

Faecal Calprotectin and a Twenty-Four-Parameter Questionnaire in Autistic Children with Gastrointestinal Symptoms

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Abstract

This study investigated potential correlation between the inflammatory marker, Calprotectin, and a S.O.S questionnaire from forty-nine Autistic children. Symptom and behavioral questionnaires were completed contemporaneously with stool sample collection.

Mixed Model data analysis showed strong correlation between some questionnaire parameters and Calprotectin. 'Need for a fixed routine' was highly significantly correlated with Calprotectin ($p < 0.00009$) with Multivariate Coefficient of 3.227, whilst paradoxically 'constipation' indicated significant change ($p < 0.02$) with negative Multivariate Coefficient (-1.584). The negative 'constipation' appears to associate with the positive 'need for a fixed routine' indicating possibility of reciprocal, independent prediction of gastrointestinal inflammation.

Results suggest that 'need for a fixed routine' and 'constipation' be included in a screening questionnaire as independent predictors of bowel dysfunction in these children.

Introduction

Autistic Spectrum Disorder (ASD) is a Pervasive Developmental Disorder (PDD) with abnormal or impaired development in reciprocal social interaction, abnormal or impaired social communication and social imagination [1-3].

There has been a considerable increase in the awareness of autism since first described by Dr Leo Kanner in 1943 [1,4,5]. However, treatment procedures that focus on the overall wellbeing of subjects affected by this condition remain underdeveloped. This applies to many aspects of the condition including the Gastrointestinal (GI) symptoms. Only recently has research highlighted the GI involvement in ASD as a potential component for assessment and treatment [6-9], although for more than a decade there have been studies undertaken on the possible link between GI abnormalities and the associated behavioral problems of ASD [8,10]. Horvath et al., (1999) after analyzing 36 autistic children suffering from GI symptoms found an incidence of reflux oesophagitis in 69%, while 88% presented with abdominal discomfort and 67% presented with chronic non-specific duodenal inflammation. Data from this study indicated that more than half of the subject population had Duodenal Paneth Cell hyperplasia and presented with abdominal pain, chronic diarrhea, bloating, sleep disturbance and/or irritability [11]. A gut-brain axis has been postulated as a link between these gut metabolic reactions and abnormal behaviour potentially indicating an important component of neural development [12,13]. This potential link has been given support by Jyonouchi et al., (2005) who postulated an intrinsic defect in the innate immune responses in ASD and thus a possible link between the GI system and behavioral symptoms mediated by immune responses [14,15].

Several authors such as Forsythe et al. [16]; Horvath et al. [11]; Jyonouchi et al. [17]; K. L. Reichelt and A. M. Knivsberg [18]; Nikolov et al. [19]; Walker et al. [9] have suggested a possible gut-brain axis that could be immunological, inflammatory or genetic in nature. A possible connection between the behavioral signs and symptoms and the gastrointestinal condition in these children has been suggested by Buie et al. [20]. Also, the lack of an appropriate low-invasive gastrointestinal treatment prompted separate part of this present study to consider addressing this lack [21].

Smith et al., (2009) reported that 35% of parents of autistic children in special schools were concerned with their child's bowel function compared with 4% of parents of children in mainstream schools. The same study also reported that the presence of constipation (25%), diarrhea (27%) and flatulence (24%) were significantly higher in children with PDD compared with a control group of

healthy subjects which was 4%, 5% and 2% respectively. Although Black et al. (2002) postulated that there was no specific GI patho-physiology in autistic children, the increased bowel symptoms in these children are suggestive of some bowel dysfunction [7,9,22,23].

There are a number of methods of determining bowel inflammation including biopsy, endoscopy, and colonoscopy and stool analysis for biochemical markers [24,25]. The least invasive, particularly important in children, utilizes faecal biochemical markers including the S100 group of proteins [26].

Calprotectin is one such marker and it is known that high levels of this S100 protein leave with the intracellular exudates fluid during various inflammatory processes [27-29] and once released from the cell it may be detected in serum and body fluids as a useful marker for inflammatory processes.

A high Calprotectin concentration is thought to be derived from either stimulated neutrophil secretion or as a result of cell death [27]. An example of high Calprotectin levels occurs in the faeces of patients suffering from Inflammatory Bowel Disease (IBD) such as Crohn's Disease (CD) and Ulcerate Colitis (UC) [30].

Currently the main application for the Calprotectin ELISA test is to differentiate between IBD and Irritable Bowel Syndrome (IBS) [31]. Calprotectin concentration levels are decreased during successful IBD therapy and higher concentration levels may be an indicator of IBD relapse [32]. Assessing faecal Calprotectin has been found to be a reliable diagnostic tool for patients who present with IBD symptoms [33]. Further, a meta-analysis by Van Rheenen et al (2010) determined that the Calprotectin prediction of inflammation in adults gave a sensitivity of 93% and specificity of 96%. The corresponding values for children and teenagers were 92% and 76% respectively. Calprotectin has been used as a non-invasive marker for identifying organic disease of the GI tract and it has been suggested that it could be used before more invasive procedures [34-36]. The faecal calprotectin ELISA has proved to be a valuable tool compared to more invasive GI tests, especially in young children [34,37] and according the NICE diagnostic guidance DG11 (2013) calprotectin is recommend as an option to distinguish between IBD and IBS.

The aim of this study was to determine whether a correlation exists between the very stable faecal GI inflammatory marker Calprotectin and the perception of parents of autistic children to the children's GI signs/symptoms. A modified Secretin Outcome Survey (S.O.S) twenty-four-parameter questionnaire [38] was used to assess parents' perceptions and a standard ELISA test used to assess Calprotectin in the stools of the children.

Material and Methods

The study is a clinical trial employing a longitudinal design with a calculated (Power Analysis) sample size of 50 subjects.

Children between 3½ and 8 years old were recruited from schools dedicated to autistic children and in response to announcements placed in local newspapers and magazines, in state schools, Universities and on the National Autistic Society research subject recruitment website.

Population

The population for the current study was composed of male and female autistic children aged between 3½ and 8 years. All subjects recruited to this study were independently diagnosed by specialist professionals prior to their inclusion in the study. All the specialist professionals in England and Wales are required to follow the DSM-4 and ICD-10 diagnostic criteria to release a Diagnostic Statement. Parents were required to provide the Diagnostic Statement, together with the statement of special educational needs provided by the ELB, prior to any contact with the researcher. The researcher did not make any contribution to the diagnostic process, thus avoiding classification bias on recruitment.

After the initial recruitment/screening questionnaire, parents were interviewed by a single senior osteopath/researcher with 16 years of clinical experience. The interview consisted of a one and half hour conversation with the mother and/or father when a thorough case history was taken. The case history was divided into pre-natal history, pregnancy, labour, medication during labour, developmental stages of the child, any accidents or surgeries, past and present medication, when a child was diagnosed as autistic, who diagnosed the child, present symptoms, GI symptoms (including colour of stools, their frequency, consistency, and smell), behavioral symptoms (flapping of hands, eye contact, communication, etc.) current diet and allergies. From this it was determined whether the subject complied with all the inclusion criteria. It is important to highlight that data analysis of parameters collected from the parental interview was not part of this study and therefore not performed. The interview was solely used to assess whether subjects complied with the inclusion criteria.

In total, 64 children were recruited to the study [Figure 1]. Ten subjects were excluded due either to a compromising underlying condition or to not presenting with GI symptoms. Further parents of autistic children completed the interview but did not comply with sending the first set of stool samples and the questionnaires necessary to participate in the study and so were classified as drop outs. The final sample therefore comprised of 49 autistic children (46 male and 3 female) aged between 3½ and 8 years.

An essential inclusion criterion was that subjects had been

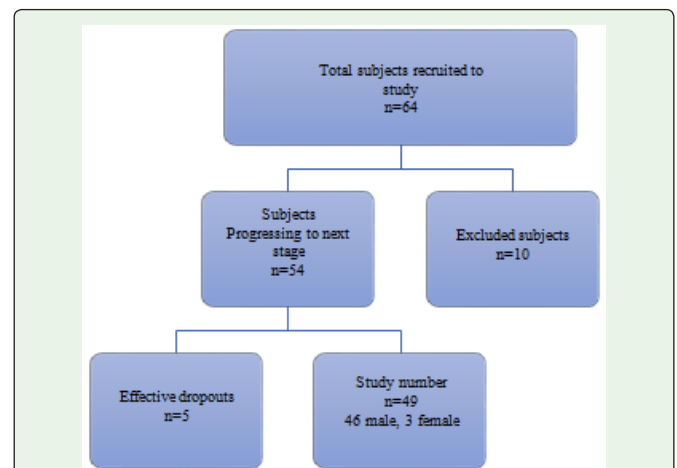


Figure 1: Flow chart indicating the recruitment process and final sample size.

Note: The flowchart indicates the number of subjects recruited, excluded subjects, dropouts, and the final subject number.

diagnosed as Autistic (ASD) by independent medical specialists prior to entry to the study. Thus, recruits needed to be enrolled at a special school for autistic children accredited by the National Autistic Society. The rationale for this requirement lay in the fact that the special schools for autistic children in England and Wales only accept children who have been given a statement of special educational needs (a legal document) by the Education and Library Board (ELB) after being assessed by an appropriate, experienced specialist. All included subjects were suffering from abnormal behaviour recognized by DSM-4 and ICD-10 Diagnostic Criteria, as well as suffering from GI symptoms e.g. abdominal distension and pain, constipation, chronic diarrhea, foul-smelling stools and/or flatulence.

Subjects were withdrawn from the study if the child developed a condition that could jeopardise general health e.g. infections.

The project was granted Ethical approval by both The British College of Osteopathic Medicine Ethics Committee and by the University of Westminster Ethics Committee.

The subjects' GI and behavioral symptoms were assessed via S.O.S questionnaires [38] completed by the parents. Laboratory analysis of collected stool samples to assess Calprotectin concentration was undertaken by a specialist, independent facility blinded to the questionnaires (Department of Clinical Biochemistry, King's College Hospital, London).

Questionnaire - Autism Research Institute Secretin Outcomes Survey Form - "S.O.S Form"

Questionnaires are useful data collection tools to analyze a patient's perception of health [39]. The current study used the S.O.S Form, a validated and standardized questionnaire [38,40-45], to measure the parents' perception of their autistic children. Benefits of using questionnaires for data collection includes the relatively low administration costs ease of response and simplicity of data analysis [39].

The health community usually uses a Likert-type or frequency scale questionnaire designed to measure attitudes or opinions. In this study, parents completed the S.O.S questionnaire regarding their assessment of the GI and behavioral symptoms of their children which consists of a 24 parameter, ten-point Likert scale, based on the Autism Research Institute Secretin Outcomes Survey Form - "S.O.S Form" [38,40-45].

The Likert scale assumes linearity of the data, measuring, for example, parameters from strongly agree to strongly disagree. It is important to demonstrate the reliability of the S.O.S questionnaires used. The most common test to analyse internal consistency and reliability of a multiple Likert scale questionnaire or survey is the Cronbach's Alpha (α) statistic. Cronbach's α score above 0.7 are considered acceptable for reliability [46,47].

Chronbach's Alpha analysis, a coefficient of reliability (or internal consistency), was conducted on the S.O.S questionnaire to evaluate reliability. The overall reliability of the S.O.S questionnaire was 0.881 on Chronbach's Alpha Scale suggesting a good internal consistency.

Procedure

All subjects followed the same procedure:

- Parents were invited to attend a meeting at their child's school to:
 - receive guidelines about the research.
 - sign the consent form authorizing the child to be part of the study
 - complete an initial recruitment/screening questionnaire about the child's history. This questionnaire was used only as a tool for the authors to determinate whether the child would fit the inclusion criteria of the research.
 - Arrange an interview with the lead author. This interview consisted of a one and half hour conversation with the parent/s when a thorough case history was taken. The case history gave important base line information and acted as a further determinant that the subject complied with the inclusion criteria.

Parents were instructed on how to collect the stool samples. They were informed of the need to answer the S.O.S questionnaire on the same occasion as the stool was collected. This resulted in matching dates for each stool sample and S.O.S questionnaire. This process was repeated eight times over ten weeks. Parents were informed that the S.O.S questionnaire was a key tool for the measurement of their child's behaviour and GI function.

Stool Samples

Calprotectin is remarkably stable in stools. It can be stored at room temperature in a suitable container for up to seven days [29]. Samples can be transported in authorized containers (Safebox) requiring no refrigeration and then stored frozen at -18°C or lower until the time of analysis. Exposure to a temperature higher than 30°C was avoided.

Stool samples were collected by parents from a potty or nappy and placed in a polystyrene screw cap tube with caps coded for identification. Parents received eight 'Safeboxes' numbered sequentially from 1 to 8 making it easy to identify the appropriate box to be sequentially dispatched. Parents were asked only to date the sample tube and the form inside the 'Safebox' which contained the subject identification. The 'Safebox' was sent to King's College Hospital where it was stored at minus 20°C for later analysis.

After the specimen collection, parents had a window of up to seven days to post the sample. In cases where the subject was not able to provide a sample to be posted on the set date, parents were asked to attempt to send a sample and complete the questionnaire within a window of two days of the predetermined set date. This maintained the matched dates for the samples sent to King's College Hospital and questionnaires posted to the lead author. This process facilitated statistical correlation analysis between sample and questionnaire.

ELISA Test Protocol

Calprotectin ELISA plates [48] were used. Authors were blind to the assay results until completion of the study. ELISA plates were purchased from Bühlmann Laboratories AG-Switzerland. The procedure has been described previously by Manz et al (2012). Faecal extracts were prepared by using 40-100 mg of faeces, avoiding any undigested solid material like fibers and seeds, were placed in a pre-weighed, empty, screw-capped tube. Pre-diluted buffer was added, 1:50, weight/volume, and the suspension mixed vigorously for 30 seconds. The suspension was then mixed for a further 30 +/-5

minutes using a shaker at approximately 1000 rpm, with a loop inside the tube as an agitator. A small amount of the mixture (1-2 mL) was then transferred to an Eppendorf tube and centrifuged at 10,000g for 20 minutes at +4°C. The supernatant was harvested and this extract used in the ELISA. A total volume of approximately 0.5 ml was taken and used either for the assay or stored, frozen. The calculations were performed by a computer linked to the ELISA reader; quality controls were included in each run. Stool concentration of calprotectin was calculated as µg/g of faeces. Values above 50 µg/g are considered a positive calprotectin test.

Data Collection Procedure

Data collected was analyzed using ‘R’ statistical package software version 2.15 [50].

Subjects’ GI inflammation was assessed using faecal Calprotectin while GI and behavioral symptoms were assessed using the twenty-four-parameter S.O.S questionnaire [38] (Table 1). This was completed by the parents on eight occasions.

A Mixed Effect Model was used to analyses the repeated observations for this longitudinal study. In this model the analysis takes into consideration missing data which could potentially have an effect on the end results, an inherent problem of longitudinal studies [51]. The Mixed Effect Model is used to correlate the repetitive measures and it also measures the within and between subject error. A mixed effect model has been employed for the analysis of the data generated by longitudinal studies [51,52] (Table 2).

Results

From a total recruitment of sixty-four autistic children fifty-

Table 1: Questionnaire parameters used to assess behavioral and gastrointestinal symptoms.

Social behaviour and communication
1. Lack of awareness and interaction with parent
2. Abnormal greeting behaviour
3. Abnormal comfort seeking
4. Can't make friends
5. Lack of awareness of social rules
6. Lack of spontaneous speech
7. Abnormal word utilization
8. Poor comprehension of verbal instructions
9. Lack of eye contact
10. Abnormal repetitive gestures
11. Need to maintain sameness
12. Need of fixed routine
13. Diarrhoea
14. Constipation
15. Poor Appetite
16. Bloating
17. Flatulence
18. Vomiting
19. Unhappy
20. Aggressive
21. Destructive
22. Spaced out/ Non-Interactive
23. Agitated
24. Disagreeable

Note: The Likert Scale of signs severity: 0= never shows this particular sign or behaviour; 1= slight/unobtrusive; 2; 3 = mild; 4; 5= moderate; 6; 7= severe; 8; 9=extreme/incapacitating.

Table 2: Raw data from Calprotectin stool samples.

	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8
Subject Number	49	49	49	49	44	42	43	35
Mean	46.2	76.8	54.2	33.5	45.8	28.6	40.8	62.9
S.D	92.8	239.6	99.3	57.8	97.2	31.2	46.7	114.0
Range	26.3-511	28.6-1582	44.7-630	25.3-381	22.6-472	24-127	42.3-236	40.5-473

Note: Mean Data has been calculated from the total data (49 subjects multiplied by 8 Samples from each subject giving a total spreadsheet of 392 cells). Table 2 shows the mean data from the total cells.

two satisfied the inclusion criteria and forty-nine entered the study [Figure 1].

Association between questionnaire data and Calprotectin adjusted by patient and sample

The data generated from the set of eight S.O.S questionnaires, from each subject, were correlated with the results of the corresponding eight sets of faecal Calprotectin. Twenty-four parameters from the questionnaires were matched with faecal sample collecting times and results. Each parameter from the questionnaire was analyzed individually using a univariate model.

Table 3 shows all the results of the univariate analysis. This is commonly used in prediction research and it includes all the univariate variables tested. The results with P value >0.15 were manually deleted and were not included in the multivariate model. Each parameter was analyzed repeatedly at several points in time and corrected by patient and by sample and correlated to Calprotectin samples. Univariate analysis is an established strategy to measure longitudinal data, single outcome variables [53]. With the aim of keeping variables in the regression model a more liberal p value of 0.15-0.25 was used. This is established as a common procedure in predictor research [54,55]. A p value of <0.15 was set as an initial filter for the univariate model analysis [56,57]. The parameters ‘Lack of awareness and interaction with parent’ (p=0.02), ‘Abnormal repetitive gestures’ (p=0.009), ‘Need to maintain sameness’ (p=0.0093) and ‘Need for a fixed routine’ (p=0.003) are significant. The parameters Constipation (p=0.108) and Bloating (p=0.086) are not significant at p<0.05, but both parameters fit the required filter criteria with p<0.15 to be incorporated in the multivariate analysis.

The data indicates a strong association between several parameters from the S.O.S questionnaire and levels of faecal Calprotectin. The parameter ‘need for a fixed routine’ was highly significant (p<0.00009), with a Multivariate Coefficient of 3.227 suggesting that autistic children who display an increase in ‘need for a fixed routine’ also show elevated Calprotectin which indicates gastrointestinal inflammation. Paradoxically, the parameter ‘constipation’ although showing a significant change (p<0.02) has a negative Multivariate Coefficient (-1.584), corresponding to a decrease in Calprotectin levels, indicating an inverse relationship between ‘constipation’ and Calprotectin. The negative value in the parameter ‘constipation’ appears to be associated with a positive value of ‘need for a fixed routine’ which may, therefore, be considered independent, but reciprocal, predictors of gastrointestinal inflammation in these autistic children.

Discussion

This longitudinal study sought to investigate twenty-four parameter questionnaire (S.O.S questionnaire) [Table 1] and determine whether a correlation existed with the biochemical marker Calprotectin [Table 2].

The study found significant correlation between the behavioral parameters ‘need for a fixed routine’ and ‘constipation’, and the inflammatory biochemical faecal marker Calprotectin. These results suggest that these two parameters, ‘need for a fixed routine’ and ‘constipation’, could be used together in an initial, standardized, screening questionnaire as a simple to use, non-invasive and inexpensive predictor of inflammatory bowel processes in young autistic children.

Table 3: Initial Univariate Model data.

Variable	Std Error	p-value
1. Lack of awareness and interaction with parent	0.90	0.02*
2. Abnormal greeting behaviour	0.81	0.35
3. Abnormal comfort seeking	0.81	0.32
4. Can't make friends	0.66	0.80
5. Lack of awareness of social rules	0.70	0.79
6. Lack of spontaneous speech	1.15	0.28
7. Abnormal word utilisation	0.67	0.69
8. Poor comprehension of verbal instructions	0.77	0.32
9. Lack of eye contact	0.92	0.57
10. Abnormal repetitive gestures	0.77	0.009
11. Need to maintain sameness	0.78	0.009*
12. Need of fixed routine	0.79	0.0003*
13. Diarrhoea	0.78	0.86
14. Constipation	0.69	0.10*
15. Poor Appetite	0.74	0.65
16. Bloating	0.76	0.08*
17. Flatulence	0.85	0.41
18. Vomiting	1.26	0.39
19. Unhappy	1.07	0.35
20. Aggressive	0.89	0.20
21. Destructive	0.82	0.36
22. Spaced out/ Non Interactive	0.79	0.14
23. Agitated	0.91	0.22
24. Disagreeable	0.82	0.01*

Note: This table includes all the Univariate variables tested as is commonly the practice in prediction research. The P values of <0.15 were manually deleted and were not included on the multivariate model. P values indicated by the asterisks were kept in the multivariate model.

Table 4: Final Univariate Model.

Variable	p set at <0.15 used as an initial filter
1. Lack of awareness and interaction with parent	0.0200
2. Abnormal repetitive gestures	0.0009
3. Need to maintain sameness	0.0093
4. Need of a fixed routine	0.0003
5. Constipation	0.108
6. Bloating	0.086

Note: Table 4 includes all the variables that satisfied the initial filter of p<0.15 in the univariate analysis of the mixed effect model. The parameters 1-4 have shown a significant correlation with the inflammatory marker Calprotectin with p<0.05. The parameters constipation and bloating although not significant in the univariate model have shown a p value <0.15 which satisfies the filter value required to be included in a multivariate model.

Table 5: Final Multivariate Model.

Variable	Multivariate Coefficient	SE	p-value
(Intercept)*	22.894636	4.023740	p set at < 0.05
Need of a fixed Routine	3.226851	0.813211	0.00009
Constipation	-1.584028	0.69109	0.02269

Note: Table 5 represents the final Multivariate model results with two significant parameters. Need for a fixed routine (p=0.00009) and constipation (p=0.02) were found to correlate with GI inflammation as assessed using Calprotectin.

* Intercept is a baseline for Calprotectin value in this multivariate model

The results from the univariate study [Tables 4 and 5], indicate a further four parameters with a possible association with Calprotectin values. Although they are not independent predictors of GI inflammation, they could be appropriately included in combination with the two multivariable parameters ‘need for a fixed routine’ and ‘constipation’.

The final set of six parameters could be used as an initial, short, standard questionnaire in clinical settings simplifying the diagnostic process enabling cost efficiency and more effectively directing subjects to further diagnostic examination as appropriate. Combining these six parameters into one single questionnaire could enable reduction of the number of parameters to be tested, from twenty-four to six, without loss of power or sensitivity. A standardized six parameter questionnaire would not replace any other form of diagnostic testing for gastrointestinal inflammation, but it could be a cost effective, non-invasive initial screen for clinical diagnosis.

These results tend to support a brain-gut axis theory that has been previously postulated [12,13,58]. The correlation between the behavioral characteristics of: ‘Lack of Awareness and interaction with parent’, ‘Abnormal repetitive gestures’, ‘Need to maintain sameness’, ‘Need of a fixed routine’, ‘Constipation’ and ‘Bloating’ and the inflammatory biochemical marker Calprotectin, may be indicative of a connection between gut responses and the classic emotional behaviour suffered by autistic children.

Limitations

One of the limitations of the study is that low spectrum autistic children usually lack the ability to describe or grade their own

symptoms. Therefore, the results from the questionnaires relied on the parents' perception of their child's symptoms and it was not possible to determine how the subject personally felt. This limitation may possibly result in parents over or under estimating the rating value on the Likert scale, potentially creating bias in the results. A blinded, independent assessor rating the parameters could possibly prevent introduction of this bias. However, the professional would have to get acquainted with the child to be able to rate the questionnaire accurately and might still not perceive changes that parents are more aware of. Moreover, this solution would not be as cost effective as the introduction of independent professionals would substantially increase the cost of the research.

Another limitation to this study was that the patients were self-referred. However, all possible measures were taken to safeguard the inclusion criteria of the research. One of them, presented in the methodology section, was that the subjects were required to present a formal diagnosis of autism and be assigned to a special school for autistic children registered by the National Autistic Society. All subjects were formally diagnosed following the same diagnostic criteria required by the DSM-4 and the ICD-10. However, children were not diagnosed by the same diagnostic centre or by the same professional and, therefore, it is not possible to know the impact of that on the results of the study. It is not known if there were any initial discrepancies in interpretation of the autism diagnosis. Future studies may benefit by using a single centre that would refer subjects to the initial screening research procedure. Participating patients could then be selected to be on the same level of the autistic spectrum and perhaps gender could also be selected and standardized. However, recruiting equal numbers of male and female subjects selected from a random sample of autistic subjects is a challenge due to the high prevalence of males diagnosed as autistic [1,59,60]. The same idea applies to the GI signs and symptoms of the subjects included in this research. Parents were self-referring their children to the study and there was no formal diagnosis from a gastroenterologist. However, according to Chandler, et al [61] and Gorrindo, et al [8] there is a high concordance between parental reporting and the gastroenterologist's evaluation of GI symptoms in autistic children. This research relied on parents' perceptions of GI signs and symptoms. Even though studies claimed high concordance between the perception of the parents and the evaluation from the medical doctor, this research might have missed subtypes of gastrointestinal conditions that could have affected the results. Possibly, this could have been prevented if a gastroenterologist were part of the project and had been given responsibility for the screening process. However, that would have imposed financial constraints beyond the scope of this research.

Conclusion

From the study results it may be possible to create a simple to use reliable, non-invasive and inexpensive screening tool capable of evaluating GI inflammation in autistic children. The final 6 item questionnaire could be easily completed by parents or guardians and could potentially facilitate initial screening of GI inflammation in autistic children.

This study attempted to explore the use of a non-invasive clinical tool, the questionnaire, in the facilitation of the analysis of possible GI dysfunction in autistic children.

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