at 3500 rpm, at 4ºC for ten minutes and filtered through syringe filter (0.22 µm).

Standards preparation

Ascorbic acid, thymol and seven other bioactive peptides (Bachem AG, Switzerland) such as, β-Casomorphine (bovine), Deltorphin II, Dermorphin, Gluten Exorphin A4, Gluten Exorphin A5, Gluten Exorphin B5 and Indolyl-3-acryloyglycine (IAG) were used as standards and their retention times were determined. Each standard was diluted to 0.5mg/mL and run in duplicates to obtain reproducible retention time. All standards were mixed to make up a final concentration of 0.5mg/mL and spiked into control urine to

determine any matrix interference. As the HPLC method was specifically designed for the detection of peptides, the substances in the urine samples having retention times similar to the standards were assumed to be peptides. The standards were also used as internal standards to ensure sufficient numbers of plates were obtained for each series of 10 runs.

Solid Phase Extraction (SPE)

Solid phase extraction of the urine samples was carried out on Reverse Phase SPE C18 Extract Clean™ columns (500mg; 8mL; Alltech, Australia) extraction cartridges facilitated with a 12-port vacuum manifold. The solid phase extraction was preconditioned by washing with 10mL of methanol, followed by 10mL flushes of milli-Q water to rinse out the methanol, then 10mL of 0.1% Trifluoroacetic acid (TFA) to equilibrate the column into the necessary conditions to optimise peptide binding during sample loading. While the cartridge remaining wet from the previous 0.1% TFA, filtered urine sample equivalent to 250nmole of creatinine was applied to the cartridge immediately with the flow going to waste container. The urines were flushed twice with 5mls of 0.1% TFA, followed by 8mL of 0.1%TFA in 0.15% Acetonitrile (CH_3CN) to remove loosely bound materials. The cartridge was heated to 40ºC and blown under nitrogen gas to dryness at the end of the final flushes. The final eluate were collected with two 5mLs flushes of 0.1%TFA in 100% CH_3CN and blown dry with nitrogen gas. The processed sample was then reconstituted with 0.1%TFA in 15% CH_3CN and immediately vortex-mixed.

High Performance Liquid Chromatography (HPLC) Analysis

Processed urine samples were analysed by HPLC using reverse phase c-18 column (Apollo C18 series, column 250 x 4.6 mm, Alltech, Australia). Sample injection volume was 20µL for extracted urines or volume equivalent to 250nmoles for un-extracted urines. The columns were run at room temperature with an automatic gradient control and a flow rate of 1.0ml/min. Column elution was monitored at 215nm, 280nm and 326nm with slope sensitivity of 0.2 and their ratio and elution position were used to estimate purity and nature of each peak respectively. The total run time was set at 100 minutes per sample and the two solvents used were:

Buffer A: 0.1% TFA in aqueous

Buffer B: 0.1% TFA in 95% CH3CN and 5% water

The elution gradient used was as follows:

Elution Gradient	Time (minutes)	Buffer A	Buffer B
Gradient 1	0-15	99%	1%
Gradient 2	15-75	Linear decrease to 60%	Linear increase to 40%
Gradient 3	75-80	Linear decrease to 40%	Linear increase to 60%
Gradient 4	80-89	40%	60%
Gradient 5	89-94	Linear increase to 99%	Linear decrease to 1%
Gradient 6	94-100	Equilibration	

Statistical Analysis

All results were analysed using SPSS 13.0 for windows.

RESULTS

Detection of opioid peptides (standards) by HPLC

Ascorbic acid, thymol and the seven opioid peptides were used as standards and their retention times were shown in Table 1. All standards were clearly evident in the chromatograms when mixed standards were spiked into control urines (Figure 2). Retention time of each standard was reproducible when placed in aqueous or urine sample.

Table 1. Retention times[Ψ] of standards analysed at 215nm/326 nm in various medium

Standards	Retention Time (mins)			
	Single in Aqueous	Mixed in Aqueous	Mixed in Control Urines (Spike)	Mean ± SD
Ascorbic Acid	5.71	5.73	5.59	5.68 ± 0.08
Gluten Exorphin A5	44.48	44.49	44.53	44.50 ± 0.03
Gluten Exorphin A4	46.87	46.73	46.78	46.79 ±0.07
IAG [a]	57.46	57.29	57.05	57.27 ± 0.21
Dermorphin	57.54	57.39	57.05	57.33 ± 0.25
Deltorphin II	61.20	61.06	61.17	61.14 ± 0.07
Gluten Exorphin B5	63.38	63.09	63.25	63.24 ± 0.14
Beta-casomorphine	64.61	64.55	64.49	64.55 ± 0.06
Thymol	87.58	86.96	86.8	87.11 ± 0.41

[Ψ] Based on mean retention time of duplicate standards

[a] IAG read at 326nm

Figure 1. Chromatogram of opioid peptides in aqueous solution.

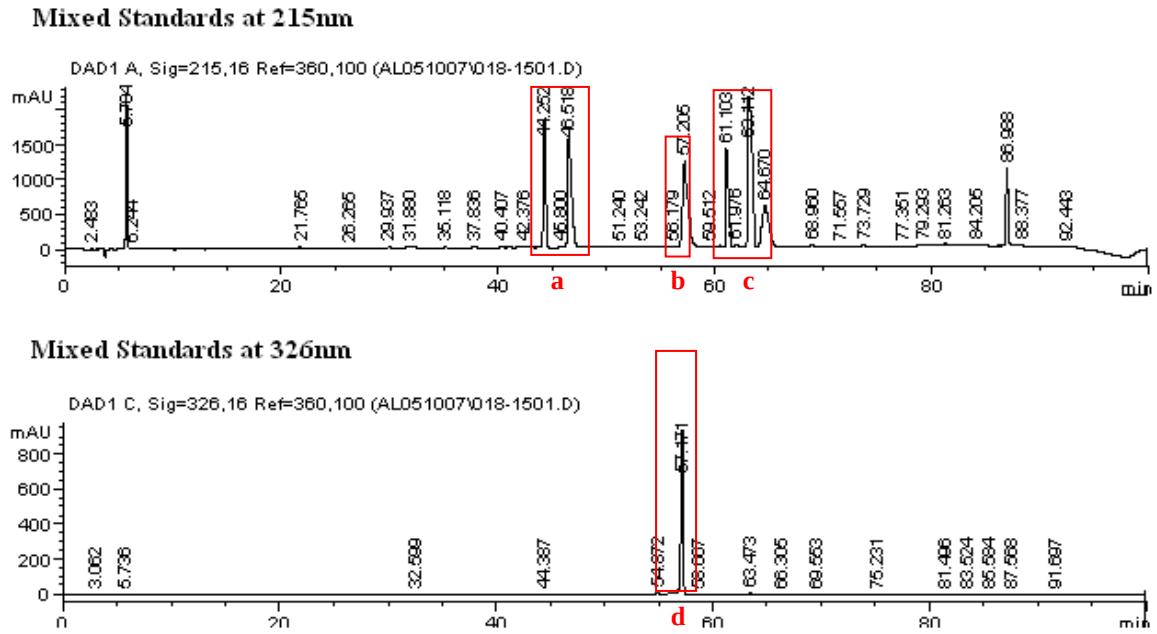

Figure 2. Chromatogram of opioid peptides spiked in control urine.

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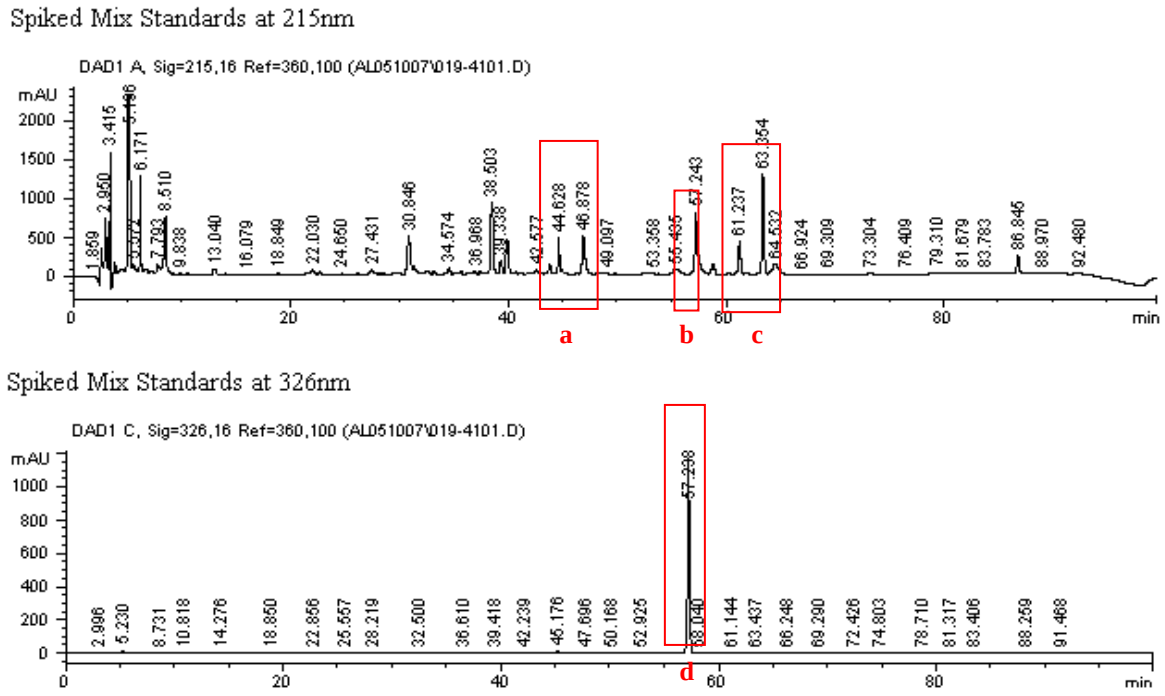


Column a. Gluten exorphin A5, Gluten exorphin A4 ; **Column b**. Dermorphin ; **Column c.** Deltorphin II, Gluten exorphin B5, Beta-casomorphin (bovine) ; **Column d.** IAG

Analysis of urine samples for the presence of opioid peptides

Chromatogram obtained from urines of control and ASD sample showed differences between each subject. A series of peaks were observed in both groups however it was not possible to distinguish between the two groups based on chromatogram alone. Thus, peptides in the urine were identified using retention time (mean ± SD) of the standards.

Figure 3a. Chromatogram at 215nm of urine from a child with ASD.

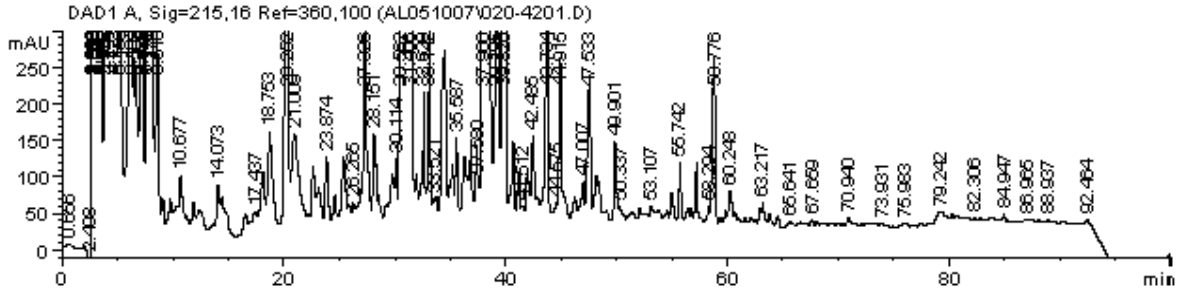
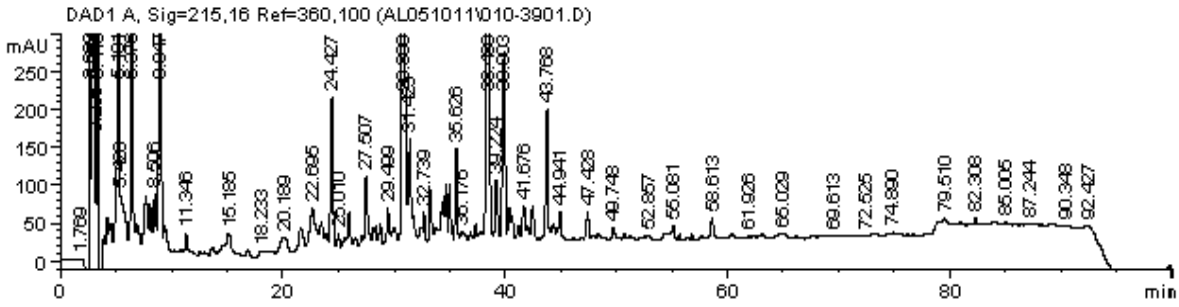


Figure 3b Chromatogram at 215nm of urine from a child without ASD.



Chromatogram from a child with ASD (Figure 1a) showed hyperpeptiduria between the 40-60 minutes region in comparison to a child without ASD (Figure 1b). However, this profile was not observed in all the ASD children involved in this study.

Figure 4a. Chromatogram at 326nm of urine from a same child with ASD.

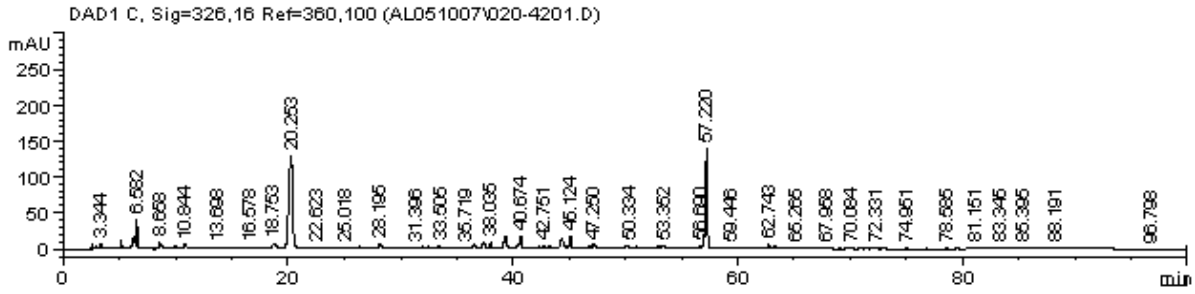


Figure 4b Chromatogram at 326nm of urine from a same child without ASD.

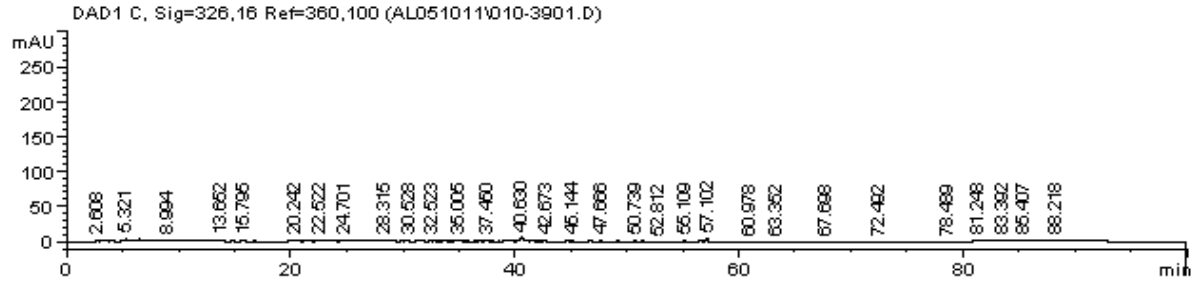
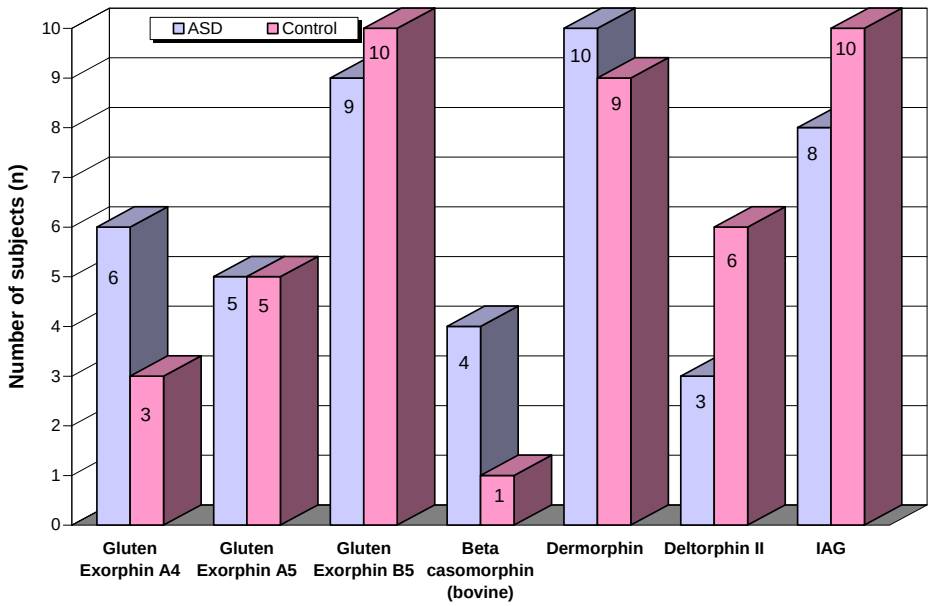


Figure 4a indicated presence of IAG (peak at 57.22 minutes) in one of the ASD child in contrast to a non ASD child (Figure 4b) which showed no peak at the corresponding time.

Figure 5. Presence of peptides in urines of the control (n=10) and ASD group (n=10)



Difference is not significant (p>0.05)

Figure 5 illustrate number of subjects who showed presence of peptides in each group. Six people from the ASD group indicate presence of Gluten Exorphin A4 as oppose to three people of the control group. Gluten Exorphin B5, Dermorphin and IAG were common peptides found in both control and ASD group. Deltorphin II, on the other hand were more likely to present in the control group. Statistical analysis using Mann-Whitney test showed that the difference for each group was not significant ($p>0.05$).

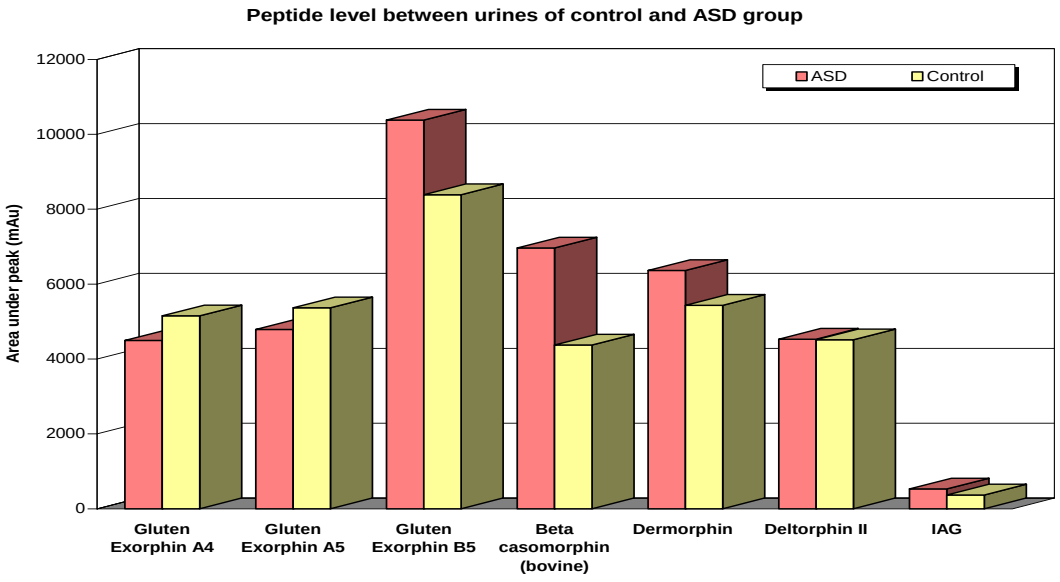
Table 2 Mean peptide level in the urines of control and ASD group

Peptides	**Mean ± SE**		**Mann- Whitney Test (P-value)**	
	ASD	**Control**	**Test 1** $^{\Psi}$	**Test 2** $^{\Omega}$
Gluten Exorphin A4	4492 ± 629	5151 ± 734	0.321	0.439
Gluten Exorphin A5	4785 ± 932	5363 ± 859	0.968	0.917
Gluten Exorphin B5	10378 ± 841	8386 ± 1730	0.650	0.369
Beta-casomorphin (bovine)	6961 ± 1340	4372 ± 0	0.112	0.480
Dermorphin	6327 ± 597	5432 ± 915	0.226	0.369
Deltorphin II	4528 ± 1502	4510 ± 1201	0.247	0.769
Indolyl-acrylolglycine (IAG)	530 ± 239	367 ± 114	0.650	0.722

$^{\Psi}$ All subjects were included

$^{\Omega}$ Subject who had none detectable level of the corresponding peptide was excluded

Figure 6. Mean peptide level between urines of control (n=10) and ASD group (n=10)



Difference is not significant ($p>0.05$)

Table 2 & Figure 6 showed that all reference peptides, except gluten exorphin A4 & A5 were increased in ASD group compared to control group. However the difference between ASD and control were not significant ($p>0.05$) based on Mann-Whitney Test. When the test were repeated by excluding those who had found no detectable peptides, the difference remained not significant.

Analysis of urine samples treated with Solid Phase Extraction (SPE)

In order to determine the recovery of the peptides after the extraction process, results of extracted urines were compared with the un-extracted urines. Chromatogram of an extracted urine (Figure 7a) showed components eluting between 0-20 minutes were eliminated. The peaks eluting between 20-60 minutes were less intense and complex.

Figure 7a. Chromatogram at 215nm of extracted urine of a child with ASD

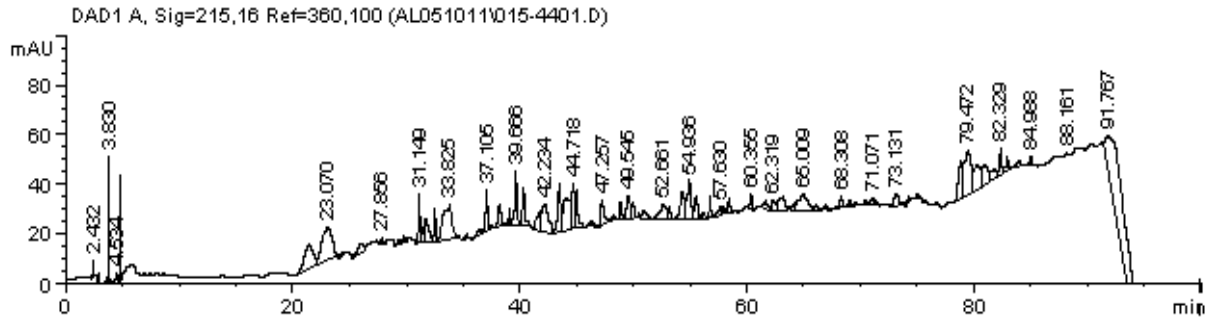


Figure 7b. Chromatogram at 215nm of non-extracted urine same child with ASD

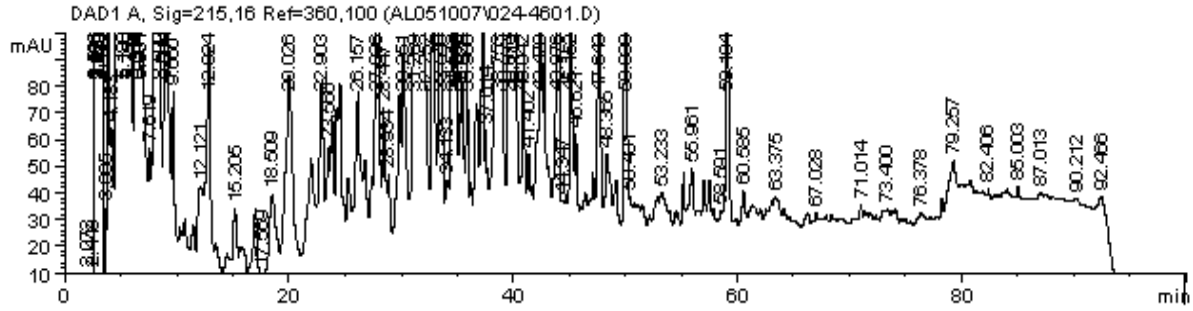


Table 3. Mean peptide level in extracted and non-extracted urines

Peptide	Mean ± SE		Wilcoxon test (P-value)
	Non-extracted	Extracted	
Gluten Exorphin A4	1703 ± 1061	0 ± 0	0.180
Gluten Exorphin A5	1601 ± 1234	261 ± 161	0.285
Gluten Exorphin B5	10461 ±1344	235 ± 92	0.043
Beta-casomorphin (bovine)	4899 ± 2051	19.4 ± 19	0.109
Dermorphin	5874 ± 752	37 ± 26	0.043
Deltorphin II	0 ± 0	76.4 ± 76	0.317
Indolyl-acrylolglycine (IAG)	787 ± 339	97 ± 46	0.043

Table 3 showed recovery of the extracted urines based on the estimated peptide level.There was a marked decrease in the peptides of the extracted urines as compared to the non-extracted ones. Deltorphin II, which was not identified previously were found in one of the extracted urines. Statistical analysis showed that the differences between the two groups were only significance for Gluten exorphin A4, Gluten exorphin B5, Dermorphin and IAG.

Effect of dietary intervention in urinary profile

The urinary profile of a ASD child prior and during elimination diet showed little difference based on chromatogram alone (Figure 8a & 8b). It was not possible to identify individual peaks of interest and determine the effect of a dietary intervention.

Figure 8a. Urinary pattern of ASD child prior to elimination diet

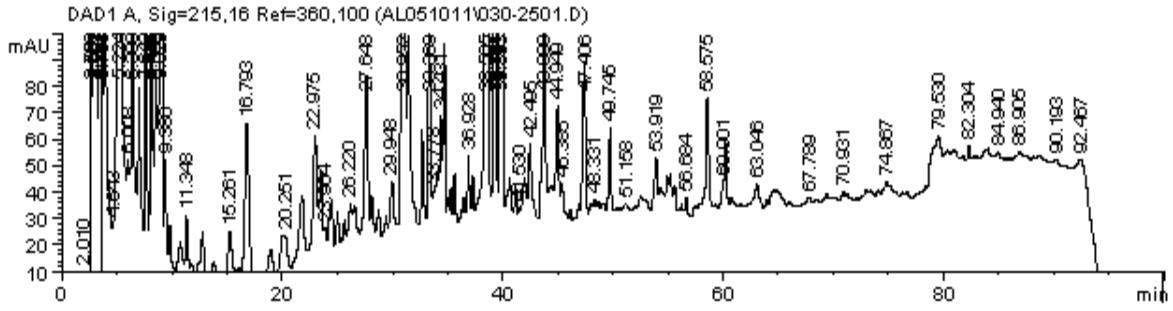


Figure 8b. Urinary pattern of same ASD child on elimination diet

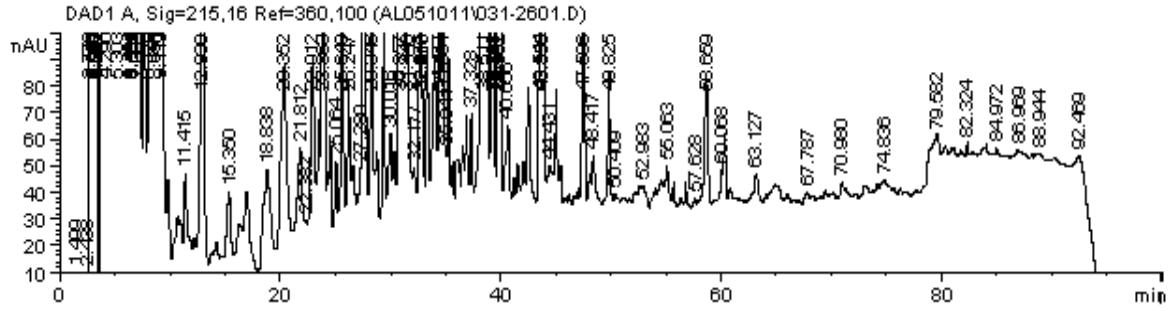
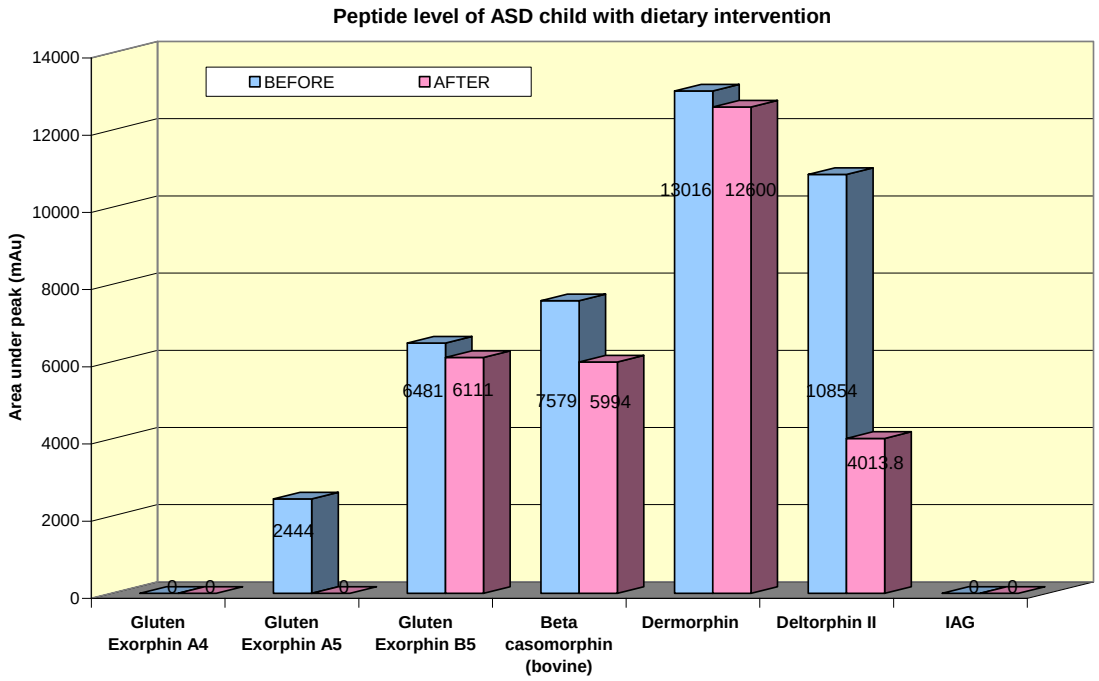


Figure 9. Peptide level of ASD child (n=1) with diet intervention



A marked decreased in peptide level was observed when individual peptide were identified based on their respective retention time (Figure 9). During elimination diet, Gluten exorphin A5, Beta-casomorphin (bovine) and Deltorphin II were excreted less as compared to other peptides. Nevertheless, only one subject were involve in this part of study and more subject would be require to validate the findings

DISCUSSION

Observation of HPLC trace pattern indicate that it is not possible to established whether the 'opioid peptide excess' exist in ASD children. Previous chromatography studies have suggested that comparison of urine profiles can be diagnostic for autism [11,12]. However, it is not possible to differentiate ASD and non-ASD group based on the urine profiles of the UV chromatograms. Evidence of hyperpeptiduria is observed in a small proportion of ASD children. Some of the ASD children somehow resembled urinary profile of the non-ASD group. A single blind study using urinary chromatogram as a diagnostic tool had results in substantial number of false positive and false negative results. It was reported that as high as 47% of ASD group was misidentified as healthy individual as against to misidentifying 33% of the healthy individual. [27].This indicates that chromatogram anomalies may not be a help of diagnostic tool.

The investigation for the presence of opioid peptides in two cohort groups implies that peptide excretion is purely individual. There were no peptides specific to autism as the excretion pattern in ASD group was no different from the control group. Dietary derived peptides such as gluten exorphin A4, A5 and B5 and Beta-casomorphin were apparent in both groups however without specific pattern indicated. Early study suggested stability of Deltorphin II and dermorpin to other gluten/ casein related peptides may be useful biomarkers for diagnosing casein/gluten intolerance form of ASD [13]. The fact that these peptides are regular excretory products in both of our study group, their capability as the biomarkers for diet-related ASD is questionable. Furthermore, **t**here was no significant difference between presence of IAG in urine of the ASD and non-ASD group. Its occurrence seems to be more frequent in the non-ASD group than the ASD group indicating it is no more than a regular metabolite of the body. Therefore, these peptides claimed to be specific to autism, is unlikely to be of help either diagnostically or as a basis for recommending therapeutic intervention with dietary manipulation.

The amount of peptides excreted in the urine of two cohort groups further indicated that the amount of excretory products is highly individual. There were no significant increased in peptides, particularly those dietary related peptides such as gluten exorphins and beta-

casomorphins. Earlier study conducted in the Allergy clinic have found no relationship between gluten and casein intake their gluten exorphin and casomorphin peptide excretion in both ASD and non-ASD group [25]. The peptides excreted in the urine may not entirely originate from dietary sources. It was suggested that the amount of peptides excreted in the urines may also be due to endogenous breakdown of protein within the body, which have a similar amino acid sequence to the peptides which are being examined. Therefore, this work questions the accuracy of previous work, whether urinary peptides can be associated to aetiology of autism. It is also questionable whether autism individual has abnormally permeable gut lining that claimed to allow greater amount of peptides to escape into the system and therefore indicated in the urine .

Previous studies have proposed that removal of these opioid peptides by exclusion of foods containing gluten and/or casein product is effective in ameliorating some behavioural symptoms in autism. Normalised urinary peptide levels have been demonstrated in children with autism on dietary intervention [23] but not in one study. Although the urinary profile of a ASD child in this study showed some reduction of dietary peptides on dietary intervention, more subjects were required to confirm this findings as only one subject were involve in this part of study.

Multiple studies have published the effectiveness of a preliminary clean up method using Solid Phase Extraction coupling with HPLC to postulate the opioid excess theory [12,13]. However, proportion of urine samples subjected to Solid Phase Extraction in this study indicated poor recovery of peptides as against to the non-extracted samples. Noticeable effect has been observed as hydrophilic compounds such as salts, amino acids, urea was vastly removed. However, there were also indications that some peptides might have been lost during the extraction process. Further confirmation is required to validate this finding.

LIMITATION AND FUTURE RESEARCH

Investigation for the presence of opioid peptides proves the complexity of human urines. Low molecular weight compound such as peptides in the urines were barely detectable by chromatogram alone unless they were presence in high concentration. Spiking of control urine with a mixture of standard peptides (2.5ug of each peptide) showed distinct peaks in this study. This may not be the case when lower concentration of peptides was applied. Peak of interest could easily obscure by other compounds in the urine, for instance, salts, urea, peptides, amino acids etc if they had similar retention time as indicated in one of the studies [18]. The study itself is therefore restraint by the detection limit of peptides in such complex biological samples.

The use of HPLC alone is inadequate for the accurate identification of peptides in the urine. The method does not allow the accurate identification of the individual compounds present in the urine. The sensitivity of these techniques is also affected by deferring abilities of individual compound to absorb UV light. The use of electrospray mass spectrometry may be incorporated as a proof of identity when the amino acids sequence eluting in the region of presumed peptides were identified.

Nevertheless, HPLC remain an excellent qualitative analysis for the purpose of this study. As this was a pilot study involved only 10 children with autism and 10 healthy children, increase number of participants would assist by enhancing the findings through providing more conclusive outcome.

CONCLUSION

The present study demonstrated that it is not possible to ascertain that the 'Opioid excess theory' exist in children with ASD due to the small sample size involved in this study. However, some preliminary finding indicates it is not possible to identify peptides specific to autism. All individual excrete peptides in their urines and the excretory pattern is extremely individual. It is not possible to adapt chromatographic methods to be a diagnostic tool for ASD or form a basis for recommending therapeutic intervention with dietary modification. It is also not possible ascertain whether IAG could be potential biomarker for ASD. Further research is required to identify the effect of dietary intervention and increase the number of participants would assist by enhancing these findings through providing more conclusive outcome.

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Appendix 5: HPLC - Standard Analysis

Retention time of standards analysed at various medium.

Standards	Individual	Combine	Spike control	Mean	Standard Deviation
Ascorbic Acid	5.71	5.73	5.59	5.68	0.08
Gluten Exorphin A5	44.48	44.49	44.53	44.50	0.03
Gluten Exorphin A4	46.87	46.73	46.78	46.79	0.07
IAG	57.46	57.29	57.05	57.27	0.21
Dermorphin	57.54	57.39	57.05	57.33	0.25
Deltorphin II	61.20	61.06	61.17	61.14	0.07
Gluten Exorphin B5	63.38	63.09	63.25	63.24	0.14
Beta-casomorphine	64.61	64.55	64.49	64.55	0.06
Thymol	87.58	86.96	86.8	87.11	0.41

Appendix 6: HPLC - Urine Analysis

Absorbance (215nm)									(326nm)
Standards	**Ascorbic Acid**	**Gluten Exorphin A5**	**Gluten Exorphin A4**	**Dermorphin +IAG**	**Dermorphin**	**Deltorphin II**	**Gluten Exorphin B5**	**Beta-casomorphin**	**IAG**
Retention Time	**5.68±0.08**	**44.5±0.03**	**46.79±0.07**	**57.33±0.25**		**61.14±0.07**	**63.24±0.14**	**64.55±0.06**	**57.27±0.21**
	5.60-5.75	**44.47-44.53**	**46.72-46.86**	**57.08-57.57**		**61.07-61.22**	**63.1-63.38**	**64.49-64.61**	**57.06-57.47**
Subject Id (ASD)									
1001	0	0	0	6656.3	4701	0	5389.3	7215.5	1955.3
1005	0	0	0	5168.6	5069.5	0	11698.9	7454.3	99.1
1009	10958.5	1634.9	0	4966.7	4562.8	0	12382.9	0	403.9
1023	3968.1	0	4874.62	9683.6	8555.1	0	12706.3	0	1128.5
1025	3137.1	6372.3	3640.8	6827.9	6479.9	0	10126.8	9824.6	348
1028	7075.2	0	6560.7	8812.4	8705.2	7184.9	0	0	107.2
1038A	10654.9	0	5785.7	8503.4	8333.4	0	12152.3	3349.8	170
1042	10291.7	6311.9	2390.5	3265.5	3240.2	0	7885.7	0	25.3
1056	6313.1	5940	3700.1	6938.2	6938.2	1985.5	12174.5	0	0
1062	8342.4	3665.9	0	6682.9	6682.9	4413	8885.7	0	0
(Control)									
4067	0	0	0	0	0	0	199	0	25.7
4074	0	7949.9	5413.1	5746.2	5008.6	5580	11821.8	0	737.6
4086	9133.9	0	0	2822.5	2719.5	0	11308.7	0	103
4102	4409.4	0	0	4380	4275.7	3574.5	12153.9	0	104.3
4103	14750.3	2674.8	0	2129	1341.9	1138.8	718	0	787.1
4107	4784.9	4884	0	4791.2	4419.4	0	3636.5	0	371.8
4114	4537.1	0	0	5274.7	4977.9	2826.3	16576.8	0	296.8
4115	3339.2	6131.4	6270.5	8142	8058.5	0	12582.8	0	83.5
4116	0	5175.7	3769	8681.9	8560.7	4238.8	7286.7	4372.2	121.2
4119	0	0	0	10568.4	9525.3	9703.7	7572.3	0	1043.1
(ASD-clean up)									
1001s	0	0	0	140	0	0	213.9	97	154.0
1005s	0	0	0	133	133	0	185.5	0	0
1009s	0	0	0	0	0	0	211.5	0	20.5
1023s	0	720	0	110	0	382	564.8	0	246.1
1025s	0	584.2	0	117.3	51.7	0	0	0	65.6

Effect of dietary intervention

Standards	Before	After
Gluten Exorphin A4	0	0
Gluten Exorphin A5	2444	0
Gluten Exorphin B5	6481	6111
Beta casomorphin (bovine)	7579	5994
Dermorphin	13016	12600
Deltorphin II	10854	4013.8
IAG	0	0

Appendix 7: Statistical Analysis -Presence of opioid peptides between ASD and Non-ASD group

Number of subjects who showed presence of peptides

Mann-Whitney Test

Ranks

Standards	Study Participants	N	Mean Rank	Sum of Rank
Gluten Exorphin A4	Control	10	9.00	90.00
	ASD	10	12.00	120.00
	Total	20		
Gluten Exorphin A5	Control	10	10.50	105.00
	ASD	10	10.50	105.00
	Total	20		
Gluten Exorphin B5	Control	10	11.00	110.00
	ASD	10	10.00	100.00
	Total	20		
Beta casomorphin (bovine)	Control	10	9.00	90.00
	ASD	10	12.00	120.00
	Total	20		
Dermorphin	Control	10	10.00	100.00
	ASD	10	11.00	110.00
	Total	20		
Deltorphin II	Control	10	12.00	120.00
	ASD	10	9.00	90.00
	Total	20		
Indolyl-acrylolglycine (IAG)	Control	10	11.50	115.00
	ASD	10	9.50	95.00
	Total	20		

Test Statistics

	Gluten Exorphin A4	Gluten Exorphin A5	Gluten Exorphin B5	Beta casomorphin (bovine)	Dermorphin	Deltorphin II	(IAG)
Mann-Whitney U	35.000	50.000	45.000	35.000	45.000	35.000	40.000
Wilcoxon W	90.000	105.000	100.000	90.000	100.000	90.000	95.000
Z	-1.314	.000	-1.000	-1.510	-1.000	-1.314	-1.453
Asymp. Sig. (2-tailed)	.189	1.000	.317	.131	.317	.189	.146
Exact Sig. [2*(1-tailed Sig.)]	.280(a)	1.000(a)	.739(a)	.280(a)	.739(a)	.280(a)	.481(a)

a Not corrected for ties.

b Grouping Variable: Study Participants

Appendix 8: Statistical Analysis – Mean peptide level between ASD and non-ASD group

Mean peptide level in the urines of control and ASD group.

Mann-Whitney Test

Ranks

Standards	Study participants	N	Mean Rank	Sum of Rank
Gluten Exorphin A4	Control	10	11.70	117.00
	ASD	10	9.30	93.00
	Total	20		
Gluten Exorphin A5	Control	10	10.45	104.50
	ASD	10	10.55	105.50
	Total	20		
Gluten Exorphin B5	Control	10	11.10	111.00
	ASD	10	9.90	99.00
	Total	20		
Beta casomorphin (bovine)	Control	10	12.10	121.00
	ASD	10	8.90	89.00
	Total	20		
Dermorphin	Control	10	13.90	139.00
	ASD	10	7.10	71.00
	Total	20		
Deltorphin II	Control	10	9.10	91.00
	ASD	10	11.90	119.00
	Total	20		
Indolyl-acrylolglycine (IAG)	Control	10	9.90	99.00
	ASD	10	11.10	111.00
	Total	20		

Test Statistics(b)

	Gluten Exorphin A4	Gluten Exorphin A5	Gluten Exorphin B5	Beta casomorphin (bovine)	Dermorphin	Deltorphin II	(IAG)
Mann-Whitney U	38.000	49.500	44.000	34.000	16.000	36.000	44.000
Wilcoxon W	93.000	104.500	99.000	89.000	71.000	91.000	99.000
Z	-.993	-.040	-.454	-1.590	-2.580	-1.158	-.454
Asymp. Sig. (2-tailed)	.321	.968	.650	.112	.010	.247	.650
Exact Sig. [2*(1-tailed Sig.)]	.393(a)	.971(a)	.684(a)	.247(a)	.009(a)	.315(a)	.684(a)

a Not corrected for ties.

b Grouping Variable: Study Participants

Mean peptide level in the urines of control and ASD group (excluded those who did not excrete peptide)

Ranks

Standards	Study Participants	N	Mean Rank	Sum of Ranks
Gluten Exorphin A4	Control	6	4.50	27.00
	ASD	3	6.00	18.00
	Total	9		
Gluten Exorphin A5	Control	5	5.40	27.00
	ASD	5	5.60	28.00
	Total	10		
Gluten Exorphin B5	Control	9	11.22	101.00
	ASD	10	8.90	89.00
	Total	19		
Beta casomorphin (bovine)	Control	4	3.25	13.00
	ASD	1	2.00	2.00
	Total	5		
Dermorphin	Control	10	9.90	99.00
	ASD	6	6.17	37.00
	Total	16		
Deltorphin II	Control	3	5.33	16.00
	ASD	6	4.83	29.00
	Total	9		
Indolyl-acrylolglycine (IAG)	Control	8	10.00	80.00
	ASD	10	9.10	91.00
	Total	18		

Test Statistics(b)

	Gluten Exorphin A4	Gluten Exorphin A5	Gluten Exorphin B5	Beta casomorphin (bovine)	Dermorphin	Deltorphin II	(IAG)
Mann-Whitney U	6.000	12.000	34.000	1.000	16.000	8.000	36.000
Wilcoxon W	27.000	27.000	89.000	2.000	37.000	29.000	91.000
Z	-.775	-.104	-.898	-.707	-1.519	-.258	-.355
Asymp. Sig. (2-tailed)	.439	.917	.369	.480	.129	.796	.722
Exact Sig. [2*(1-tailed Sig.)]	.548(a)	1.000(a)	.400(a)	.800(a)	.147(a)	.905(a)	.762(a)

a Not corrected for ties.

b Grouping Variable: Study Participants