Effectiveness of visceral osteopathic treatment on gastrointestinal indicators and behaviour patterns in autistic children: using questionnaire and biochemical markers to measure outcomes

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EFFECTIVENESS OF VISCERAL OSTEOPATHIC TREATMENT ON GASTROINTESTINAL INDICATORS AND BEHAVIOUR PATTERNS IN AUTISTIC CHILDREN; USING QUESTIONNAIRE AND BIOCHEMICAL MARKERS TO MEASURE OUTCOMES

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Declaration

The work presented in this thesis is the work of the author. I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. This thesis is entirely my own work. Where any material could be construed as the work of others, it is fully cited and referenced, or acknowledgement given where appropriate. This work will not be submitted to any other university, or similar institution, for any qualification until after the outcome of the current application to the University of Westminster is known.

Abstract

Introduction. The precise aetiology of autism is unclear, however recent studies link autism with gastrointestinal (GI) dysfunction. Although there is no specific treatment, the potential to ameliorate the behavioural and GI problems of autistic children is of interest. This study aimed to evaluate inflammatory markers in faecal samples taken from autistic children with GI dysfunction before, during and after visceral osteopathic techniques (VOT), and to link these findings with contemporaneous questionnaires. These data assessed whether GI status could be reliably determined from a single sample, whether VOT affected behaviour and clinical signs, and whether there was any association between biochemical markers and the questionnaire. **Methods**. Faecal samples were analysed for three biochemical markers, calprotectin, M2-pyruvate kinase and lactoferrin. Forty nine children, between 3½ and 8 years old, and independently diagnosed as autistic by specialist professionals were recruited. Questionnaires using a 10 point Likert scale assessed behavioural parameters and clinical signs throughout the 18 week study period, before, during and after VOT. **Results**. Due to intra-individual biological variability, analysis of single faecal samples over time did not give a consistent readout of marker levels. The questionnaire showed significant improvement in symptoms and behaviour during treatment, specifically, reduction in vomiting ($p<0.001$), and poor appetite ($p<0.05$), and an increase in eye contact ($p<0.05$). Analysis of an inflammatory marker, together with the questionnaire, showed a highly significant association of 'need for a fixed routine' ($p<0.0001$) and 'constipation' ($p<0.02$) parameters with calprotectin, and showing multivariate coefficients of 3.227 and -1.584 respectively. **Discussion and Conclusion**. VOT ameliorates GI symptoms in these autistic children and a standardised questionnaire could include 'need for a fixed routine' and 'constipation' as independent predictors of their bowel dysfunction. This study uniquely used biochemical markers to assess symptomatic changes before, during and after VOT.

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List of Abbreviations

AAP	American Academy of Paediatrics
ABA	Applied Behaviour Analysis
ADHD	Attention Deficit Hyperactivity Disorder
ADI-R	Autism Diagnostic Interview Revised
ADOS	Autism Diagnostic Schedule
ANOVA	Analysis of Variance
ASD	Autistic Spectrum Disorder
BSPGHAN Nutrition	British Society of Paediatric Gastroenterology Hepatology and
CAM	Complementary and Alternative Medicine
CD	Crohn's disease
CDC	Centres for Disease Control and Prevention
CNS	Central Nervous System
CSCD	Centre for Social Communication and Disorder
DNS	Did Not Show
DSM –IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
DSM-V	Diagnostic and Statistical Manual of Medical Disorders, 5th edition
ELB	Education and Library Board
ELISA	Enzyme Linked Immunosorbent Assay
ENS	Enteric Nervous System
ESDM	Early Start Denver Model
GERD	Gastroesophageal Reflux Disease
GI	Gastrointestinal
IBD	Inflammatory Bowel Disorder
IBS	Irritable Bowel Syndrome
ICD-10	International Classification of Diseases, 10th edition
IPAN	Intrinsic Primary Afferent Neuron

LD	Learning Disability
MRC	Medical Research Council
M2-PK	Tumour M2-Pyruvate Kinase
NCCAM	National Centre for Complementary and Alternative Medicine
NICE	National Institute for Health and Care Excellence
OPS	Osteopathic Practice Standard
PDD	Pervasive Developmental Disorder
PDD-NOS	Pervasive Developmental Disorder not otherwise specified
PPIs	Proton Pump Inhibitors
PRT	Pivotal Response Treatment
PST	Phenol Sulphur Transferase
RA	Rheumatoid Arthritis
TEACCH	Treatment and Education of Autism and Communication Handicapped Children
UC	Ulcerative Colitis
VIP	Vasoactive Intestinal Peptide
VOT	Visceral Osteopathic Technique

List of Abstracts and Presentations

Publications

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Chapter 1 - Introduction

1.1 Autism

Autism is a multifactorial condition with unclear aetiology and no specific treatment. Studies have been undertaken regarding a possible link between autistic behavioural dysfunction and gastrointestinal (GI) signs and symptoms. Some studies claim that there is a possible gut-brain axis, where the worsening of behavioural symptoms may possibly be due to inflammatory gut reactions meditated by immunological signals (Jyonouchi et al., 2005a, Reichelt and Knivsberg, 2009, Forsythe et al., 2010).

Over the past 15 years an increased awareness has developed amongst researchers of the number of children with autism suffering from GI symptoms. According to Horvath et al. (1999), children with autism often suffer from reflux oesophagitis (69%), chronic gastritis (42%) and chronic duodenitis (67%). A recent study suggests that intestinal biopsies from autistic children with GI symptoms display a characteristic genetic profile distinct from that of control, non-autistic children with similar symptoms (Walker et al., 2013). Nikolov et al. (2009) reported a link between the worsening of behavioural symptoms and GI signs and symptoms, specifically diarrhoea and constipation. The same study also reported that autistic children suffering from GI problems had a tendency to present with higher levels of irritability and anxiety, and were more socially withdrawn compared with children with autism who had no GI symptoms (Nikolov et al., 2009). A consensus paediatric report undertaken by the Paediatric Gastrointestinal Unit in Boston, USA, concluded that the behaviour of children with autism may have an underlying medical condition, which may be GI in nature (Buie et al., 2010).

Currently, studies suggest that the management of GI symptoms have the potential to assist with some of the behavioural symptoms seen in children with autism (Buie et al., 2010). However, there remains a lack of satisfactory low-invasive treatment that could potentially ameliorate the challenging behaviour and GI signs and symptoms of children with autism (Furuta et al., 2012).

The current research aimed to investigate the effects of visceral osteopathic techniques VOT, a low-invasive method of manipulative treatment, on the GI and behavioural signs and symptoms of children with autism. GI signs and symptoms, such as constipation, diarrhoea, bloating, abdominal pain, poor appetite, flatulence and vomiting, and behavioural patterns typical in children with autism, were assessed before, during and after VOT treatment via questionnaires and the analysis of three biochemical markers.

The rationale for the use of VOT on the abdominal area of autistic children suffering from GI complaints was based on evidence, new at the time of the start of this study, linking the amelioration of behavioural patterns in children with autism after medical intervention for their GI dysfunction (Horvath, 2000, Horvath and Perman, 2002, Buie et al., 2010, Furuta et al., 2012). Exploration of the use of standardised VOT on the abdominal area of autistic children suffering from GI problems coupled with analysis of the behavioural and GI signs and symptoms before, during and after its application is a novel approach to this problem.

The first attempt to study the effects of VOT on GI and behavioural signs and symptoms of autistic children dates back to 2002 when a pilot study, by the same author, was performed on 13 autistic children suffering from GI dysfunction (Bramati-Castellarin and Janossa, 2002). The concept of the pilot study was developed from a lack of alternative or complementary interventions available focusing on the GI dysfunction of autistic children.

Historically, positive results have been associated with application of VOT to the abdominal area of subjects with GI dysfunction (Ernst, 1999, Finet and Williame, 2000, Lamas et al., 2009, Attali et al., 2013). However, VOT has never before been applied specifically to autistic children. The subjects who participated in the pilot study in 2002 were selected from special schools for autistic children. All subjects were treated using VOT and a modified Secretin Outcome Survey (S.0.S) questionnaire was used to measure possible changes in the GI signs and symptoms and behavioural patterns of the selected children. The results of the pilot study suggested positive GI and behavioural responses after application of VOT. These positive results led the

author to develop this pilot protocol, introducing biochemical markers in an attempt to quantify any changes experienced by the patients.

1.2 History of Autism

Autism was first described in 1943 by Dr Leo Kanner, an American child psychiatrist at Johns Hopkins University (Kanner, 1943, Aarons and Gittens, 1999). He employed the Greek word ‘autos’, meaning ‘self’, to describe the condition, which is marked by children being engrossed within their own world (Kanner, 1943). Later, Kanner and Eisenberg (1957), in an attempt to clearly define the condition, included a number of behavioural features which remain relevant today since they describe the condition in its classic form Table 1-1 lists the behavioural features observed by Kanner when Autism was first described.

Table 1-1: Kanner’s Classical Behavioural Features of Autism.

Inability to develop relationships
Delay in the acquisition of language
Non-communicative use of spoken language, after it develops
Delay echolalia - the repetition of words and phrases
Pronominal reversal - a child rarely uses the pronoun ‘I’
Repetitive and stereotyped play
Maintenance of sameness
Good rote memory - autistic children may show remarkable feats of memory and rote learning
Normal physical appearance

(Compiled from Kanner (1943))

An epidemiological study undertaken in 1993 by Wing suggested that the core deficit in autism is social in nature (Wing, 1993b). This study indicated that the difference between an autistic child and another with learning impairment is that autistic individuals have an observable social impairment. The study also suggested that there is no clearly defined limit to the disorder. Consequently, clinicians may encounter individuals who do not conform to the classical

description of autism as described by Kanner (Wing, 1993b). Wing highlighted three areas of social difficulty that an autistic individual may display, and described these as forming 'the triad of impairments of social interaction' which she divided into three areas: relationships, communication and imagination (Figure 1-1: (Wing, 1993b).

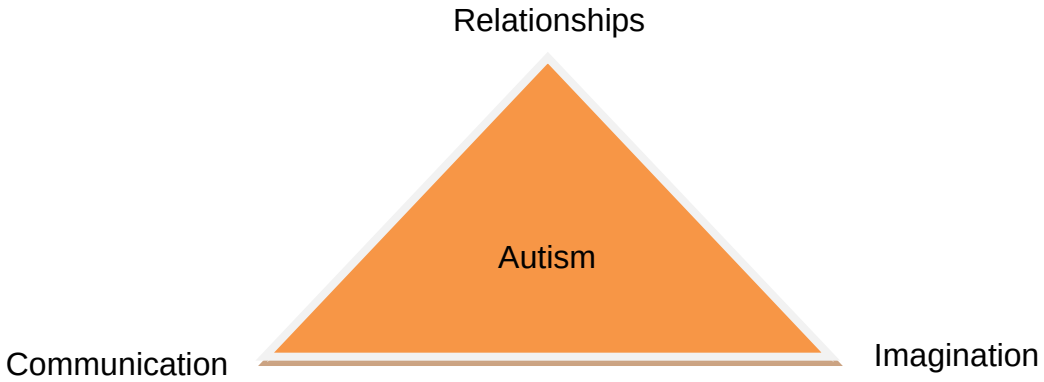


Figure 1-1: The triad of impairment of social interaction

Since autism was first described, researchers have tried to establish criteria with which to clearly diagnose the condition. The original diagnostic criteria were based on the nine requisite points defined by Kanner (see Table 1-1). If a child presented with only eight out of these nine requisite features, then he/she would not be diagnosed as autistic (Kanner, 1943, Aarons and Gittens, 1999).

1.3 Current Classification and Definition

Autism or autism spectrum disorder (ASD) is classified as a pervasive developmental disorder (PDD), with abnormal or impaired development in reciprocal social interactions, abnormal or impaired social communication, and social imagination (Wing, 1993a, Aarons and Gittens, 1999). This classification is utilised in both the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM –IV) and the International Statistical of Diseases and Related Health Problems, 10th edition (ICD-10), that describe the international standardised classifications for psychiatric diseases and disabilities (World Health Organization, 2004).

Both classifications define autism as a PDD and not a ‘child schizophrenia or psychosis’ as suggested in the past (Treffert, 1970). These classifications are used as a diagnostic tool, yet they do not explain either the causes or the true nature of the disorder neither of which are clearly understood.

Researchers use DSM IV and the ICD10 as criteria to classify autistic individuals who possess a deficit in verbal communication and non-verbal behaviour, such as eye contact, facial expression, body posture and gestures, thereby making general and sustained social interaction difficult. Individuals who have verbal skills may lack the ability to initiate or sustain a conversation with others (Wing and Wing, 1971, Wing, 1993a, Waterhouse et al., 1996, Aarons and Gittens, 1999, Kent et al., 2013). Speech may present with echolalia, the repetitive use of language regardless of meaning, as well as abnormal pitch, intonation, rate and rhythm. Older children or adults, who have gained some interpersonal interactions, may still show a disturbance in their comprehension of language, and demonstrate an inability to understand questions, directions or jokes (Wing, 1998).

Imaginative play is often absent and a child will not engage in games or routines of early childhood (Wing, 1993b) but instead may perform repetitive mimicking actions, for example by copying the actions of television commercials and repeating them over and over again. Individuals may insist on following a specific routine or sequence and show resistance or distress over trivial changes to this routine. Autistic individuals present with a failure to develop relationships, and have little or no interest in establishing friendships (American Psychiatric Association, 1994), indeed they may show a lack of emotion and reciprocity, and therefore, an individual with this disorder may not notice another person’s distress (Wing, 1993b).

Individuals typically demonstrate restricted, repetitive and stereotyped patterns of behaviour, interest and activity, and repetitive motor mannerisms are characteristically present (e.g. clapping of hands, finger flicking or rocking, dipping and swaying the whole body) (Wing, 1993b). Similarly, they may show a fascination for spinning objects, opening and closing doors, or they may become fixated on some inanimate object. Relatively normal development is

sometimes reported during the first two years of life, although parents of autistic children usually report regression in language development after the child has acquired five to ten words (Wing, 1993b).

1.4 Diagnosis

No single test has been developed that clearly diagnoses autism, thus a gold standard for diagnosis is yet to emerge (Matson et al., 2012). Currently, the diagnosis of an ASD is determined via a series of parental interviews and diagnostic observation scales, following the DSM-IV and ICD-10 diagnostic criteria (American Psychiatric Association, 2000, World Health Organization, 2004).

The observational schedule and structured interview currently used are the Autism Diagnostic Schedule (ADOS) and the Autism Diagnostic Interview Revised (ADI-R) respectively, the latter being a complement to ADOS (Le Couteur et al., 1989, Lord et al., 1994, Lord et al., 2000, Gotham et al., 2007, Luyster et al., 2009). Both instruments are used to recognise patterns of behaviour present early in life, usually before the age of 36 months.

A revised diagnostic description of autism and autism-related disorders, encompassing them under a single umbrella description, was published in May 2013 (American Psychiatric Association, 2013). This revised version replaces the DSM-IV. According to the American Psychiatric Association, the classification of mental disorders, and also of autism, have been modified (American Psychiatric Association, 2013), and the new criteria employed will create a better link between DSM-5 and the ICD. ICD-10 is currently in the process of being revised and the 11th edition has a provisional publication date of sometime in 2014. According to Swedo et al. (2012), classifying autism as a neurodevelopmental disorder may encourage earlier diagnosis; however, other studies disagree with this change in classification. McPartland et al. (2012) suggested that the specificity of the new diagnostic criteria for ASDs, at 60%, is quite high, whilst the sensitivity of the new diagnostic criteria for ASD subgroups, such as Asperger's and PDDs not otherwise specified (PDD-NOS), and cognitive ability is very low, raising questions concerning the

validity of the new ASD classification by the scientific community (McPartland et al., 2012). Swedo et al. (2012) were highly critical of the comments made by McPartland et al. (2012), and the Neurodevelopmental Work Group supports the decision to alter the diagnostic checklist in the latest edition of the DSM (Swedo et al., 2012). At present, there is a lack of research using the new diagnostic criteria and therefore it is not yet possible to draw a conclusion on its validity.

Although the American Psychiatric Association has announced changes in the classification of PDDs, the current study used the DSM-IV and ICD-10 as the standard for the classification of diseases as these were in use at the time the study was designed and the data collected.

1.5 Current treatment and/or Management of Autistic Symptoms

As yet, there is no defined protocol in place for treatment and/or management of autism. However, in 2014, NICE released the quality standard 51 (NICE, 2014), an updated quality standard recommendation for the assessment, diagnosis and treatment of children, young people and adults diagnosed as autistic. The document follows the criteria of the Autism Act released in 2009 which requires that each local authority in England and Wales creates provision in health and social care for individuals diagnosed as autistic (NICE, 2014).

NICE (2014) quality standard 51 recommends that individuals diagnosed as autistic should receive personalised care developed and implemented by the individual's carer, their family, and the autism team. The needs of autistic individuals vary according to where they fall within the autistic spectrum. Some individuals require a wider range of support than others. Hence, quality standard 51 was developed to ensure the best outcome possible for individuals diagnosed as autistic, based on their personal needs.

Over the past 15 years, the increased awareness of and research into autism has helped broaden the intervention modalities to help with managing

symptoms associated with the condition. However, there is not yet a cure and no specific treatment developed for autism. At the present time, there is little empirical evidence to support the effectiveness of any treatments and/or management modalities used to help minimise the symptoms of autism.

1.5.1 The Current Approach to Treatment of Autism

Psychological interventions are usually implemented in a variety of settings. Some children have intervention in educational settings where behavioural therapy aims to implement strategies of socialisation and integration as well as focusing interpersonal skill development, acquisition of language, play and other skills (Hess et al., 2008).

The private sector also offers group and individual treatment programmes for autistic children where positive reinforcement is used to promote attention and behavioural imitation especially in non-verbal autistic individuals (Levy et al., 2009).

According to Ingersoll et al. (2007), parental mediated interventions have also demonstrated to be an important aspect of behaviour management. It is suggested that parents become positive collaborators in the intervention as long as they learn how to apply the method suggested to their autistic child.

1.5.1.1 Behavioural Intervention Methods

The Early Start Denver Model (ESDM) (Rogers et al., 2003, Rogers et al., 2006, Rodger et al., 2010) is an early intervention approach for children age 12 months to 48 months of age. This intervention has been developed specially for young children, toddlers and preschool autistic children and aims to focus on individual profiles of strengths and weaknesses and on play-based routines to improve parent-child and teacher-child relationships as well as language (Rogers et al., 2006, Dawson et al., 2010). The ESDM model has been shown to have positive results in ASD children after 12 months of 15-25 hours of therapy weekly (Vivanti et al., 2014). Dawson et al. (2010) also suggests a significant improvement in cognition, language skills and adaptive abilities in autistic children after ESDM intervention.

Applied Behaviour Analysis (ABA) (Foxx, 2008) is another type of behavioural intervention that uses positive reinforcement, such as a reward, as a tool to aid behavioural changes. ABA can be applied in different settings such as schools and everyday situations (Foxx, 2008, Mohammadzaheri et al., 2014). Mohammadzaheri et al. (2014) compared the effects of ABA and Pivotal Response Treatment (PRT) and suggested that PRT is more effective in improving social communication. PRT is a behaviour intervention similar to ABA therapy; however it uses preferred toys and activities as stimuli rather than the artificial stimuli such as cards that are used in ABA. Mohammadzaheri et al. (2014) suggests that the use of natural stimuli, creating a more interesting teaching environment, may possibly be the reason why PRT is more effective than ABA. Treatment and Education of Autistic and Communication Handicapped Children (TEACCH) (Schopler, 1994, Panerai et al., 2002) recognises autism as a lifelong condition. It aims to help individuals with autism to live a more independent life, focusing on an individual's strengths and abilities (Schopler, 1994, Panerai et al., 2002).

1.5.1.2 Occupational Therapy

The objective of occupational therapy for autistic individuals is the same as for any other patient who needs help in performing daily tasks and personal care. The treatment is patient centred and varies according to their needs but is usually focussed on enhancing sensory processing issues, behaviour and motor coordination (Rodger et al., 2010, Ashburner et al., 2013, Ashburner et al., 2014).

1.5.1.3 Medication

Medication may be prescribed to autistic individuals either to address co-morbid symptoms such as attention-deficit-hyperactive disorder, and including anxiety and depression or as an adjunct to educational, behavioural and developmental treatments. Recently 'Risperidone' and 'Aripiprazole', both antipsychotic antidepressant drugs approved by the U.S. Food and Drug Administration, have been used in the treatment of irritability in ASD patients (Jesner et al., 2007, Robb et al., 2011). However, as irritability is not a core

symptom of autism, researchers have not yet come into a consensus with regard to the effectiveness of these drugs (Jesner et al., 2007, Robb et al., 2011, Rossignol and Frye, 2014). Other medical symptoms experienced by ASD children such as seizures and tics are usually treated with appropriate medication.

1.5.2 Complementary and Alternative Approaches to Treating Autism

According to Hanson et al. (2007), parents often use complementary and alternative medicine (CAM) to treat autistic symptoms because of concerns about the safety and efficacy of the standard prescription medicines; even though medication has only a minor role in the treatment of autism (Wong and Smith, 2006).

Wong and Smith (2006), investigated the use of CAM therapy in autistic children as compared with typical unaffected developing children. The study found that 52% ($p=0.024$) of the parents of autistic children reported using, or had used, at least one CAM therapy. Parents of autistic children use CAM therapy to treat a variety of symptoms such as: general symptoms (35%), concentration or attention (19%), relaxation (23%), GI problems (15%), sleep disorders (12%), communication/speech (12%), tactile sensitivity (4%), and also to maintain general health (8%) (Wong et al., 2006).

CAM therapies encompass a wide range of philosophies and beliefs. The National Centre for Complementary and Alternative Medicine (NCCAM) defines CAM as ‘a group of diverse medical and health care system practices and products that are not presently to be part of ‘conventional medicine’. NCCAM divided CAM therapies according to the following categories: alternative medical systems (e.g. acupuncture), biological based therapies (e.g. diets), manipulative and body based therapies (e.g. osteopathy and chiropractic) (Wong et al., 2006, Hanson et al., 2007). Examples of these therapies employed during management of ASD are given below.

1.5.2.1 Acupuncture

Acupuncture has been reported to be effective on some of the symptoms of ASD. Acupuncture may influence the neuroplasticity of the brain and thereby have a therapeutic role (Hong et al., 2014, Li et al., 2014).

According to Wong (2010) acupuncture to the tongue may improve language, social communication and cognition in autistic children after one or two courses of treatment. The same author suggests that electro-acupuncture also has a beneficial effect on autistic symptoms (Wong et al., 2014). However, there is no consensus as to the effects of such therapy on autism; necessitating the need for more controlled trials.

1.5.2.2 Massage and/or Touch Therapy

A typical symptom experienced by autistic children is sensory hypersensitivity. Silva et al. (2007) showed improvement in social skills in 13 autistic children of 3-6 years old through following a series of Qigong massage treatments.

Silva and Schalock (2013) also demonstrated that tactile sensitivity improved after a series of Qigong massage treatments and Cullen et al. (2005) demonstrated that touch therapy improved the ability of autistic children to accept touch.

To date, there are no published research papers regarding the effects of other manipulative CAM therapies such as osteopathy and chiropractic on the management of autism.

1.5.2.3 Diet

ASD children may have feeding difficulties and/or may be very selective when choosing what to eat; raising concerns with parents (Williams and Seiverling, 2014). Food allergy and/or sensitivity may be a contributory cause of this food selectivity or avoidance (Shattock and Whiteley, 2002, Shattock et al., 2004).

According to several studies (Elder et al., 2006, Alpert, 2007, Elder, 2008, Hsu et al., 2009, Pennesi and Klein, 2012), a gluten/casein free diet may improve some of the core symptoms of autism.

1.5.2.4 Hyperbaric Oxygen Chamber - Non CAM Therapy

A randomized double-blind controlled trial has been conducted (Rossignol, 2007, Rossignol et al., 2007), and confirmed the positive effects on autistic individuals of hyperbaric oxygen therapy previously reported in anecdotal studies and in studies without adequate controls.

Rossignol et al. (2009) also suggests that 40 sessions of oxygen treatment in a hyperbaric chamber results in the amelioration of receptive language, eye contact and sensory cognitive awareness. Even though the results of the studies are promising, more randomized controlled trials should be performed to validate the effectiveness of this therapy.

The NCCAM do not accept hyperbaric oxygen chamber therapy as part of CAM, even though parents refer to it anecdotally as an alternative or complementary therapy.

1.6 Gastrointestinal Problems in Autistic Children Compared with Unaffected Developing Children

A recent research paper compared the frequency of GI problems between autistic children, children with developmental delay and typically developing children (n=960) (Chaidez et al., 2014). The research suggests that there is a higher frequency of GI symptoms in autistic children and developmentally delayed children, compared with unaffected developing children (Chaidez et al., 2014). The research also suggested a correlation between frequency of GI symptoms and maladaptive behaviour. The lack of research on the frequency of GI symptoms in typical developing children was also highlighted by Chaidez et al. (2014)

A national health survey performed between 2006 and 2010 by the Center for Disease Control and Prevention in the United States concluded that children with learning disability (LD) had a higher prevalence of medical conditions compared with children without LD (Schieve et al., 2012). The LD population included both autistic children and children with concurrent medical conditions, such as GI problems. The study concluded that children with autism were 70% more likely to suffer from colitis/diarrhoea compared with the intellectual disability group, twice as likely to suffer from GI dysfunction than the attention deficit hyperactivity disorder (ADHD) group and seven times more likely to experience GI dysfunction compared with typical developing children (Schieve et al., 2012).

1.6.1 General Paediatric Population: Guidelines for Management of Commonly Encountered Gastrointestinal Symptoms

GI symptoms can be distressing for both children and parents, resulting in anxiety and inability to cope with everyday tasks.

The American Academy of Paediatrics (AAP), NICE, the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition and the British Society of Gastroenterology have formulated guidelines for management of paediatric GI symptoms. The guidelines attempted to clarify common misconceptions, inadequate knowledge or insufficient knowledge amongst professionals. The recommendations formulated to date have been based on individual GI symptoms such as abdominal pain, chronic constipation, acute diarrhoea and oesophageal reflux (American Academy of Pediatrics Subcommittee on Chronic Abdominal Pain, 2005, The British Society of Gastroenterology, 2005, NICE, 2010, NICE, 2014).

1.6.1.1 Abdominal Pain

The American Academy of Pediatrics Subcommittee on Chronic Abdominal Pain (2005) state that:

‘In clinical practice, it is generally believed that pain that exceeds 1 or 2 months in duration can be considered chronic’.

Although abdominal pain may be distressing for the child and the parents, it is usually benign in nature. According to the AAP, most children displaying abdominal pain suffer from functional abnormalities in the enteric nervous system (ENS) also known as 'gut brain' (American Academy of Pediatrics Subcommittee on Chronic Abdominal Pain, 2005, Forsythe et al., 2010). ENS interacts with the central nervous system (CNS) in a bidirectional fashion and regulatory loss of this circuit plays an important role in the pathogenesis of the abdominal pain (Forsythe et al., 2010). Research suggests that abdominal hyperalgesia may be the result of changes in the intraluminal pressure, as well as to inflammatory processes in the gut that may cause sensitization of the afferent nervous system (Mertz, 2002, American Academy of Pediatrics Subcommittee on Chronic Abdominal Pain, 2005, Jyonouchi et al., 2005b).

In the opinion of AAP the most common cause of chronic abdominal pain is functional in nature (American Academy of Pediatrics Subcommittee on Chronic Abdominal Pain, 2005). Pain can be categorized as functional dyspepsia, irritable bowel syndrome, abdominal migraine or functional abdominal pain syndrome. Treatment is recommended to attempt to establish normal gut function. Medications such as antispasmodic agents, smooth muscle relaxants and laxatives may be prescribed for a short period of time and the effect on the pain monitored. According to AAP (2005), parental education and stress reduction play an important role in the treatment of functional abdominal pain (American Academy of Pediatrics Subcommittee on Chronic Abdominal Pain, 2005).

1.6.1.2 Chronic Constipation

The diagnosis of constipation in the general paediatric population is based on clinical signs and symptoms and is defined as a delay or difficulty in defecation, present for more than two weeks. Constipation is a common childhood problem and the prevalence is dependent upon the diagnostic criteria used at the time of the diagnosis. Therefore, the reported incidence may vary between 5 and 30% of the paediatric population (NICE, 2010). The aetiology of constipation is not fully understood but contributory factors are: pain, fever, dehydration, diet and fluid intake, psychological issues, toilet

training, medication and family history. According to NICE (2010) clinical guideline 99, the signs and symptoms of childhood idiopathic constipation are: infrequent bowel activity, foul smelling, excessive flatulence, irregular stool texture, passing enormous stool or frequent small pellets, withholding or straining, soiling, abdominal pain, distension, discomfort, poor appetite, lack of energy, unhappy, angry or irritable mood and malaise. NICE (2010) clinical guideline 99 suggests that autistic children, and those with cerebral palsy and Down's syndrome are more likely to develop chronic constipation.

It is recommended that management of chronic constipation is undertaken according to the severity of the case (Constipation Guideline Committee of the North American Society for Pediatric Gastroenterology and Nutrition, 2006, NICE, 2010). In cases of impacted stools with no red flags, use of medication such as laxatives is advised. Dietary modification and adequate fluid intake are also recommended. If, after going through the recommended guidance, the child is still suffering from symptoms of chronic constipation, enema is the last recommended treatment option (Constipation Guideline Committee of the North American Society for Pediatric Gastroenterology and Nutrition, 2006, NICE, 2010).

1.6.1.3 Acute Diarrhoea/ Gastroenteritis

The British Society of Gastroenterology (2005) compiled guidelines on the diagnosis, treatment and management of chronic diarrhoea. According to these guidelines, chronic diarrhoea may be defined as:

'the abnormal passage of three or more loose or liquid stools per day for more than four weeks and/or daily stool weight greater than 200g/day'.

The patients should be screened for infections via blood testing and stool analysis. Patients should also undergo investigation for laxative abuse, and, according to the guidelines, this should be part of the early investigation. Intervention should follow the investigation and rehydration is an important part of management (Sandhu et al., 2001, The British Society of Gastroenterology, 2005).

1.6.1.4 Irritable Bowel Syndrome (IBS) - Functional Gastrointestinal Disorder

The ROME Committee (ROME III, 2006) defines IBS in children as abdominal discomfort or pain that improves with defecation and its onset is associated with frequency of stool and with change in faecal form. These symptoms must not be associated with evidence of inflammatory, anatomic, metabolic or neoplastic processes. Diagnosis is reached through taking a careful case history; not only from the patient but also from the parents or carers.

1.6.1.5 Reflux

Reflux is the passage of gastric contents into the oesophagus. Clinical manifestation in the paediatric population includes the following symptoms: poor weight gain, vomiting, dysphagia, abdominal or substernal pain, oesophagitis and respiratory disorder (Rudolph et al., 2001). Diagnosis of reflux through physical examination of infants and older children and taking a case history is sufficient to initiate management of the condition; according to the North American Society for Pediatric Gastroenterology and Nutrition (2001). Management may be complicated by anatomical abnormalities such as pyloric stenosis, hiatus hernia and oesophageal stricture. Management of straightforward cases may involve dietary changes and sleep repositioning with elevation of the head and chest in a supine position in infants or left side positioning in case the older children. Acid suppressants may be recommended such as proton pump inhibitors (PPIs). Surgery may be necessary in the case of anatomic abnormalities (Rudolph et al., 2001).

1.6.1.6 Inflammatory Bowel Disease – IBD

The guidelines formulated by the British Society of Paediatric Gastroenterology Hepatology and Nutrition (BSPGHAN) on inflammatory bowel disease (Crohn’s disease (CD) and ulcerative colitis (UC)) in the paediatric population (Sandhu et al., 2010) provide a consensus based on evidence of the diagnosis, treatment and management of the condition. Guidelines advise that laboratory investigations such as blood tests, stool

culture and levels of biochemical markers, such as calprotectin and lactoferrin, may become important tools for diagnosis and monitoring of the condition. BSPGHAN advocate investigations such as endoscopy and colonoscopy to confirm the diagnosis in children suspected of having CD and UC.

The treatment for IBD consists of enteral nutrition and administration of corticosteroids to induce and maintain remission.

1.6.2 Autistic Paediatric Population: Recommendations for Management of Commonly Encountered Gastrointestinal Symptoms

In 2010, the American Academy of Paediatrics issued recommendations for primary care providers for the management of GI problems in ASD children (Buie et al., 2010). These recommendations were compiled after collating the suggestions of clinical paediatric gastroenterologists with experience in caring for autistic children. This was an attempt to raise awareness of the management of GI conditions in the paediatric autistic population (Buie et al., 2010). However, evidence based guidelines for management and treatment of GI conditions in autistic children are yet to be formulated.

The quality standard 51 (NICE, 2014) recommends that people who are referred for autistic diagnostic assessment are also assessed for coexisting health and mental conditions. This is to avoid coexisting conditions going unrecognised and/or untreated. Quality standard 51, recommends that physical assessment of autistic individuals should include consideration of GI conditions such as constipation, altered bowel habits and faecal incontinence that could potentially trigger challenging behaviours in this population.

The recommendations for management and treatment of GI problems in autistic children have been divided into four main categories; chronic abdominal pain, constipation, chronic diarrhoea and oesophageal reflux (Buie et al., 2010). The primary care practitioner can evaluate GI disorders in autistic children. However, assessment and diagnosis may be challenging. Unpredictable behaviour displayed by some autistic individuals, along with lack of verbal communication and aggressive behaviour such as biting,

pushing spiting and screaming are limitations to the examination process (Buie et al., 2010).

1.6.2.1 Chronic Abdominal Pain

In cases where verbal communication is a limitation factor, the care provider should be aware of behaviours that may indicate abdominal discomfort; such as pressing or tapping on the abdominal area. Changes in sleep patterns, self-injurious behaviour and aggression may also be indicative of pain. Parental education is an important factor on management of chronic abdominal pain and parents should be informed that, although pain is real, it may not be indicative of serious disease (Buie et al., 2010).

The recommendations for autistic children with alarming abdominal pain follow the same advice as the guidelines for unaffected developing children with similar pain. Further testing is advised in cases of involuntary weight loss, deceleration of growth, significant vomiting and chronic or severe diarrhoea.

1.6.2.2 Constipation

Sensory processing abnormalities may cause stool withholding in ASD children. Buie et al. (2010) states that autistic children may suffer from stool retention, unnoticed by parents, teachers or carer, as well as to determine whether the condition is functional or organic, a thorough case history should be taken in conjunction with a physical examination of the abdomen (look for distension, palpable liver or spleen and palpable mass) and the rectum (check the rectum tone for stool impaction). Challenging behaviour displayed by some ASD patients means that a rectal examination may not be feasible. Therefore, the rectal tone may not be assessed to help identify stool retention and occult mass. In these cases, it is recommended that an X-ray of the abdominal area be taken. However, a systematic review by Reuchlin-Vroklage et al. (2005) yields conflicting results regarding the use of radiography to diagnose stool impaction. Pharmacotherapy for treatment of constipation in ASD patients is generally the same as for unaffected children. Behavioural management such ABA, TEACH or ESDM may also be useful as a

therapeutic tool for the treatment of constipation in ASD children. See section 1.5.1.1- Behavioural Intervention Methods.

1.6.2.3 Chronic Diarrhoea

There are, as yet, no clinical guidelines for the evaluation and treatment of chronic diarrhoea in ASD children. The standard paediatric approach is used for ASD children. Food allergies, celiac disease, parasites and IBDs (CD and UC) are the most common causes of chronic diarrhoea. Relevant diagnostic tests should be performed. In an attempt to raise awareness of the incidence of GI issues in the autistic population, Buie et al. (2010) recommends that clinicians should exercise particular care in clinical judgement when treating ASD patients with chronic diarrhoea.

1.6.2.4 Gastroesophageal Reflux Disease (GERD)

The same clinical and practical guidelines used for the general paediatric population suffering from GERD are recommended for ASD children. The differences lie in the behavioural changes seen in ASD children when in pain. ASD children may express discomfort through erratic, aggressive and/or self-harming behaviour. Clinical examinations such as barium enema and endoscopic examination using a nasal probe are challenging for ASD children. Such investigations should therefore, be carried out under general anaesthesia. In the case of strictures, mal-rotation or oesophageal cancer, treatment of ASD children should follow the same guidelines as have been put in place for the general paediatric population.

1.7 Epidemiology

Epidemiological studies show that the concordance in monozygotic twins is significantly greater than that in dizygotic twins, and it is a significant genetic factor in diagnosing infantile autism. The published ratio of male to female cases ranges from 2:1 to 4:1 (Wing, 1993b), and the total prevalence of autism ranges from 2 to 5 per 10,000 individuals (Aarons and Gittens, 1999), although this number can vary from 3 to 16 per 10,000 according to the

diagnostic criteria used within a study (American Psychiatric Association, 1994). It is suggested that an improvement in the diagnostic procedure and a greater awareness of the condition may have influenced this reported increase (Kurita, 2006, Williams and Brayne, 2006), although the considerable rise in incidence cannot be explained by improved diagnostic criteria alone (Hertz-Picciotto and Delwiche, 2009).

A review to clarify the reasons for the differences in reported findings was undertaken in 1993 by The Centre for Social and Communication Disorders (CSCD) in Kent. The CSCD gathered information from 16 epidemiological studies over a 25 year period, which used Kanner and Rutter's diagnostic criteria and the 3rd edition of the DSM (Wing, 1993b). These sets of criteria for the diagnosis of infantile autism have commonalities as they include social interactions and communication, and repetitive stereotyped behaviour as essential factors. Each of the 16 studies also covered geographically defined populations. The conclusion of the CSCD study revealed a strong possibility that individual interpretations of the diagnostic criteria may have caused discrepancies affecting the epidemiological results. According to the CSCD study, it was not possible to determine either precise male to female ratios or the prevalence of ASDs per 10,000 individuals (Whitehouse, 2013). This finding may reflect an implied geographical or socio-economic influence in this condition (van der Ven et al., 2013).

1.8 Clinical Features of Autism

1.8.1 Onset

According to the DSM-IV (American Psychiatric Association, 2000) and the majority of clinicians and researchers, the abnormal development of autistic individuals is most apparent before the ages of 30 - 36 months (Wing, 1993b, Wing, 1998, American Psychiatric Association, 2000). The severity of the symptoms may become less immediately obvious during the pre-school years and resistance to being touched and held may decrease with age. Even so, the inability to play reciprocally, to treat humans as humans rather than objects, and distant behaviour may persist into adulthood (Seltzer et al., 2003).

1.8.2 Behaviour

The Centres for Disease Control and Prevention (CDC) advocate that children should be screened to assess their natural development using a series of normal milestones that are classified into four areas: social and emotional; language and communication; cognitive and movement/physical development (Council on Children With et al., 2006). Studies suggest that children are equipped with primitive responses that help them integrate into society from birth (Farroni et al., 2002, Simion et al., 2007, Vouloumanos et al., 2010) and problems in these primitive areas can be classified as delayed or abnormal. One of the most apparent symptoms in autism is the failure to reach social and emotional milestones, resulting in the adoption of abnormal behaviours. Several studies describe the main behavioural symptoms of autistic children as the failure to develop eye contact, the failure to develop social interaction, and the failure to develop communication skills (Gillberg and Coleman, 1992, Wing, 1997, Wing, 1998, Tanguay, 1999, American Psychiatric Association, 2000, Wing, 2002, World Health Organization, 2004).

1.8.3 Communication

Individuals who fit the DSM-IV and ICD-10 diagnostic criteria for autistic disorders may experience delayed onset in speech development (Gillberg and Coleman, 1992, American Psychiatric Association, 2000, World Health Organization, 2004). Subjects may present with communication abnormalities; such as echolalia (parrot-like copying of other people's speech) or delayed echolalia (repetition of words in a stereotyped way) (Wing, 1997). The rhythm and melody of speech may also be altered, with the intonation of the voice being high-pitched or monotonous ('robotic' speech) (Wing, 1998). Some autistic children develop a large vocabulary, and whilst this may not be used for conversation with other people, they may learn to follow instructions given in a familiar social context (Wing, 1997); however, they may not understand the same instruction if the social context is changed. Autistic individuals may become confused with personal pronouns and frequently there is the substitution of 'I' for 'you' (Rapin and Dunn, 1997, American Psychiatric Association, 2000, World Health Organization, 2004). They may also have

difficulty interpreting other people's tone of voice, body posture and facial expression during a conversation (Tanguay, 1999), and may fail to initiate, pursue, or terminate conversations (Rogers et al., 2003).

Some children demonstrate a compromised ability to decode verbal stimuli; resulting in verbal auditory agnosia, meaning that they do not understand verbal languages and thus fail to acquire speech (Klein et al., 2000). A recent study suggested that a deficit in communication may appear in infants who are later diagnosed as autistic as early as six months of age (Shic et al., 2014). According to Shic et al. (2014), autistic children may not be able to regulate their responses to social stimuli and they present with a marked reduction in attention to social facial expression, either static (non-verbal) or dynamic (verbal). This lack of attention may later affect the normal development of communication skills.

1.8.4 Impairment of Imagination and Repetitive Stereotyped Activities

Autistic children do not develop 'pretend' play and imaginative activities in the same way as normal children; toys are purely objects that provide a physical sensation (Wing, 2002). Children may appear to be engaged in imaginative activities but prolonged observation reveals that they are repeating the same sequence of events over and over again, and at times are merely repeating games from a television programme or stories that have been read to them (Rapin and Dunn, 1997, Wing, 1997, Wing, 1998, Wing, 2002). Other forms of repetitive activity involve staring at lights, twisting and turning objects, and switching lights on and off (Gillberg and Coleman, 1992).

1.8.5 Sensory Motor Symptoms

Ming et al. (2007) assessed the prevalence of motor impairment in 154 ASD children of between 4 and 18 years old, and suggested that motor impairment is more frequent in ASD children compared with children developing normally. Autistic individuals may also present with a deficit in sensory motor control, with a delay in movement execution, joint laxity, hypotonia, clumsiness and apraxia (the inability to make skilled movements with accuracy) (Stoit et al., 2013).

Reduced sensory motor control features in ASD may include abnormal posture and reduced coordination of the upper limbs, as well as clumsiness and a lack of balance. The unstable gait of autistic individuals is due to a lack of symmetry and a reduced stride length (Gillberg and Coleman, 1992, Page and Coleman, 1998, Jansiewicz et al., 2006). The prevalence of sensory motor symptoms as described by Ming et al. (2007), range from toe walking, finger flicking, flapping of arms and hands, running in circles and self-injurious behaviour (e.g. head banging and biting), all of which become more apparent when a child becomes excited. Ming et al. (2007) found hypotonia to be the most common symptom, affecting 51% of the cohort studied; however, this symptom was less apparent in older autistic children, suggesting that hypotonic symptoms may improve over time.

1.8.6 Epilepsy

Epilepsy is the abnormal discharge of neurons within the brain and it occurs more frequently among autistic and mentally handicapped children than in the general population (DeLong, 1999, Besag, 2002). About a third of autistic children will have at least two unprovoked epileptic seizures before reaching adulthood (Rapin and Dunn, 1997), and their onset in autistic individuals may be suggestive of brain dysfunction and intellectual compromise (Tuchman and Cuccaro, 2011, Robinson, 2012). The frequency of epileptic fits may increase at puberty, possibly due to hormonal changes, possibly resulting in the regression of developmental features already acquired (Brooks-Kayal, 2010, Tuchman and Cuccaro, 2011).

1.8.7 Gastrointestinal Symptoms

Patients with an autistic disorder may present with GI symptoms, such as abdominal distension and pain, constipation, chronic diarrhoea, foul-smelling stools and/or flatulence (Shattock and Savery, 1996, Lewis, 1998, Jyonouchi et al., 2005a, Jyonouchi et al., 2011). Recent studies suggest that a cognitive deficit in autistic children may be linked to these GI symptoms (Horvath, 2000, Horvath and Perman, 2002, Koves et al., 2004, Valicenti-McDermott et al., 2008). Nikolov et al. (2009) reported a link between the worsening of behavioural symptoms and GI symptoms, specifically diarrhoea and

constipation. The same study also reported that autistic children suffering from GI problems have a tendency to present with higher levels of irritability, anxiety, and are more socially withdrawn compared with autistic children without GI symptoms (Nikolov et al., 2009). A consensus paediatric report undertaken by the Paediatric Gastrointestinal Unit in Boston, USA, concluded that the behaviour of children with autism may have an underlying medical condition, that could be GI in nature (Buie et al., 2010).This issue will be discussed in greater detail in section 1.10, Autism and the Gastrointestinal Link.

1.8.8 Toxicity or Allergic Symptoms

Food containing gluten and/or casein appears to be the main allergen affecting autistic children (Lewis, 1998, Shattock and Whiteley, 2002, Shattock et al., 2004). A recent report comparing autistic individuals (with or without GI complaints) with aged matched healthy controls suggested that autistic individuals (with or without GI symptoms) possess increased levels of IgG antibodies to gliadin; therefore, this does not exclude the possibility of an increased prevalence of celiac disease amongst autistic individuals (Lau et al., 2013).

Some authors suggest that autistic individuals may present with abnormal gut permeability, leading to ‘leaky gut syndrome’ and behavioural disorders (Shattock and Savery, 1996, Shattock and Whiteley, 2002, Shattock et al., 2004, Jyonouchi et al., 2005a, Jyonouchi et al., 2005b). It is suggested that peptides derived from gluten and casein containing foods may ‘leak’ through an abnormally permeable gut epithelium and then enter the central nervous system (CNS), thereby generating abnormal physiological symptoms, such as behavioural changes, mood swings and GI symptoms (Shattock and Whiteley, 2002, Shattock et al., 2004, Jyonouchi et al., 2011). See summary of autism symptoms Table 1-2.

Table 1- 1-2: A Summary of the Symptoms of Autism

Behaviour	Failure to develop eye to eye contact; failure to develop social interactions; failure to develop communication skills; lack of pretend play and imaginative activities; repetitive stereotyped activities (staring at lights, twisting and turning objects and switching lights on and off over and over again).
Communication	Echolalia (parrot-like copying of other people's speech); altered rhythm and melody of speech; high-pitched or monotonous speech, confusion over use of personal pronouns (substitution of 'I' for 'you'); failure to initiate, pursue, or terminate conversations; difficulty interpreting other people's tone of voice, body posture and facial expression during a conversation; failure to acquire speech.
Sensory Motor	Joint laxity and hypotonia; clumsiness and apraxia (inability to make skilled movements with accuracy); toe walking; finger flicking; flapping of arms and hands; running in circles; self-injurious behaviour.
Neurologic	A third present with epilepsy
Gastrointestinal	Abdominal distension and pain; constipation; chronic diarrhoea; foul-smelling stools; and gaseousness.
Toxic/Allergic	Food intolerance; sensitivity to food containing gluten and/or casein.

Compiled from (World Health Organisation, 1992, American Psychiatric Association, 1994, Wing, 1997), Tanguay (1999).

1.9 Aetiological Hypothesis

Several theories concerning the cause of autism have been suggested. A controversial aetiological hypothesis defining autism in psychological terms was first proposed by Kanner and Eisenberg (1957). Kanner initially suggested that autism was a primary response to a lack of maternal warmth, but this hypothesis is unsubstantiated and has not been supported (Kanner, 1943).

A more recent study hypothesises that there are two forms of autism. The first is as a result of bilateral brain damage during early life, possibly involving the temporal lobes and resulting in dysfunction in language, social skills, and organised activity. The second is an idiopathic form, based on Kanner's original description of autism, that is also related to familial psychopathology and low serotonin levels in the left hemisphere of the brain, resulting in symptoms such as irritation, anxiety, withdrawal from social contact, diminished cognition and attention (DeLong, 1999). According to DeLong (1999), a high functioning autistic individual may have a disease process affecting only one hemisphere, whilst a low functioning individual may have low serotonin synthesis in the left hemisphere, resulting in a wide functional deficit of cortical association areas and thus causing a profound cognitive disability (DeLong, 1999).

1.9.1 Genetics

The sibling recurrence figures of autism are between 2% and 5% (Aarons and Gittens, 1999), and this percentage has led to investigations into the possibility of the condition having a genetic cause. One genetic study has claimed an association between chromosomal abnormalities and an autistic phenotype (DeLong, 1999), involving a duplication and deletion of the 15q chromosomal region (Waterhouse, 2000). Cook et al. (1998) suggested that there is disequilibrium between an autistic disorder and the GABRB3 155CA-2 gene, that is located on chromosome 15q11-13. However, a further study that recruited a large number of families with at least two children diagnosed as autistic, gave conflicting results (Rish et al., 1999). This later study, used the DSM-IV diagnostic criteria and did not identify a chromosomal region with significant evidence of linkage, but instead indicated that the most likely location for some genetic abnormality would be on chromosome 17q (Rish et al., 1999). Wang et al. (2009) analysed 10,000 subjects with European ancestry and identified genetic variants on chromosome 5p14.1, which affects the morphology and function of the brain, possibly resulting in the clinical features of autism. There is clearly a need for more studies in this area to clarify whether or not autism has a genetic component.

1.9.2 Allergies

Ongoing investigations are looking at a possible link between allergies and autism (Shattock and Savery, 1996, Shattock and Whiteley, 2002, Shattock et al., 2004, Jyonouchi et al., 2005b, Valicenti-McDermott et al., 2006, Valicenti-McDermott et al., 2008, de Theije et al., 2014). The theory of allergy-induced autism focuses on testing various family members of autistic individuals who have a history of asthma, eczema, hay fever and migraines (Srinivasan, 2009, Lyall et al., 2013), and autistic behaviour is considered to be the result of an intolerance to certain foods and/or chemicals (Shattock and Savery, 1996, Shattock and Whiteley, 2002, Shattock et al., 2004). Such food intolerances may bring on symptoms such as bad catarrh, diarrhoea, bloating, stomach pains, asthma and possibly petit mal epilepsy and deterioration of behaviour. There is evidence that children suffering from autism have a lack of the enzyme phenol sulphur transferase (PST) (Alberti et al., 1999, Harris et al., 2000). This enzyme is involved in the metabolism of hormones, neurotransmitters and a range of toxic molecules (Alberti et al., 1999, Harris et al., 2000), and a lack of PST may lead to leaky gut syndrome, where improperly metabolised proteins could escape into the bloodstream (Shattock and Savery, 1996, Shattock and Savery, 1997, Shattock and Whiteley, 2002, Shattock et al., 2004). Other studies support this hypothesis and add that, if PST is dysfunctional, then the body would accumulate abnormal levels of serotonin, dopamine and noradrenaline (Rimland and Baker, 1996). In addition, phenolytic compounds, which are present in foods containing artificial colourings, preservatives and salicylates, can disrupt metabolic processes resulting in physical symptoms that are also associated with autism, such as excessive thirst, night sweating, facial flushing and reddened ears (O'Reilly and Waring, 1993, Alberti et al., 1999). Each of these metabolic disruptions may lead to abnormal biochemical actions, particularly in the brain (O'Reilly and Waring, 1993, Rimland and Baker, 1996).

Le Couteur et al. (1988) and Shattock and Savery (1996) supported the hypothesis that autism may be the consequence of the action of opioid peptides of exogenous origin, which affect neurotransmission within the CNS, disrupting cognition, mood and behaviour. Whiteley et al. (1999) claimed that

increased levels of peptides may be the result of the incomplete breakdown of foods containing gluten (found in wheat, barley, rye, etc.) and casein (found in milk and dairy products) (Shattock and Savery, 1996). Together with an increased permeability of the gut wall, it is possible that opioid peptides cross into the bloodstream, and these toxic metabolites may easily reach the CNS and possibly cross the blood brain barrier, resulting in the abnormal behaviour demonstrated by autistic individuals (Gillberg, 1995, Shattock and Savery, 1996, Shattock and Savery, 1997, Shattock and Whiteley, 2002, Shattock et al., 2004).

1.9.3 Environmental Toxicity

A recent meta-analysis suggested a potential association between ASDs and environmental toxins (Rossignol et al., 2014). Rossignol et al. (2014) supported the idea that some individuals may be more susceptible to genetic and environmental factors during periods of neurodevelopment, which in turn may contribute to the development of ASDs. However, this systematic review demonstrated that several of the studies were poorly designed, thereby prompting the need for more and better epidemiological studies to be conducted.

1.9.4 Gastrointestinal

One study analysed the structure and function of the upper GI tract of 36 patients with autism who also presented with GI symptoms (Horvath et al., 1999). Of the cases studied, 25 showed signs of reflux oesophagitis, 15 chronic gastritis, and 24 chronic duodenitis. Twenty-two of the 25 children with reflux oesophagitis also had symptoms such as night-time awakening, with irritability and signs of abdominal discomfort (or pushing on the abdomen), similar to those typically reported by non-autistic children suffering from reflux oesophagitis. Fifteen of these children also presented with chronic inflammation of the gastric mucosa and 21 had low levels of carbohydrases, a possible cause of flatulence and diarrhoea. The study concluded that these findings may contribute to the evidence of GI involvement in behavioural problems of non-verbal autistic subjects (Horvath et al., 1999).

An interesting anecdotal case study has led to a new avenue of research into ASDs and further evidence of GI involvement. An autistic boy, who was non-responsive, non-verbal, not toilet trained and sleeping poorly, was referred for endoscopy to investigate the cause of his stomach problems (Rimland and Baker, 1996, Rimland, 1998). Within days of the procedure the boy showed remarkable signs of recovery, such as an improvement in eye contact, calmer behaviour and improved sleeping and continence (Rimland and Baker, 1996, Rimland, 1998). The only explanation for both the behavioural and GI symptom changes was postulated to be the pharmacokinetics of the anaesthetic used during the procedure (Rimland and Baker, 1996, Rimland, 1998). The anaesthetic contained the hormone 'secretin' extracted from porcine pancreas. The boy's response to the endoscopy procedure led researchers to investigate further the effects of intravenous secretin infusion on children suffering from ASDs.

This dramatic improvement in behavioural symptoms in the boy who received the dose of anaesthetic containing secretin may be explained by the relationship of secretin to its biochemical family members, particularly vasoactive intestinal peptide (VIP) (Bayliss and Starling, 1902). Studies have suggested that increased levels of secretin in the bloodstream stimulate VIP binding site receptors within the brain. This activation may produce a positive effect and regulate energy levels in memory-forming neurons. However, while a single case is insufficient to establish a direct behavioural and GI response link to secretin infusion, nevertheless, a series of studies using secretin infusion in autistic children have transpired.

One such study compared three ASD children with non-autistic children following intravenous administration of secretin (Horvath et al., 1998). The study reported that, within five weeks of the secretin infusion, there was an increase in pancreaticobiliary fluid and amelioration of GI symptoms, social behaviour and communication in the children with ASD, Horvath et al. (1998) suggested that the GI symptoms and a physiological lack of secretin possibly play a role in the pathogenesis of the disease, whilst conversely, Owley et al. (2001) reported a lack of efficacy for secretin infusions on the behaviour of

ASD children after a randomised placebo-control trial. Williams et al. (2012) reviewed 16 randomised studies with placebo groups on the use of secretin infusion in ASD patients. These studies found no evidence that either single or multiple secretin infusions ameliorated the symptoms of ASD.

Although autism has been the focus of many investigations over recent decades, it remains a disorder with no clear aetiology and no effective treatment. Some hypotheses focus on the possibility of autism being linked with GI abnormalities (Horvath et al., 1999, Horvath and Perman, 2002, Chen et al., 2010, Chen et al., 2011), whilst others have claimed that the underlying GI dysfunction in autistic children is characteristic of the immunological and inflammatory dysfunctions seen in patients suffering from inflammatory bowel disorders (IBDs) (Horvath et al., 1999, Furlano et al., 2001, Torrente et al., 2002). Jyonouchi et al. (2005a) confirmed this in their study which indicated that there was an intrinsic defect of the innate immune response suggesting a possible link between GI and behavioural symptoms mediated by immune responses. There is growing awareness of the possibility of a link between gut metabolic reactions and abnormal behaviour, indicating that the 'gut-brain axis' may be central to neural development (Reichelt and Knivsberg, 2009). These studies provide a basis for further research into the relationship between the brain and the GI system, the so called 'brain-gut' or 'gut-brain' axis and its role in neurological disorders.

Autism remains a syndrome with no clear aetiological explanation, although there have been attempts to determine the cause of the disorder by different professional groups. Psychologists are tempted to explain the disorder based on their understanding of the mind, geneticists rely on their understanding of human genes, whilst similarly, nutritionists, physiologists, neurologists, paediatricians and GI specialists all seek explanations pertaining to their own field. Specialists have given noteworthy insights into the disorder, but a satisfactory aetiological explanation of the condition still remains beyond our current understanding. Perhaps, a more holistic approach, using combined professional expertise, may facilitate the formulation of an accurate aetiological theory.

1.10 Autism and the Gastrointestinal Link

Anecdotal reports from parents of autistic children with GI abnormalities in have been reported for some time; however, it was not until the late 1990s when D'Eufemia et al. (1996) published a paper suggesting an association between GI abnormalities and autism that the research community began to study a possible link. It has since been suggested that imbalances or abnormalities in the GI system may possibly have a direct influence on the abnormal behaviour of children diagnosed as autistic (D'Eufemia et al., 1996, Horvath et al., 1999, Ibrahim et al., 2009, Nikolov et al., 2009).

The mechanism of this connection is not yet clear, although it has been suggested that the enteric nervous system with its neuro-regulatory pathways may play an important role in this intrinsic puzzle (Furness, 2000).

1.10.1 The Brain-Gut axis Anatomy

Neural information travelling from the brain to the gut and from the gut to the brain use the terms “gut-brain” and “brain-gut” interchangeably.

The GI system has its own nervous system, the enteric nervous system (ENS), consisting of a complex web of neurons connected to the CNS via the vagus nerve. The vagus nerve contains sensory and motor fibres and is the longest cranial nerve. It exits the skull via the jugular foramen and passes through the neck, thorax and abdomen to synapse with the ENS (Powley, 2000, Drake et al., 2010). It is suggested that the complex ENS neuronal circuit may affect CNS operations via vagus afferents creating a bidirectional circuit, that may also flow from the CNS and affect the ENS (Powley, 2000, Mayer, 2011).

The ENS is part of the autonomic nervous system and can operate independently of the brain and spinal cord; it has been referred to as a ‘second brain’ (Bayliss and Starling, 1899, Gershon, 1999). The neurons of the ENS are divided into two types: the myenteric plexus and submucosal plexus (Furness, 2000). The myenteric plexus is located between the circular and longitudinal layers of the muscularis externa and contains both

parasympathetic and sympathetic nerve fibres that regulate motility of the GI tract (Furness et al., 1999, Furness, 2000, Snell, 2010). The submucosal plexus is located in the submucosa, between the circular muscular layers of the muscularis mucosae and has a secretory controlling function (Furness, 2000, Clerc et al., 2002).

The ENS operates as a parallel circuit regulating somatic, autonomic, neuroendocrine, and pain associated with stress and emotional states (Mayer and Tillisch, 2011).

1.10.2 ENS Neurons

The intrinsic primary afferent neurons (IPANs), the interneurons and the motor neurons are a set of neurons responsible for the coordinated autonomic activity of intestinal motility (Furness et al., 1999, Furness, 2000, Bornstein et al., 2004, Lomax et al., 2005).

1.10.2.1 IPANs

IPANs are neurons embedded within the gut wall that generate intrinsic reflexes, resulting in the propulsive movements of the gut muscles, changes in the blood flow of the gut and modulation of the secretion of water and electrolytes (Furness, 2000, Clerc et al., 2002). IPANs react to three types of stimuli; chemical changes in the intestinal lumen, mechanical distension of the mucosa, and mechanical force in the external musculature via muscle stretch or contraction (Kunze et al., 1999, Clerc et al., 2002).

1.10.2.2 Interneurons

Interneurons are responsible for the propulsive reflexes within the gut, and transmit ascending and descending information within the ENS (Bornstein et al., 2004).Enteric motor neurons can be divided into four types; excitatory muscle motor neurons, inhibitory muscle motor neurons, motor neurons to the muscularis mucosae and motor neurons to the striated muscle of the oesophagus (Furness, 2000).

1.10.3 The ENS or 'Second Brain'

The vagus nerve provides a steady transmission of signals between the brain and the gut, and signals from the gut to the brain maintain homeostasis and allow responses to changes caused by disease. The gut sends warning information to the brain regarding contaminated foods, chemical poisoning, allergens and pathogens, and the brain responds with well-known symptoms, such as nausea, abdominal pain, dizziness and vomiting (Gershon, 1999). Visceral pain is poorly localised through small diameter unmyelinated fibres and thinly myelinated fibres that convey both visceral and somatic pain sensations via the dorsal horn to the spinal cord, resulting in viscero-somatic reflexes. Information from the viscera also travels to the CNS via the vagal visceral afferent pathway (Grundy, 2004). These afferent signals regulate gut motility, secretion and blood flow.

This well-orchestrated system is part of the integrated physiology for the maintenance of health; however, it is not yet fully understood which of these 'brains' is leading the signalling information. According to some studies, conditions such as an ulcer may have their root cause within the CNS, as anxiety could potentially influence GI function and its effects may result in damage to the lining of the gut (Mayer et al., 2006, Goehler et al., 2007). Psychological stresses may potentially threaten GI homeostasis by affecting the gut-brain axis, consequently becoming a precursor of functional GI diseases, such as IBDs and IBS. Konturek et al. (2011) state that several areas of gut physiology could be affected by stress, including gut mobility, GI secretion, gut permeability, blood flow in the gut mucosa, and visceral sensitivity. These factors were also implicated in a study on the effects of stress on functional dyspepsia, where the authors suggested that psychological distress is directly linked to GI symptoms (Aro et al., 2009). According to Blomhoff et al. (2001), the co-morbidity of the GI system and anxiety has a direct effect on severity and duration of IBS symptoms, possibly explaining the mind and body relationship. Blomhoff et al. (2001), showed that increased activity in the gut-brain axis affects the ENS and the CNS nerve receptors and it seems that phobic anxiety hyper-activates the visceral frontal region of the cortex influencing IBS symptomatology (Blomhoff et al., 2001).

The gut-brain axis has also been proposed in a recent study where depression-like symptoms were induced in mice. According to Park et al. (2013), symptoms of depression alter both colonic motor activity and the microbial profile, most likely via the hypothalamic-pituitary axis.

A variety of psychological symptoms have been reported, such as changes in mood and cognition that may be a result of ENS imbalances; consequently, the root cause of some behavioural disorders could potentially be the GI system rather than the CNS. Infection and inflammation of the GI system may also affect mood (Goehler et al., 2007); therefore, it is possible that epigastric symptoms resulting from pathogens could generate anxiety, rather than the other way round (Gershon, 1999, Goehler et al., 2007). A study using a mouse model suggested that alteration in microbial composition through dysbiosis, may potentially contribute to psychiatric disorders. The study suggested that changes in the normal microbial balance in healthy mice may alter their brain chemistry, thus altering their behaviour. The authors further suggested that the gut-brain axis influences the brain biochemistry in mice and is directly linked to behavioural changes (Bercik et al., 2011). The same observation has also been described in other studies using animal models that have implicated infection and inflammation of the gut in behavioural changes, such as anxiety; which is mediated via vagal sensory neurons (Goehler et al., 2005, Lyte et al., 2006). Anxiety may be a warning signal of a GI infective state in mice Goehler et al. (2007).

The information emanating from the brain to the GI and from the GI to the brain is very much interconnected, and imbalances within this bi-directional axis may disrupt the ENS and the CNS, resulting in GI and behavioural dysfunction (Mayer, 2011). However, the mechanism of this gut-brain intercommunication and the potential effects of its imbalance is, as yet, poorly understood. Some studies suggest that the constant passage of information to or from the ENS may sensitise specific nerve fibres to targeted organs and induce neuroplastic changes. In turn, these sensitised pathways may be induced by psychological stresses, such as mood changes, stress and

anxiety, leading to chronic symptoms, even though no pathogen is present (Mayer and Tillisch, 2011).

Understanding the neuroplasticity of the ENS may help clarify the pathophysiology of GI diseases and guide therapeutic mechanisms (Vasina et al., 2006). According to Lomax et al. (2005), neuroplasticity is a feature of inflammation and is also of paramount importance in the restoration of gut function. It may be possible that the adaptive changes within the neural circuit can be set to help the body to regain its homeostatic balance. A review by Mawe et al. (2009), shows that neurophysiological changes are only part of the picture in the alterations that occur within the gut during inflammation. However, understanding the neuroplastic changes within the ENS may help to identify therapeutic means with which to restore gut function (Katsui et al., 2009, Mawe et al., 2009).

1.10.4 Autistic Behaviour and the Connection to the Gut

Resonating with the research outlined above, recent studies have demonstrated a strong connection between the ENS and autistic behaviour. A recent report suggested that the disturbed behaviour of autistic children may have an underlying medical condition, including GI disorders (Buie et al., 2010). The report recommended conducting a systematic GI investigation in autistic children similar to that used in non-autistic individuals when they present with GI symptoms (Buie et al., 2010).

D'Eufemia et al. (1996) published one of the first papers to suggest that autistic children have an imbalance in intestinal permeability. Follow up studies suggested a possible interaction between the damaged gut and the brain of an autistic individual. Such an interaction occurs through lymphocytes and cytokines crossing the damaged lining of the gut into the circulation and then passing across the brain-blood barrier into the brain itself (Furlano et al., 2001, Torrente et al., 2002, Ashwood et al., 2003, Lau et al., 2013). ENS neurons and the enteric glial cells are important for the motility of the gut and also essential in maintaining the homeostatic balance, and influencing motility and inflammatory processes (Bassotti et al., 2007). According to de Theije et

al. (2011) the gut-brain axis interconnection is via an inflammatory process that may change the neural homeostasis and result in the exacerbation of the severity of autistic behaviour.

The so called 'gut-brain axis' within autism has been reviewed by White (2003), who suggested that the GI pathology may be central to the aetiology of autism. Another study concluded that GI symptoms may be a co-morbidity of autism, and some of the autism behavioural symptoms may be the result of the interaction between the brain and GI function (Valicenti-McDermott et al., 2006). The bidirectional axis that has been suggested between the brain and gut could potentiate or even generate behavioural symptoms in autistic children. In turn, this could influence neuroplastic changes in either the CNS or non-neuronal elements (Gershon, 1999, Dong and Greenough, 2004, Vasina et al., 2006). The possibility of a gut-brain axis co-morbidity in autism has only recently been postulated but it is receiving increasing research attention.

1.11 Markers used to Assess Gastrointestinal Inflammation

1.11.1 Faecal *Markers Selection*

Kings College Hospital, a centre of excellence for measuring faecal biochemical markers, was approached by the researcher to discuss the use of gastrointestinal markers. Several meetings took place with the senior consultant biochemist, Professor Roy A Sherwood, prior to the selection of appropriate faecal markers that could be used as outcome measures.

The research was granted collaboration with the King's College Hospital (see letter Appendix 10) and was provided with facilities and high level expertise in measuring faecal inflammatory markers. All biochemical analysis of samples was performed by a senior Biomedical Scientist in the Department of Clinical Biochemistry's at King's College Hospital.

Following discussions with Professor Sherwood, calprotectin was selected as the main marker and M2-PK and lactoferrin as adjuncts. All three markers are stable and show good reproducibility and have high levels of specificity and

sensitivity for detecting gastrointestinal anomalies (see sections 2.19; 2.20 and 2.21) (Bunn et al., 2001, Summerton et al., 2002, Fagerberg et al., 2003, Kane et al., 2003, Szarszewski et al., 2003, Koss et al., 2005, Loughlin et al., 2005, Ahmed et al., 2007, D'Inca et al., 2008, Langhorst et al., 2008, Gonzalez-Chavez et al., 2009, NICE, 2013). In faecal samples calprotectin and lactoferrin indicate GI inflammation whilst M2PK indicates cell proliferation but is also used as a marker for IBDs and IBS (see table 1-3). These markers are reliable indicators of IBDs and IBS (Bunn et al., 2001, Kane et al., 2003, Fagerberg et al., 2005, Chung-Faye et al., 2007, Langhorst et al., 2008).

Table 1-3 - Faecal Biochemical Markers Reliability

Faecal Biochemical Markers	Calprotectin	M2-PK	Lactoferrin
Diagnostic Sensitivity	80%	81%	77%
Diagnostic Specificity	100%	50%	85%

Legend to the table 1-3: Represents levels of sensitivity and specificity of calprotectin, M2-PK and lactoferrin (Bunn et al., 2001, Fagerberg et al., 2003, Kane et al., 2003, Fagerberg et al., 2005, Chung-Faye et al., 2007, Langhorst et al., 2008).

Faecal calprotetin is used to assess IBDs and non IBDs (IBS) in ASD children (Sandhu et al., 2010, NICE, 2013). According to the NICE diagnostic guidance 11, lower bowel symptoms are more often related to IBS. According to the ROME criteria III (ROME III, 2006), IBS is a functional bowel condition which can affect quality of life although it does not develop into a serious pathology (see section 1.6.1.4). Alternatively, persistent lower bowel symptoms for more than 6 weeks may potentially be the result of a more serious condition, such as IBD, which requires careful investigation (see section 1.6.1.6).

This research was not designed to investigate IBDs but used calprotectin only as a marker to assess and follow any inflammatory change during application of VOT, so the premise for adherence to the NICE further investigation/treatment guidelines was not met. According to guidance 11 (NICE, 2013), raised levels of calprotectin require further investigation such as blood tests, i.e. C-reactive protein and full blood count. Also, if the symptoms

are persistent for more than 6 weeks and calprotectin analysis is positive then the recommendation is an exploratory colonoscopy. This diagnostic and/or treatment strategy was not part of the current study so the Ethics Committee neither considered nor approved any such course of action in this study population.

1.11.2 Calprotectin

Calprotectin, also known as MRP8/14, is a heterodimer of two calcium binding protein elements that belongs to the S100 protein family (see Figure 1-2) and it is found abundantly within the cytoplasm of neutrophils (Striz and Trebichavsky, 2004, Stroncek et al., 2005). Within neutrophils it constitutes approximately 30% to 60% of the total cytosolic proteins (Olafsdottir et al., 2002, Stroncek et al., 2005). It is found in lower amounts (~1% of total cytosolic proteins) in some monocytes (Yui et al., 2003, Stroncek et al., 2005) and may also be occur in macrophages at sites of acute infection (Stroncek et al., 2005).

It is known that high levels of calprotectin exist within the intracellular fluid during various inflammatory processes (Striz and Trebichavsky, 2004, Lundberg et al., 2005), and upon its release it can be detected within serum and other body fluids as a useful marker of inflammatory processes, such as UC, CD and IBD (Striz and Trebichavsky, 2004, Lundberg et al., 2005, Stroncek et al., 2005).

It has been reported that the plasma concentration of calprotectin can be 10 fold higher in patients suffering from rheumatoid arthritis (RA) and blood calprotectin levels are often elevated in patients suffering from CD, cystic fibrosis, multiple sclerosis, and in patients who have undergone major surgery (Yui et al., 2003). A significant increase in calprotectin concentration has been found to be present in extracellular fluid from local inflammatory sites, which has either been secreted from stimulated neutrophils or released as a result of cell death (Yui et al., 2003). A further example of a high extracellular concentration of calprotectin is in the faeces of patients suffering from an IBD such as CD or UC (Lundberg et al., 2005, Stroncek et al., 2005), where the

median calprotectin level in subjects suffering from CD is 31 mg/L (normal range 0.5 mg/L – 50 mg/L) and in subjects suffering from UC is 116 mg/L (normal range 0.5 mg/L – 50 mg/L) (Summerton et al., 2002).

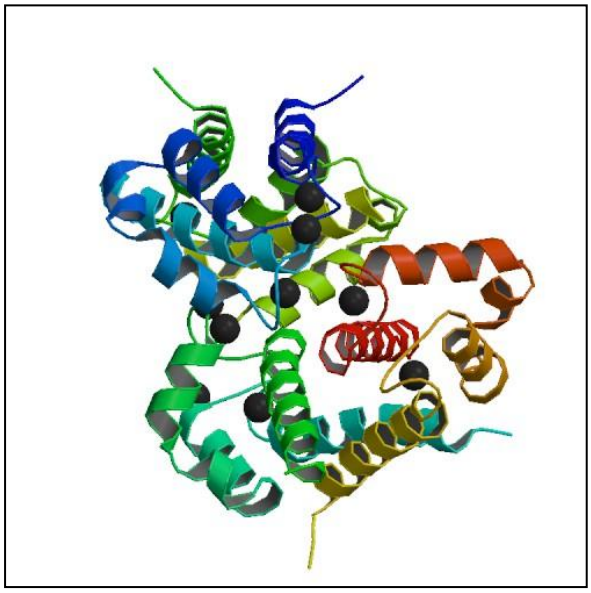


Figure 1-2 - Crystal structure of human calprotectin

Protein Data Bank (http://www.rcsb.org/), DOI:10.2210/pdb1xk4/pdb

Table 1-4 Calprotectin reference ranges for healthy and diseased patients

Healthy subjects	0.5 – 50 mg/L
Active IBD	500 – 50,000 mg/L
Non-active IBD	150 – 500 mg/L
IBS	1 – 150 mg/L

Legend to table 1-4: Table representing the ranges of calprotectin for healthy subjects, IBD and IBS subjects (Lundberg et al., 2005). A result of > 50mg/l is considered a positive calprotectin analysis.

The calprotectin enzyme-linked immunosorbent assay (ELISA) has been viewed as a very good diagnostic tool, since an elevated calprotectin level correlates with an inflammatory disease state (Bunn et al., 2001). Faecal calprotectin has high levels of specificity (100%) and sensitivity (80%) for children with GI disorders (Bunn et al., 2001); therefore, detecting the level of calprotectin in faeces can be used as a non-invasive screening test for identifying organic diseases of the small intestine or large bowel. The faecal

calprotectin ELISA has proved to be a valuable tool compared to more invasive GI tests, especially in young children (Bunn et al., 2001, Fagerberg et al., 2005).

Calprotectin is remarkably stable in stools, and samples can be kept in a suitable container at room temperature for up to seven days (Lundberg et al., 2005), although long-term storage until analysis should be at -18 °C or below. Exposure to temperatures greater than 30°C should be avoided (Buhlmann Laboratories, 2011).

The main applications of the calprotectin ELISA are (Tibble et al., 2002) :

- Differentiation between organic IBD and irritable bowel syndrome (IBS).
- The detection of a decrease in faecal MRP8/14 concentrations during successful therapy for IBD.
- The detection of an increase in faecal MRP8/14 as a short term indicator for IBD relapses.

1.11.3 Tumour M2 Pyruvate Kinase

M2-PK, a dimeric isoform of pyruvate kinase (see Figure 1-3), has been identified as a metabolic marker for various tumours, such as colorectal (Loughlin et al., 2005), pancreatic, GI, lung and breast amongst others (Allocock, 2004, Ahmed et al., 2007, Oremek et al., 2007). It is a novel marker that reflects metabolic activity of tumours and has become a useful tool in diagnosis and detection of a range of tumours (Eigenbrodt, 2001). M2-PK can be measured from both blood or stool samples and analysis of stools for M2-PK has been used primarily to screen and detect colorectal cancer. It is a very stable marker, can be stored at 25°C for 48 hours and even very small amounts can be easily detected (Allocock, 2004, ScheBo® · Biotech AG, 2011).

A recent study has reported that faecal M2-PK as a marker has a sensitivity of 92% for the detection of colorectal cancer, 60% for the detection of large polyps and 25% for the detection of small polyps. M2-PK specificity in all of

these cases was 92% (Koss et al., 2005). Faecal M2-PK is an enzyme that is released by the tumour itself and its detection has been used increasingly as a tumour marker. Ultimately, detection of M2-PK may replace more invasive methods of tumour detection (Loughlin et al., 2005).

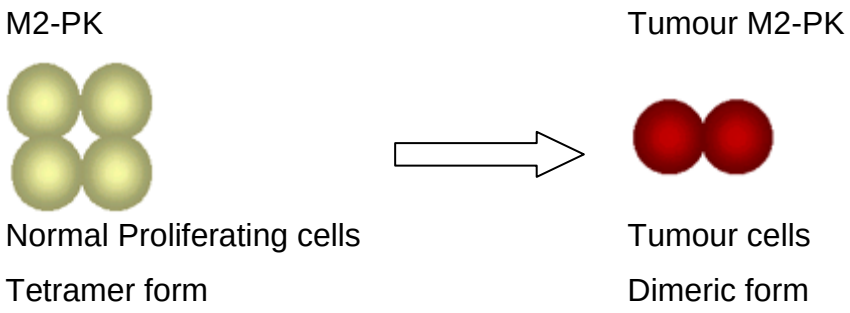


Figure 1-3 M2-PK Formation. Schematic illustrating the Tumour Metabolome-Metabolic Database (www.metabolic-database.com)

A recent report from King's College Hospital suggested that faecal M2-PK could be used as a non-invasive marker to differentiate IBD from functional bowel disorders (Chung-Faye et al., 2007). This report postulated that dimeric M2-PK that occurs in tumour cells would be increased in IBD owing to the rapid cell turnover (cell proliferation) and so the study aimed to determine the value of M2-PK for UC, CD and functional bowel disorders (Chung-Faye et al., 2007). It was found that faecal M2-PK has a sensitivity of 81% and specificity of 50% in the detection of IBD and the values for the different diseases ranged from34.3 to 62.6 U/mL for UC27.3 to 59.9 U/mL for CD and 1.4 to 20.8 U/mL for functional bowel disease (e.g. IBS)

The study concluded that faecal M2-PK is a novel marker that can be used to differentiate IBD from functional bowel disorders and that it correlates well with calprotectin measurements that are already validated.

1.11.4 Lactoferrin

Lactoferrin is a 703-amino acid iron binding glycoprotein present in mucosal secretions and in the secondary granules of neutrophils and its structure is shown in

(Levay and Viljoen, 1995, Walker et al., 2007). Is has been suggested that lactoferrin is a possible marker for assessing IBD activity, and therefore is a

potentially non-invasive marker of IBDs such as CD and UC, and a discriminative marker between IBD and IBS (Sidhu et al., 2010a).

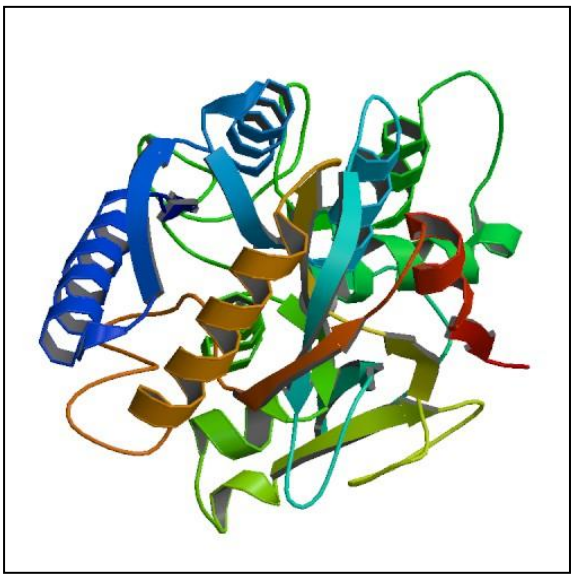


Figure 1-4: Structure of the recombinant N-terminal lobe of human lactoferrin
Protein Data Bank (http://www.rcsb.org/), DOI:10.2210/pdb1lct/pdb

Lactoferrin can be detected in mucosal secretions, such as saliva, tears, vaginal secretions and breast milk, all of which are in contact with environmental pathogens (Walker et al., 2007, Gonzalez-Chavez et al., 2009). In addition, several studies have claimed that faecal lactoferrin is a surrogate marker for IBD and a non-invasive marker for UC and CD (Walker et al., 2007, Sidhu et al., 2010a). Lactoferrin has the ability to respond to several changes in the homeostatic balance, with its biological functions ranging from anti-bacterial, anti-viral, anti-parasitic and anti-fungal to anti-inflammatory (Gonzalez-Chavez et al., 2009, Jenssen and Hancock, 2009). Laboratory analysis of tissue from the intestinal lumen of subjects diagnosed with IBDs shows the accumulation of polymorphonuclear neutrophils (Baveye et al., 1999). The innate immune response of the activated intestinal lumen is to release pro-inflammatory proteins, and one of these released proteins is lactoferrin (Levay and Viljoen, 1995).

A recent study evaluated lactoferrin levels in faecal samples from 170 children, mean age 13.4 years, in order to assess IBD disease activity. The study suggested that faecal lactoferrin testing is a non-invasive reliable

marker within the paediatric population (Walker et al., 2007). Over the last decade, several studies have suggested that lactoferrin has high levels of specificity and sensitivity for distinguishing between IBDs and IBS. A study by Kane et al. (2003) suggests that faecal lactoferrin was 90% sensitive for diagnosing active IBD and 100% specific in discarding IBS as a potential diagnosis, whilst Langhorst et al. (2008) found that lactoferrin sensitivity was 85% and specificity was 77% for IBS patients.

One study analysed the specificity and sensitivity of lactoferrin in CD compared to the use of capsule endoscopy, a novel wireless imaging device, as a form of diagnosing IBD, and suggested there was a correlation between faecal lactoferrin levels and capsule endoscopy ($p=0.03$). Lactoferrin sensitivity and specificity was reported to be 71% and 100%, respectively. However, only seventeen patients were recruited for this study (Sidhu et al., 2010a). A further study by Sidhu et al. (2010a) analysed the sensitivity and specificity of faecal lactoferrin in differentiating between IBD and IBS, and recruited 137 subjects with IBS, 126 with UC, 104 with CD and 98 healthy subjects. This study suggested that lactoferrin has a higher discrimination for IBD sufferers compared to IBS with 67% sensitivity and 96% specificity.

Faecal lactoferrin is stable in stools and can be stored at room temperature for seven days before being frozen for later analysis (TechLab®, 2008).

1.12 Questionnaires

Questionnaires are useful data collection tools to analyse a patient's perception of health (Rattray and Jones, 2007). The current study used a validated standardized questionnaire to measure the parents' perception of their autistic children before, during and after application of VOT. Benefits of using questionnaires for data collection include the relatively low administration costs, ease of response and simplicity of data analysis (Rattray and Jones, 2007).

The health community usually uses a Likert-type or frequency scale questionnaire designed to measure attitudes or opinions. In this study,

parents completed a questionnaire regarding their assessment of the GI and behavioural symptoms of their children using a 24 parameter, ten point Likert scale, based on the Autism Research Institute Secretin Outcomes Survey Form – "S.O.S Form". This is a validated and standardized questionnaire (Rimland, 1998, Brudnak et al., 2002, Unis et al., 2002, Esch and Carr, 2004a, Erickson et al., 2005b, Sturmey, 2005, Williams et al., 2005).

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 questionnaires used for studies. The most common test to analyse internal consistency and reliability of a multiple Likert scale questionnaire or survey is the Cronbach's α statistic. Cronbach's α scores above 7 are considered acceptable for reliability (De Vellis, 2003, Field, 2009).

1.13 Visceral Osteopathic Manipulation and the Gastrointestinal Tract

The lining of the GI tract is regarded as a sensory organ similar to the skin, and it contains three types of sensory detectors that are controlled and/or monitored by the ENS: neurons, local endocrine cells, lymphatic cells (Furness et al., 1999, Smid, 2008).

Constant signals from the enteric plexus are sent locally to the lining of the GI tract to control motility, secretion, tissue defence, and vascular perfusion, and also to other digestive organs, such as the stomach, gall bladder and pancreas, and to the CNS (Willard, 1997, Furness et al., 1999). This integrated system responds to changes in the GI luminal content (Furness et al., 1999). Usually this system protects the body against bacteria, toxic substances or harmful entities that may enter the digestive system via food intake; however, the system's response is not always well adjusted which may cause disease or dysfunction.

'The enteric nervous system is composed of two ganglionated plexus. The larger myenteric plexus, situated between the circular and the longitudinal

layers of the muscularis externa, contains the neurones responsible for motility and for mediating the enzyme output of adjacent organs. The smaller submucosal plexus contains sensory cells to the motor neurons of the myenteric plexus, as well as motor fibers that stimulate secretion into the gut lumen.' (Gershon, 1999)

1.13.1.1 The Neurons

There are three levels of sensory information that are necessary for proper functioning of the GI tract. Sensory afferent neurons are responsible for local reflexes in the gut wall, and form a short loop interconnecting the gut mucosa, submucosa or muscle to the enteric ganglia. Afferent neurons from the gut mucosa to the pre-vertebral ganglia along the aorta (Willard, 1997) participate in the intra-abdominal reflex arcs. Finally, the third level involves sensory neurons that project from the gut wall to the brainstem via the vagus nerve or to the spinal cord via the thoracic, lumbar and pelvic splanchic nerves (Willard, 1997, Furness et al., 1999, Gershon, 1999).

The first level of sensory neurons responsible for local reflexes in the gut are the IPANs (Furness et al., 1999, Gershon, 1999, Smid, 2008), and these have the important task of generating reflex responses to the intestinal content. These reflexes cause mixing and propulsive movements of the gut muscles, local changes in the blood flow and modulation of the secretion of water and electrolytes. A study using the ileum of guinea pigs has shown that IPANs also respond to tension in the muscles of the gut wall, and it is suggested that these types of neurons are stretch-sensitive with activation occurring in response to muscle stretching (Furness et al., 1999, Kunze et al., 1999, Clerc et al., 2002, Smid, 2008). Thus, either passive or active stretching of the muscles of the gut wall seems to result in the firing of the IPANs. This information is then conveyed to other neurons of the ENS, and the information integrated resulting in appropriate changes in mixing and propulsive activity of the gut, water and electrolyte transport, local blood flow, and possibly endocrine secretions. Dysfunction of the GI system may cause disruption to this integrated system; however, visceral manipulation techniques may stimulate the IPANs via the stretch reflex and consequently may help to

rebalance the function of its integral parts, including the second and third levels of sensory afferent neurons (Furness et al., 1999, Gershon, 1999).

Other fibres that may be involved are the peripheral afferent fibres of the vagus nerve. The intramuscular arrays and intraganglionic lamina endings have a mechano-sensory function and respond to muscle tension elicited via passive muscle stretching. Intramuscular arrays are sensory receptors and the intraganglionic lamina endings are suggested to be associated to connective tissue that responds to sheering forces generated from stretching during muscle contractions (Grundy, 2002, Grundy, 2004).

1.13.1.2 Endocrine Cells

The endocrine system is directly dependent on the cardiovascular system for hormonal transport to distant sites; delivering control mechanisms for the secretion of hormones; and hormone concentrations which influence the magnitude of effect at the site of action (Furness et al., 1999). Alterations in blood flow at a site responsible for hormonal release may cause disruption to the hormone secretion rate; therefore, the function of hormone target cells may be compromised causing an imbalance in the entire body system. For this reason, increasing blood flow to a dysfunctional area may result in a change in the functional regulation of an endocrine cell (Furness et al., 1999, Stone, 1999).

1.13.1.3 Lymphatic Cells

The most important functions of the lymphatic system are to maintain fluid balance and to protect the body from potential infection (Kuchera and Kuchera, 1994). Whenever injury occurs, the subsequent inflammation brings about an increase in vascular perfusion, capillary filtration, and lymph production to the area. The greater the lymphatic flow through the body, the greater the contact between body defences and toxins (Kuchera and Kuchera, 1994, Wallace et al., 1997, Huff et al., 2008). Movements of the internal organs, such as through respiration and abdominal peristalsis, have a positive effect on the pumping action of the lymphatic system, thereby enhancing its

function (Huff et al., 2008). This can be achieved via direct pressure on the lymphatic channels via visceral soft tissue manipulation, resulting in increased lymphatic flow (Wallace et al., 1997, Finet and Williame, 2000, Huff et al., 2008).

Inadequate lymphatic drainage results in the development of tissue oedema which compresses lymphatic channels, the vascular tree, and adjacent neurological structures, potentially reducing their function (Kuchera and Kuchera, 1994, Finet and Williame, 2000). Increased congestion leads to the stasis of interstitial fluids and changes in tissues, further compromising function. Visceral osteopathic manipulation may promote decongestion in an organ or region of the body (Wallace et al., 1997). A clinical trial undertaken on the effects of osteopathic lymphatic pump treatment involving patients suffering from pneumonia suggested that manual stimulation of the pulmonary area may reduce infection by enhancing immunity (Huff et al., 2008). Osteopathic lymphatic pump treatment increases the circulating leukocytes, resulting in the attenuation of bacteria within the lung tissue, and may therefore enhance the lymphatic system (Huff et al., 2008).

It is important for professionals in manual therapy to recognise the importance of the enteric system. The function of this network of neurons, lymphatic cells and endocrine cells may be the clue to formulating an effective visceral treatment protocol for functional bowel disease.

‘Three systems for detecting changes in the tissues are located in the wall of the intestine: neural, endocrine and immune detecting systems. Neural sensations are conveyed by extrinsic and intrinsic primary afferent neurones. Endocrine messages are in the form of hormones released from cells in the mucosal epithelium. The hormones enter the circulation and thus act at remote sites, but they also act locally, on nerve endings, epithelium, microvasculature, and cells of the immune system. Immune messages are conveyed by local activation of lymphocytes and augmented by circulating lymphocytes that are activated by antigens from the lumen.’ (Furness et al., 1999)

1.13.2 Rationale for the Use of VOT

Visceral osteopathy is used to optimise blood and lymphatic supply to the internal organs. The viscera are a collection of organs in the abdominal cavity that generally respond to the internal physiological motion guided by the involuntary movement of the diaphragm in respiration; the internal motility of the viscera such as peristaltic movement; cardiac movement and blood and lymph circulation; and via skeletal movement such as walking, running or exercising. This motion and motility and the influence on the viscera is constantly present throughout life (Barral and Mercier, 2006).

The internal visceral physiological balance may be disrupted or impaired by internal adhesions and/or fixation that, according to the basic concepts of visceral osteopathy, could result in visceral functional impairment (Bove and Chapelle, 2012, Chapelle and Bove, 2013). VOT aims to help restore a visceral physiological balance via low invasive techniques to the abdominal area (Barral and Mercier, 2006).

A recent study, using rats, analysed the effects of visceral manipulation after abdominal surgical intervention (Bove and Chapelle, 2012). The aim of the study was to determine the effect of visceral manipulation in preventing and managing post-surgical visceral adhesions. Thirty rats were assigned to one of three different groups; the lysis group, the preventative group and the control group. Rats from each group were given caecal abrasion surgery. In the lysis group, rats received one visceral manipulation at day 7 post-surgery and were then euthanized. In the preventative group, rats received visceral osteopathic manipulation post-operatively once a day until euthanasia at day 7. The control group received no visceral treatment and were also euthanized on day 7. Bove and Chapelle (2012) suggest that there were no changes in the number of adhesions between the three groups of rats, however the severity of the adhesions, measured on a scale from 0-4 by a blinded assistant, showed a significant decrease between the lysis and preventative groups where the severity of the lysis scores was reduced from 1.9 to 0.6 respectively ($p<0.01$). The authors hypothesised that the effect of visceral manipulation improved fluid movement and inhibited fibroblast migration, thus

reducing adhesion formation at the injury site (Bove and Chapelle, 2012). This study suggests a positive post-surgical effect of visceral osteopathic manipulation.

Another study on the effectiveness of visceral osteopathic techniques (VOTs) was performed on IBS patients, again with positive results. Attali et al. (2013) considered 31 IBS patients in a randomized cross-over placebo controlled study. The qualitative effects of depression, constipation, diarrhoea, abdominal distension and abdominal pain using visual analogue scales (VAS), and rectal sensitivity using a distension balloon, before and after treatment were evaluated. The treatment group received general visceral osteopathy sessions as well as locally applied techniques in specifically sensitive areas of the abdomen and gentle manipulation to the sacral area. The placebo group received treatment in the same areas of the abdomen with a light, non-therapeutic, pressure. The authors reported that VOT ameliorates diarrhoea, abdominal distension and pain as well as rectal sensitivity. The positive effects of the therapy were long lasting and the symptom scores continued to be low at one year follow-up with no further treatment.

The positive effects of VOT on IBS patients were also suggested by Hundscheid et al. (2007). This study randomised patients into two groups, the standard care group and the osteopathic group assigning 19 and 20 patients respectively. Patients allocated to the osteopathic group reported a 68% improvement in overall symptoms with one patient (5%) being completely pain free after the intervention. The symptomatic changes between the groups showed a statistically significant improvement in the osteopathic group ($p<0.006$). Bove and Chapelle (2012) indicated that visceral massage attenuated post-operative ileus. The treatment significantly increased GI transit, augmented cumulative faecal pellet discharge, and shortened the time interval of first faecal discharge post-surgery. According to the authors the positive effect of the massage may possibly have improved peristaltic activity.

1.13.3 Non-Specific Effects of VOT

One of the challenges of VOT is to produce measurable isolated effects. The clinical nature of the technique cannot isolate specific tissues; therefore the application of the techniques may be postulated to affect blood supply, lymphatic system and organ system function. In general VOT affects motility and mobility, circulation fluids, sphincter and muscular spasms, hormonal and chemical production, immunity and the psyche (Barral and Mercier, 2006).

1.13.4 Effectiveness of VOT in other Conditions

According to Huff et al. (2008) the lymphatic pump technique, a type of visceral osteopathy applied to the thoracic cavity region and used by osteopaths when treating oedema patients, mobilizes inflammatory mediators into the lymphatic circulation. The authors tested the thoracic and intestinal lymph of dogs both at rest and after thoracic lymphatic pumping. The study suggests an increase in thoracic and intestinal influx of cytokines and chemokines after the application of the technique. The authors support the idea that the increased influx of cytokines may enhance immunity, possibly facilitating the clearance of infection (Huff et al., 2008).

Another study, by the same group, (Hodge et al., 2010) has tested lymphatic pumping techniques in rats and measured lymphatic flux. The cisterna chyli of the rats were cannulated and measurements of lymph flux were taken four minutes pre-lymphatic pump technique (LPT) and at four minutes and ten minutes post-LPT. The results revealed an increased number of lymphocytes released into the lymphatic circulation. The group also found that gut lymphocytes were mobilized into the cisterna chyli and thence to the circulation. These studies provide experimental support for the clinical application of VOT. According to the authors, VOT may enhance the distribution of antigens and antibodies and may be clinically effective in treating and controlling infection. Huff et al. (2008), demonstrated that LPT is also effective in patients with chronic pneumonia. According to the authors LPT increases the circulatory leukocytes that are mobilized in the lungs, killing bacteria and reducing tissue damage.

1.14 Relevance of VOT in this Current Study

The rationale for applying VOT to autistic children suffering from GI signs and symptoms rests with its holistic and low invasive approach. The use of touch and mobilisation of organs in an attempt to restore function has the potential to affect the ENS and possibly help to restore the gut-brain function (Mayer and Tillisch, 2011). It has been suggested that psychological stressors may result in GI symptoms. Autistic children are in a constant state of 'stress' purely due to the nature of the condition itself. Any positive effect of VOT on intestinal function has the potential to decrease GI signs and symptoms and this may be translated positively into a reduction in typical autistic behaviour.

1.14.1 Challenges of VOT Application on Autistic Children

The major challenge of application of VOT in autistic children is the use of touch as a form of treatment. Autistic children are hyper-sensitive to touch. According to Cullen et al. (2005) disruption of the primary senses can have a negative effect on the world around autistic children and their parents. It can be translated as touch avoidance with hyper-sensitivity to touch and touch defensiveness resulting in challenging behaviour.

Touch is a primary sense and it is essential to a child's development. According to Escalona et al. (2001) and Field et al. (1997), massage has a positive role in decreasing touch avoidance in autistic children. Cullen et al. (2005) studied the effects of parental touch on autistic children as a form of therapy. Parents were trained for eight weeks to apply touch therapy to their child. Baseline measurements were collect via a semi-structured phone interview before the initiation of sessions and again at week 16 of the treatment period. The authors observed that autistic children dictated the terms of how and when they should be touched by their parents, making spontaneous touch a challenging experience. The parents very aware of the possibility that their child would not be able to cooperate during the touch therapy sessions, creating apprehension and further concerned at not being able to comfort their own child in cases of pain or distress. However, after 16 weeks of touch therapy, autistic children and their parents reported a sense of

closeness. The child was able to request touch therapy at home; in turn creating a more positive home environment. Parents reported that the children were more tolerant to touch and appeared to have improved verbal communication and were calmer and more relaxed. In contrast to previously held beliefs, autistic children enjoyed the touch therapy experience.

1.14.2 Gap in the Literature

According to Buie et al. (2010) the management of GI symptoms in autistic children may improve some of the behavioural symptoms. However, to date, there are no known published studies on the use of a low-invasive treatment that could potentially ameliorate the challenging behaviour and GI signs and symptoms of children with autism (Furuta et al., 2012).

The first attempt to study the effects of VOT on GI and behavioural signs and symptoms of autistic children dates back to 2002 when a pilot study was performed on 13 autistic children (Bramati-Castellarin and Janossa, 2002). The concept of the pilot study was developed from a lack of alternative or complementary evidence in this area. Historically, positive results have been associated with application of VOT to the abdominal area of general population subjects with GI dysfunction (Ernst, 1999, Finet and Williame, 2000, Lamas et al., 2009, Attali et al., 2013). However, VOT had not been applied specifically to autistic children before the commencement of this study. The results of the pilot study suggested positive GI and behavioural responses after application of VOT. These positive results led the author to develop the pilot protocol, with the added introduction of biochemical markers in an attempt to objectively quantify any changes.

The aim of the present study attempted to address the gap in the literature by studying the effect of VOT on autistic children suffering from GI signs and symptoms using both questionnaires and biochemical marker analysis.

1.15 Aims and Objectives

The aims and objectives of this research were to investigate the effect of visceral osteopathic techniques (VOT) on the behaviour and GI signs and symptoms of autistic children.

A. GI inflammation was assessed using:

- Levels of calprotectin as a primary biochemical marker of inflammation, which was assessed using stool analysis.
- Levels of tumour M2-pyruvate kinase (M2-PK) as a second biochemical marker, which were then correlated with the results for calprotectin.
- Levels of lactoferrin as a third biochemical marker, which were correlated with the results for calprotectin and M2-PK.

B. Behavioural and GI activity was assessed utilising a 10 point scale questionnaire, 'Autism Research Institute Secretin Outcomes Survey Form, the 'S.O.S Form', which was given to parents before and after treatment (Appendix 5).

1.15.1 Null Hypothesis:

After six weeks of VOTs autistic children will not experience:

1. Changes in GI symptoms
2. Changes of behavioural symptoms
3. Measurable changes in faecal calprotectin levels
4. Measurable changes in faecal M2-PK levels
5. Measurable changes in faecal Lactoferrin levels

1.15.2 Experimental Hypothesis:

After six weeks of VOTS autistic children will experience:

1. Changes in GI symptoms
2. Changes of behavioural symptoms
3. Measurable changes in faecal calprotectin levels
4. Measurable changes in faecal M2-PK levels
5. Measurable changes in faecal lactoferrin levels

Chapter 2 - Methodology

This chapter outlines the aims and objectives of the research before moving to explain the research design, data collection and statistical analysis.

2.1 Objectives of the Current Study – Brief Overview

The objective of the current study was to investigate the effect of VOT on GI signs and symptoms as well as behaviour patterns in autistic children. Two outcome measures were used during the study, specifically questionnaires, given to parents to assess GI signs and symptoms and behaviour patterns, and three biochemical markers of inflammation present in faeces (calprotectin, M2-PK and lactoferrin). Each was measured before, during and after application of VOT. The only intervention was therapeutic VOT applied to the abdomen of the subjects. The methodology remained consistent throughout to maintain statistical power.

Each subject was examined and screened at the start of each treatment session to determine whether there were any issues, contraindications or adverse events associated with the previous treatment or research protocol. The normal procedure for osteopathic care followed the Osteopathic Practice Standard (OPS) for the duration of the research programme. The safety and welfare of the subjects was always of paramount importance.

2.1.1 Justification of Methods

The study aimed to address the lack of low-invasive treatments to autistic children suffering from GI signs and symptoms. The possible connection between changing behaviour signs and symptoms and the gastrointestinal condition in these children, as well as the lack of an appropriate low-invasive gastrointestinal treatment led the researcher to study the possible effects of application of VOT in these autistic children suffering from GI signs and symptoms. This methodology sought to investigate a possible link between changing behavioural symptoms and the GI system as suggested by Buie et al. (2010). Several authors such as Horvath et al. (1999), Jyonouchi et al.

(2005a), Nikolov et al. (2009), Reichelt and Knivsberg (2009), Forsythe et al. (2010), Walker et al. (2013) have also suggested a possible gut-brain axis that could be immunological, inflammatory or genetic in nature.

The methodology chosen for this study took into consideration the complexity of behavioural patterns displayed by autistic children and their strong preference to mantain routine and sameness (Wing, 1997, Wing, 1998, American Psychiatric Association, 2000). Only a small number of quantifiable data collection tools are available which conform with minimal enviromental changes. Hence, questionnaires and faecal biochemical markers were chosen for this study due to their low invasive nature, the ability of producing meaningful data and their compliancy with the Ethics commitee constraints when assessing and treating autistic children.

The questionnaires were used to measure the parent's perception of behavioural and GI signs and symptoms, whilst the biochemical markers objectively assessed GI inflammation. The outcome measures were considered before, during and after application of VOT. Other data collection tools such as blood tests, endoscopy, X-Rays and MRI were considered to be higly invasive by the Ethics Commitee and beyond the financial scope of this research.

Questionnaires are useful data collection tools to analyse a patient's perception of health (Rattray and Jones, 2007). The current study used a validated, standardized questionnaire to measure parents' perception of their autistic children before, during and after application of VOT. The benefits of using questionnaires for data collection include the relatively low administration costs, ease of response and simplicity of data analysis (Rattray and Jones, 2007).

Following several meetings with Professor Roy Sherwood, senior consultant at the Department of Clinical Biochemistry, King's College Hospital, London it was decided to use three faecal biochemical markers to assess GI inflammation in this cohort. Professor Sherwood advised the use of calprotectin as a main marker and M2-PK and lactoferrin as adjunct markers

(Sherwood et al., 2005, Chung-Faye et al., 2007, Sherwood, 2012). All three markers display high levels of stability and reproducibility as well as good sensitivity and specificity for IBDs and IBS (Kane et al., 2003, Szarszewski et al., 2003, Koss et al., 2005, Loughlin et al., 2005, Lundberg et al., 2005, Ahmed et al., 2007, Ewald et al., 2007, Oremek et al., 2007, Walker et al., 2007, Langhorst et al., 2008, Gonzalez-Chavez et al., 2009, Sidhu et al., 2010b, Zippi et al., 2010, Aomatsu et al., 2011, Day et al., 2012, Manz et al., 2012, Yamamoto et al., 2013) and are therefore appropriate markers to assess levels of GI inflammation.

2.2 The Development, Evaluation, Implementation and Design Process of the Current Study

The Medical Research Council created guidance for the development, evaluation and implementation of research design for complex interventions resulting in the “MRC Framework for the Development and Evaluation of RCTs for Complex Interventions to Improve Health” (Medical Research Council, 2000). These guidelines are intended to advise researchers about choosing the appropriate methods of research when considering a complex research intervention to improve health. The principles originally applied to the current study during the development, evaluation and analysis stages are in accordance with ‘Developing and evaluating complex intervention: new guidance’ (Medical Research Council, 2006). The MRC guidance was published after the current study was designed and undertaken but is referenced to illustrate that the methodology published is a formalisation of basic principles previously in existence. Methodology used in the current study followed clear guidelines for clinical safety. The application of osteopathic techniques followed the principles enshrined within the OPS (General Osteopathic Council, 2012). Before any assessment, intervention or measurement was undertaken a systemic review was made to evaluate the viability of such a study.

2.2.1 Piloting and Evaluation of the Intervention

A pilot study was performed with 13 autistic children between the ages of 3 ½ and 8 years. This pilot study acted as a trial for the viability of the VOT treatment with autistic children. In addition, this pilot study trialled the recruitment process, the pre-assessment questionnaires and osteopathic case history taking, the modified S.O.S questionnaire and the VOT procedure. The effectiveness of the pilot study (Bramati-Castellarin and Janossa, 2002) informed the design of the current study.

The results from the pilot study informed on the effect of group size and variability as well as on the rate of recruitment. A refinement arising from the pilot study was the inclusion of biological markers to measure possible GI changes via objective, experimental, quantitative assessment.

2.3 Research Design: Before and After Intervention Study of Effectiveness

This research study was structured as a clinical trial employing a before and after treatment study of effectiveness with repeated measures design. The before and after treatment study was chosen due to its high sensitivity in measuring treatment effectiveness in each individual subject and its avoidance of inter-subject variability (Kabat-Zinn et al., 1992, Rosner, 2011), enabling the analysis to focus more precisely on treatment effects. The design is economical with the subjects acting as their own controls thus requiring a smaller total number of subjects and eliminating the necessity for additional ethical approval for experimentation on ‘normal’ children; the controls are directly related and matched to the study set.

There are some disadvantages to this type of research design. One is the carryover effect of the treatment before the effect of the previous treatment has worn off. This design flaw was minimised by allowing a week between each treatment procedure in an attempt to avoid carryover effects; standard practice in osteopathic clinical research studies (Licciardone et al., 2013). Another disadvantage of this type of research design is the learning effects

that may possibly occur after using a measuring tool such as a questionnaire. Subjects may become test wise and therefore present pattern improvement. This is addressed in the discussion (4.3.4).

2.4 Ethical Approval

A proposal for a double blind controlled trial was rejected by the ethics committee as it was considered unethical to expose the children to a 'no treatment' or 'sham treatment' control.

A modified proposal constructed as a before and after Intervention study, with each subject acting as their own control (baseline) was submitted to the ethics committee and this project structure was granted ethical approval by both the British College of Osteopathic Medicine Ethics Committee and by the University of Westminster Ethics Committee as a 'Before and After Intervention Study of Effectiveness' (see Appendix 1).

A proposal to assess behavioural changes by an independent psychologist who was blind to the techniques was not feasible due to cost constraints and it was not, therefore, possible to use a blinded external professional to assess behavioural patterns before and after intervention.

The ethical approval that was granted did influence the final study design and this is addressed in the discussion.

2.5 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-IV and ICD-10 diagnostic criteria to release a Diagnostic Statement. Parents were asked to provide the Diagnostic Statement given to them, prior to any contact with the researcher. The

researcher did not make any contribution to the diagnostic process, thus avoiding classification bias on recruitment.

2.5.1 Inclusion Criteria

Autistic children suffering from classic signs and symptoms, including:

1. Abnormal behavioural symptoms characteristic of autism as recognised by the DSM-IV and ICD-10 diagnostic criteria.
2. GI symptoms, including abdominal distension and/or pain, constipation, chronic diarrhoea, foul-smelling stools and/or flatulence.

2.5.2 Exclusion and Withdrawal Criteria

1. Autistic children without GI symptoms.
2. In the event of a child being unable to cooperate or being distressed.
3. In the event of parental withdrawal of consent to treatment.

In the event that a child developed a condition that could put their health at risk, e.g. serious infections, epilepsy.

2.6 Recruitment Sites

The main recruitment process was via special schools for autistic children (see table 2-1). Ten special schools accredited by the National Autistic Society were randomly selected, from London and the South East of England, and were contacted via formal invitation of the head teacher. Of the ten randomly selected special schools, four accepted the invitation to be part of the research recruitment process. (Letters and background information provided in Appendix 2).

Following the first encounter with the head teacher, the researcher was granted permission to give a talk to the parents during a school parent's meeting to present the research background. At the presentation, the parents were given the research guidelines as well as the inclusion and exclusion criteria for the study. Parents who considered that their autistic child

presented with GI dysfunction were then selected to respond to an initial questionnaire; used to establish the fulfilment of the inclusion criteria.

Table 2-1 Recruitment sites and subjects number

Recruitment Site	Subject per site	Percentage subject per site
School 1	8	17%
School 2	6	12%
School 3	4	8%
Private Practice	31	63%
Total	49	100%

Subjects were also recruited in response to announcements placed in local newspapers and/or magazines (Angel & Urchins); Schools, Universities (Westminster University and The British College of Osteopathic Medicine clinical area) and the internet (Talk Autism website – forum for parents)(Appendix 4). Subjects recruited from this process were treated at a private practice and required to fulfil the same inclusion and exclusion criteria as the subjects recruited from the special schools for autistic children. One of the criteria for subjects recruited via these announcements was that they 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 (which is a legal document) by the Education and Library Board (ELB) after being assessed by an appropriate specialist.

The parents were required to provide a copy of the Diagnostic Statement together with the statement of special educational needs provided by the ELB. Appropriately anonymised examples of the statements provided by the ELB and the Diagnostic Statement are provided in Appendix 3.

The recruitment advertisements are provided in Appendix 4.

2.7 Recruitment Process, Screening and Consent

After the initial recruitment/screening questionnaire, an interview questionnaire (Appendix 5) was completed by parents in order to obtain a child's case history and to evaluate whether osteopathic treatment was safe and appropriate. This 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), behavioural symptoms (flapping of hands, eye contact, communication, etc.) current diet and allergies. From this it was determined whether the subject complied with all of the inclusion criteria and a decision was made as to whether it was safe for the subject to be treated using VOT.

Written and oral guidelines were provided explaining the research and the treatment procedure.

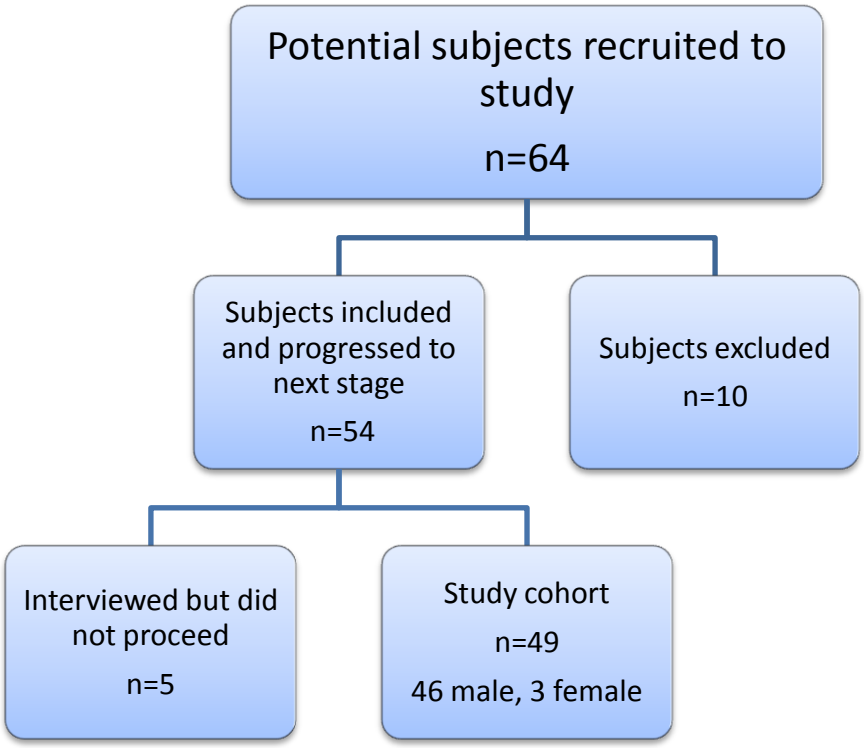
A signed consent form authorising the child to be part of the study was obtained for each study participant.

2.8 Sample Size

In total, 64 children were recruited to the study (Figure 2-1). Ten subjects were excluded due either to a compromising underlying condition or to not presenting with GI symptoms. A further five 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 (male and female) aged between 3½ and 8 years. Of these, 18 subjects were recruited and treated at three different schools for autistic children in the London area, while the remaining 31 were treated at a private practice in west London. All 49 subjects were treated by the same practitioner

using the same range of techniques. The initial period consisted of no treatment (control/baseline period) in order to determine a baseline for each specific subject. A placebo was not used in this study; to minimise any potential additional challenge to the children. It was also deemed ethically inappropriate to use a placebo in children with a learning difficulty/disability.

Figure 2-1 Flow chart indicating the recruitment process and final sample size



2.9 Overview of the Study Periods

The study comprised of three periods, Period I – control (baseline), Period II – treatment, and Period III – rest and post-treatment; which were considered together.

2.10 Control Period (Baseline) – Period I of the Study

The subjects acted as their own controls; the control/baseline period covered six weeks during which time no treatment or intervention was given, but weekly questionnaires were completed and stool samples collected. During this control/baseline period the researcher had no contact with the subjects

except by text message reminders to complete the questionnaires and to post the stool samples. Examples of the reminders sent to the subject can be seen in section 2.24.

Four questionnaires were completed during the first six weeks of the clinical trial; at weeks 1, 3, 5 and 6 before the treatment period commenced, in order for parents to become familiar with the questionnaire and to act as a baseline control for responses (questionnaires 1-4). At least three stool samples for calprotectin/M2-PK/lactoferrin analysis were collected during this period, and the Safeboxes™ (Royal Mail)(Figure 2-15) utilised for their transport were clearly labelled 1-4 to identify that the samples came from the control period.

The baseline data generated from this period (control/baseline) were then compared with the results obtained during treatment (treatment period) and following treatment (post-treatment period).

2.11 Treatment Period – Period II of the Study

Four questionnaires were completed during the six week treatment period of the clinical trial; at weeks 8, 9, 10 and 12 of the study (questionnaires 5-8). At least three stool samples for calprotectin/M2-PK/lactoferrin analysis were collected during this period, and the Safeboxes™ utilised for their transport were clearly labelled 5-8 to identify that the samples came from the treatment period. Parents were instructed in how to collect their child's stool samples for the assessment of faecal markers in the same way as in Period I. A timetable for stool collection (to be used for faecal marker analysis) was agreed upon and given to the parents before the commencement of the osteopathic treatment sessions.

The intervention comprised of six osteopathic sessions, using VOT to the abdominal area. The interventions were a week apart.

It was arranged for a parent to be present with the child for the physical osteopathic examination and follow up treatment sessions. At every treatment session, the parent was asked whether there were any changes in the GI

and/or behavioural symptoms of their child following the previous session. The parent was also asked if there had been any changes in their child's diet or medication, or if any accidents or infectious diseases had occurred during the week following the previous session. This monitoring during every treatment was also designed help to highlight any trends. All of the information provided by the parents was recorded on the treatment notes, with the case history.

2.12 The Rest and Post-Treatment Period – Period III of the Study

During the five week rest/post-treatment period there was no intervention. During this five week period one questionnaire was collected, six weeks after completion of the treatment period (questionnaire 9), at week 18 of the study. One stool sample for calprotectin/M2-PK/lactoferrin analysis was collected during this period and the Safebox™ utilised for transport was clearly labeled 9 to identify that the sample came from the post-treatment period. A summary of the study design is shown in Figure 2-2 and a summary of the questionnaire and stool collection timeline is provided in Table 2-2.

Figure 2-2 Summary of Study Design

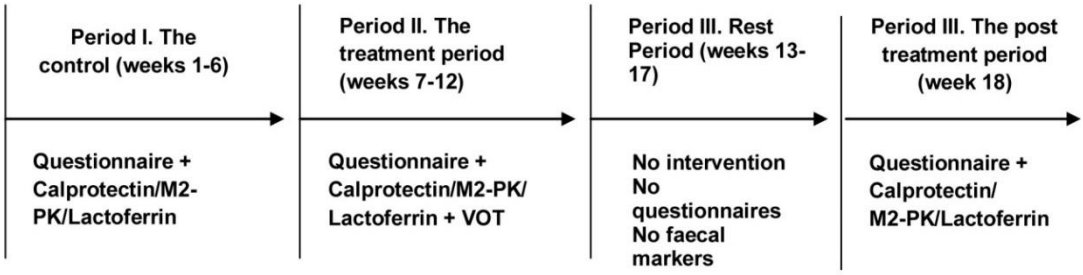


Table 2-2: Timetable for questionnaire completion and faecal sample collection

Study Period	Weeks	Questionnaire/Faecal sample number
Period I. The control/baseline period	1-6	1-4
Period II. The treatment period	7-12	5-8
Period III. The post- treatment period	18	9

2.13 Visceral Osteopathic Techniques (VOT) – Intervention

All subjects were treated using VOTs over the entire abdominal region, including the duodenum, ileo-caecal valve, sigmoid colon, pancreas and general abdominal regions. The treatment protocol was designed using standard osteopathic techniques. Although the treatments were performed over the entire abdominal area, the treatment focused on the classical regions of the landmarks listed in Table 2-3. It is accepted that this anatomical picture of the abdomen is generic and not an accurate illustration of individual anatomy. However, since the entire abdominal region is treated, each of the specific areas will be included within the treatment regimen.

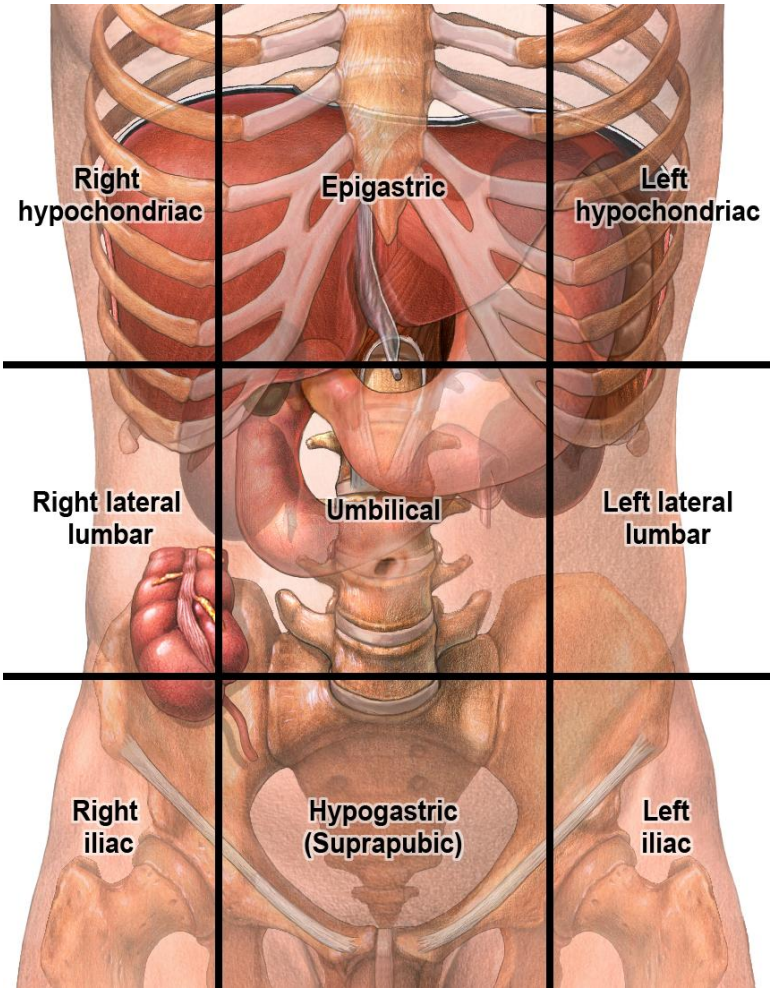


Figure 2-3: The nine abdominal pelvic regions

Legend to Figure 2-3: Illustration giving a general anatomical overview of the nine regions of the abdomen. There is no attempt to predict the location of the abdominal pelvic organs accurately.

2.13.1 VOT Protocol

The intervention was composed of a set of VOT techniques applied to the abdominal area by a single senior osteopath/researcher (the practitioner) with 13 years of experience in osteopathic practice. Table 2.3 lists the standardized VOT used in the study. Six VOT sessions were proposed for the study.

Table 2-3: List of standardised VOTs used in the study

Mobilisation Target	Abdominal Area	Position
Ilio-caecal Valve	Between right lateral lumbar legion	Supine or side lying
Duodenum	Umbilical region	Supine
Ligament of Treitz	Umbilical region	Supine
Pancreas	Epigastric region	Supine
Sigmoid colon	Left lumbar region	Supine or side lying

Legend to Table 2.3: Represents a list of standardised VOTs used during the treatment period of the study, Period II. All techniques were applied to the abdominal area (Stone, 1999, Barral and Mercier, 2005, Barral and Mercier, 2007).

The time allocated for each consultation was half an hour (30mins), timed from the moment the subject entered the treatment room until they left. The initial 10 minutes was used for meeting and greeting the patient and the parent as well as taking brief notes of the preceding week and to allow enough time for the autistic child to become accustomed to the presence of the practitioner.

The practitioner spent the initial 10 minutes acclimatising the patient, with the intention of palpating, examining and treating the abdominal area. Patients often displayed unpredictable and/or aggressive behaviour towards the osteopath, such as spitting, biting, pushing, hair pulling and scratching and the nature of the treatment took these behaviours into account. All measures were taken to maintain the safety of both patient and practitioner.

Each VOT session lasted 20 minutes. At the beginning of each session the practitioner adopted a global visceral osteopathic palpatory technique aiming

to treat the areas around the ileo-caecal valve, the duodenum, the ligament of Treitz, the pancreas and sigmoid colon.

The visceral techniques chosen for this research project aimed to increase circulation, detoxification (biochemical turnover), peristalsis and gut emptying, in addition to aiding neuro-regulatory responses via the ENS as outlined in chapter one, (1.10.1) and thereby positively influencing the GI and behavioural function of the subjects.

2.13.2 VOT Techniques used in the Current Study

The VOT sequence used in this study is an illustration of the treatment procedure. Owing to the complexity of each case, the researcher had to adapt the positions and sequence of the VOT treatment to suit each patient; taking into account challenging behaviour exhibited by high end sufferers. Auditory, visual or tactile props were needed to attract a child's attention to the treatment. Treatment took place variously under the couch, with the patient sitting on their mother's lap, or in a standing position. Some of the subjects displayed distinctive behaviour, such as jumping up and down on the couch, flapping their hands, running in circles or needing a specific toy to play with during a session, as well as aggressive actions such as biting, kicking and spitting. A major determining factor for the sequence of the techniques was the individual's behaviour, with patient and practitioner safety being of paramount importance. However, the techniques described in Section 2.13 were all used in the treatment sessions, although not necessarily in any fixed or specific order. To date, there is no evidence to support a particular treatment sequence, nonetheless, in this study, the specific order of the sequence of treatment was noted on the case history for each subject for future record.

The order of treatment was determined by the individual, for example, some patients had faecal impaction in the sigmoid colon and so this area was chosen as the starting point for treatment. It was common for children to need to empty their bowels urgently during treatment, which tends to support the treatment strategy.

2.13.2.1 Ileo-caecal Valve Region Technique

The patient lies in the supine position (Figure 2-4). Using both hands, the practitioner will mobilise the ileo-caecal region out of the right iliac fossa. Gentle motion will be applied diagonally towards the patient's left shoulder (see arrow in Figure 2-4). The same technique can be applied for a side lying or sitting position (Figure 2-5 and Figure 2-6, respectively).

Figure 2-4: Ileo-caecal valve region technique supine position

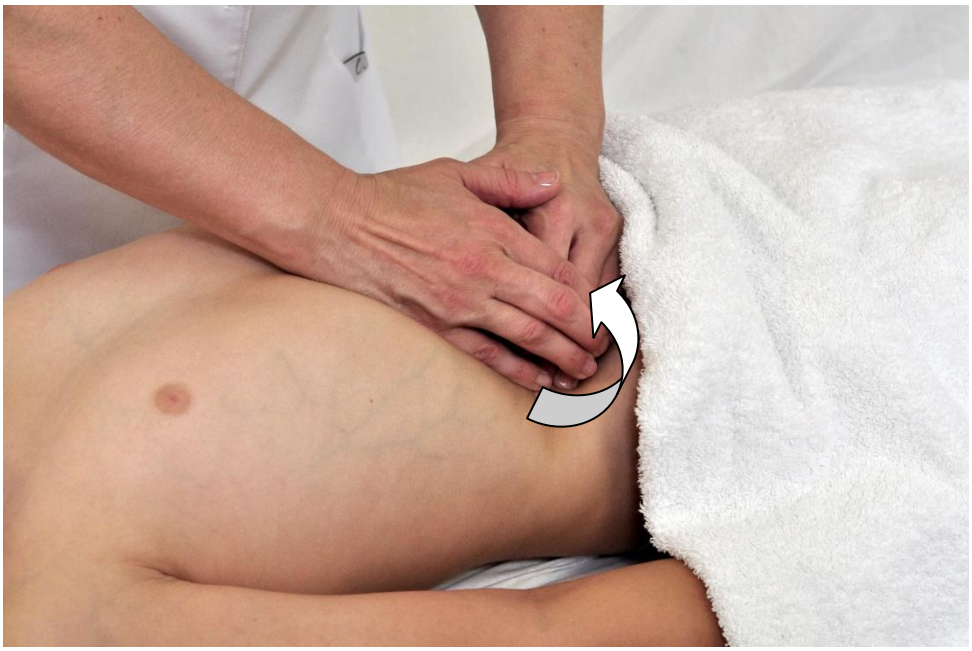


Figure 2-5: Ileo-caecal valve region technique side lying position

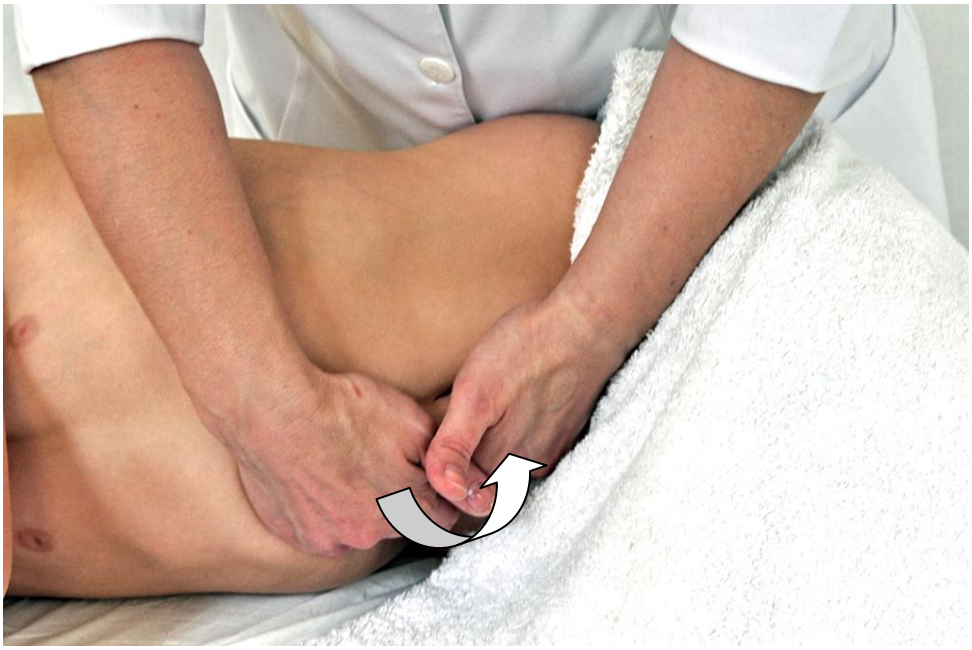
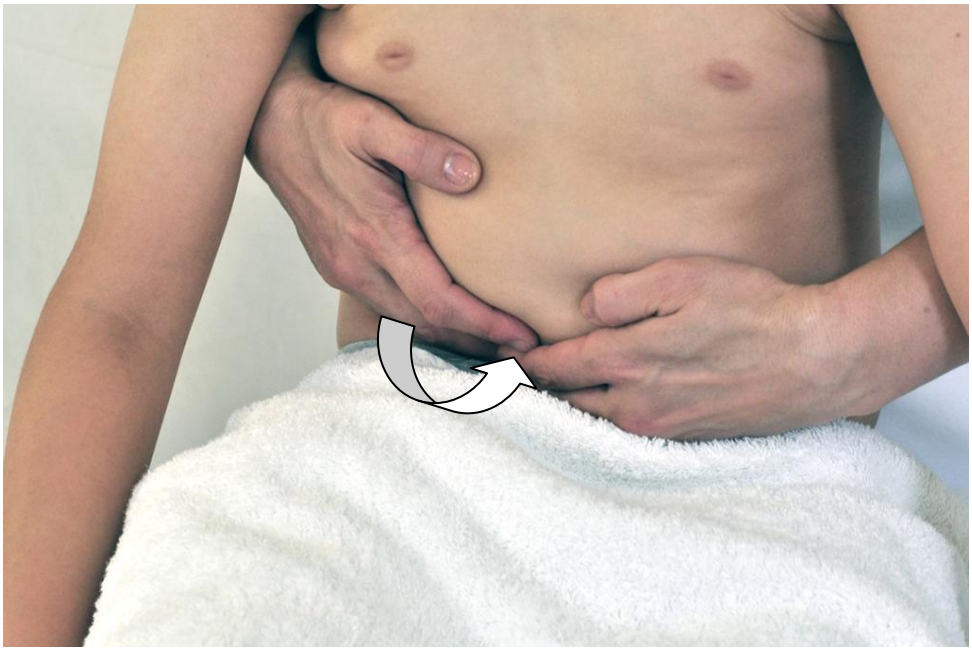


Figure 2-6: Ileo-caecal valve region technique sitting position

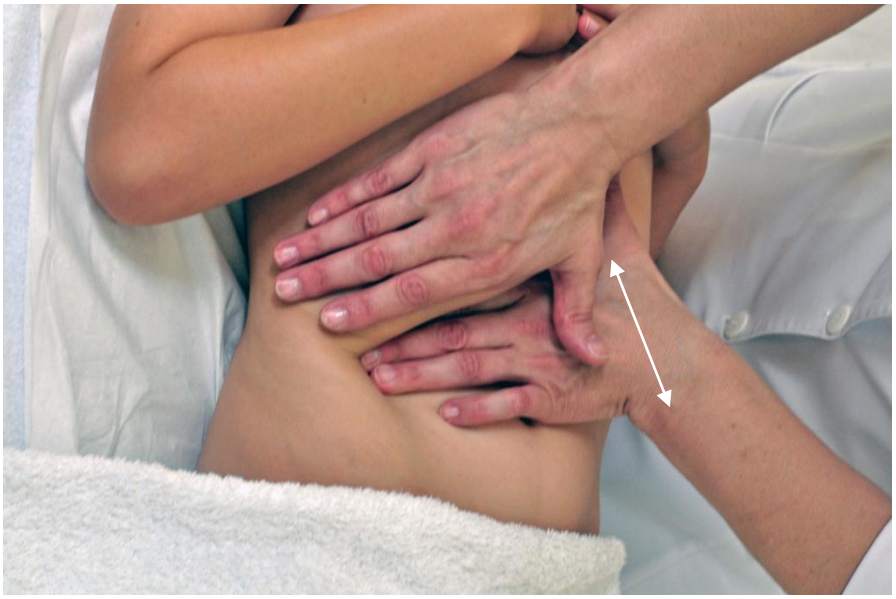


2.13.2.2 Mobilisation of the Duodenum – D2 Region in the Supine Position

The practitioner stands by the patient's left side, and using the right hand, applies an anterior posterior pressure on the ribcage of the patient thereby creating slack tissue (Figure 2-7). The practitioner's left hand palpates the

duodenal region at the D2 level and gentle backwards and forwards motion is applied (see arrow in Figure 2-7).

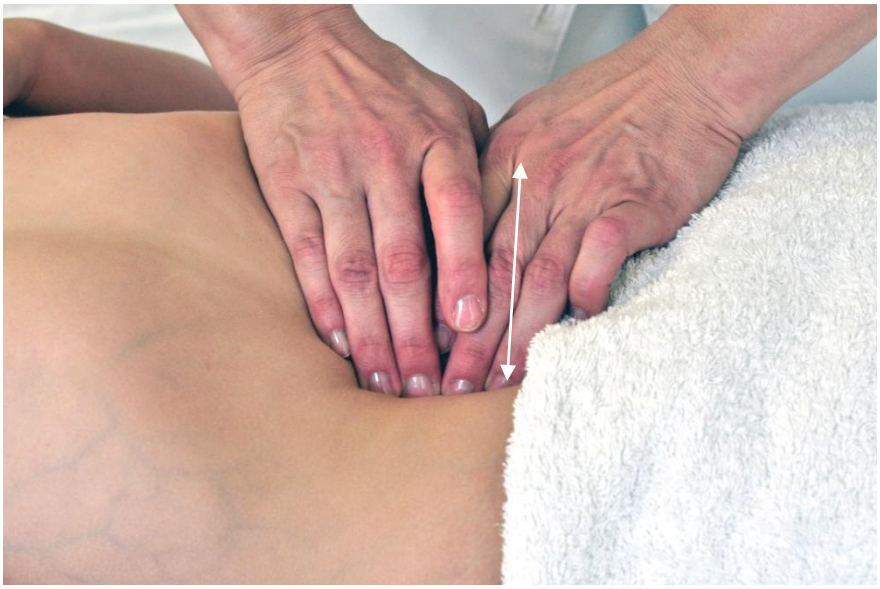
Figure 2-7: Duodenum region technique



2.13.2.3 Mobilisation of the Ligament of Treitz Region in the Supine Position

The practitioner will be at the patient's left side and will then move his/her fingers across towards the patient's right side over the ligament of Treitz region. With the practitioner using both hands, gentle backwards and forwards motion is applied (see arrow in Figure 2-8:).

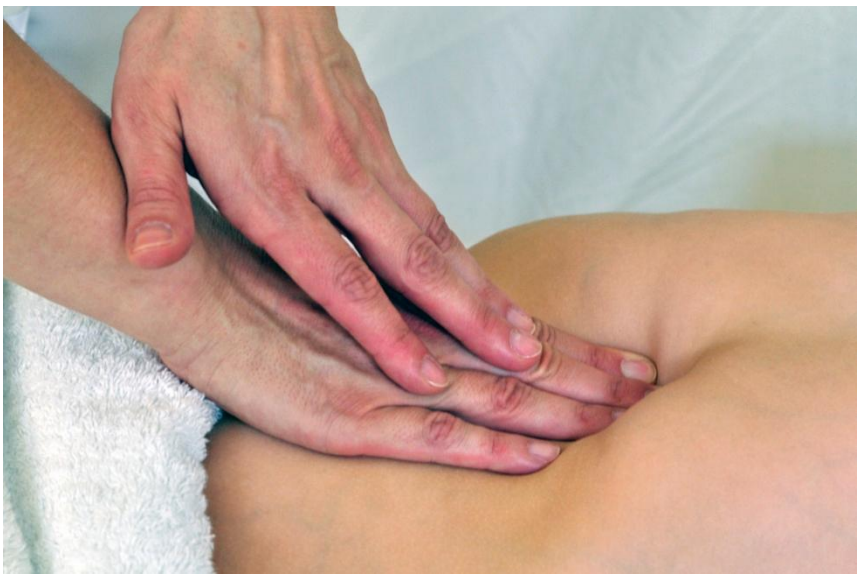
Figure 2-8: Ligament of Treitz region technique



2.13.2.4 Mobilisation of the Pancreatic Region in the Supine Position

The practitioner is by the patient's right side and places his/her right hand towards the pancreatic region. Gentle circular clockwise motion will be applied using both hands (see Figure 2-9).

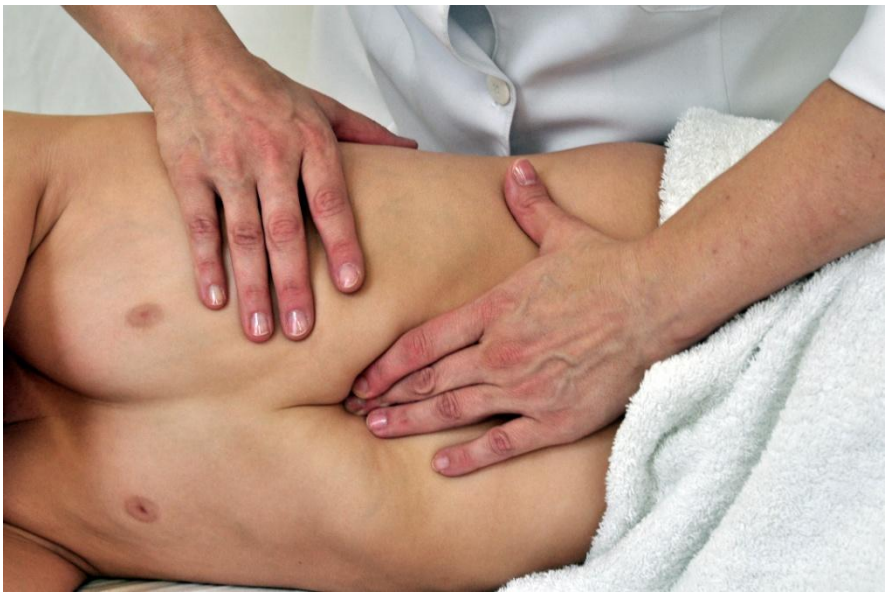
Figure 2-9: Pancreatic region technique in the supine position



2.13.2.5 Mobilisation of the Pancreatic Region in the Side Lying Position

The same technique is applied to the pancreatic region in the side lying position. The practitioner is stationed behind the patient and gentle circular clockwise motion is applied using one hand (see Figure 2-10).

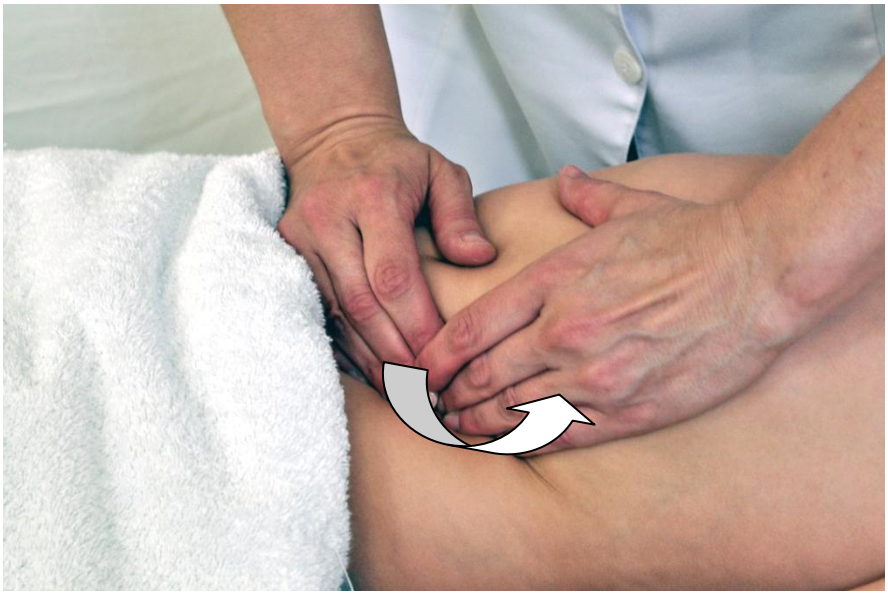
Figure 2-10: Pancreatic region technique in the side lying position



2.13.2.6 Sigmoid Colon Region Technique in Supine Position

This technique is applied with the patient lying supine. Using both hands the practitioner will mobilize the sigmoid colon region out of the left iliac fossa. Gentle motion will be applied diagonally towards the patient's right shoulder (see arrow in Figure 2-11).

Figure 2-11: Sigmoid colon region technique in the supine position



2.13.2.7 Sigmoid Colon Region Technique in the Side Lying Position

The practitioner's position is to the side of the patient, facing their head unless otherwise stated, whilst the patient will lie on his/her right side. Using both hands the practitioner will gently mobilise the sigmoid colon region out of the left iliac fossa. Gentle motion will be applied diagonally towards the patient's right shoulder (see arrow in Figure 2-12) the same technique can be applied in a sitting position (see Figure 2-13).

Figure 2-12: Sigmoid colon region technique in the side lying position

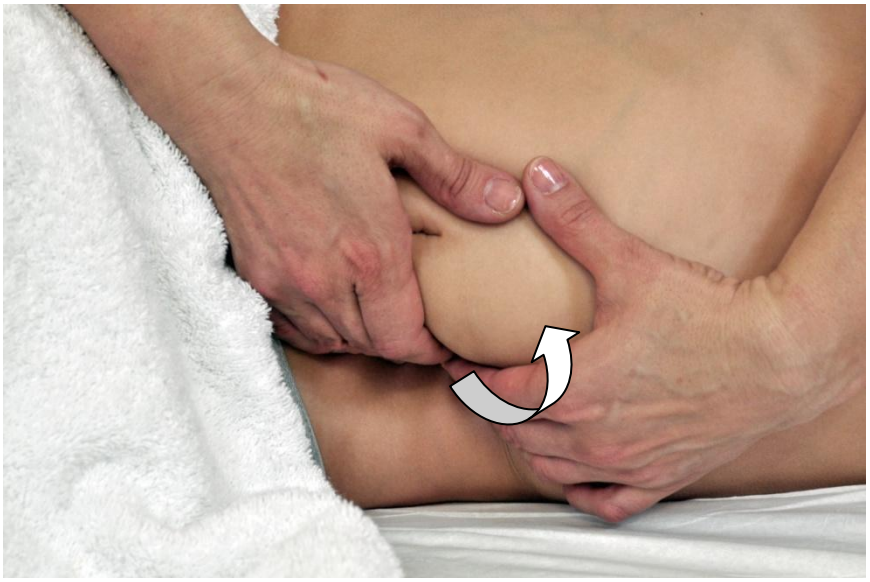
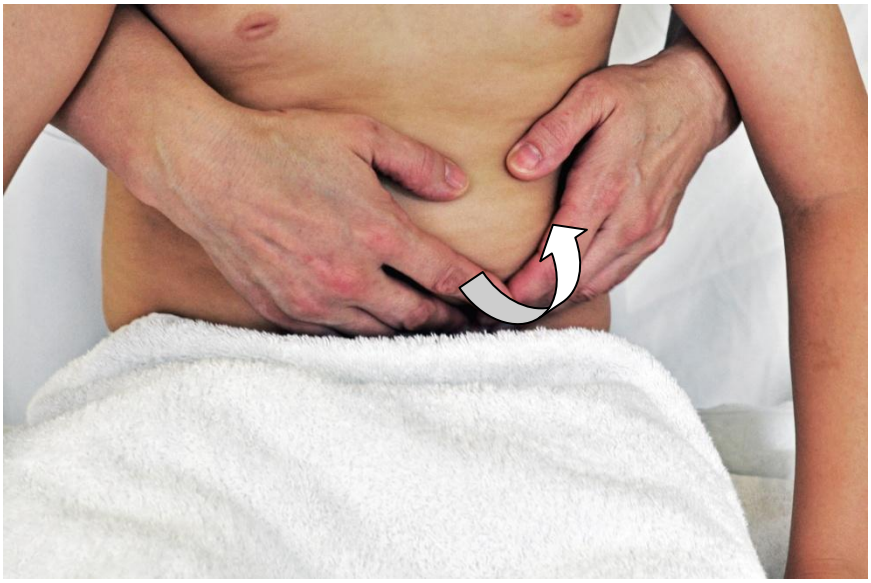


Figure 2-13: Sigmoid colon region technique in the sitting position



2.14 Quality Control and Fidelity

The VOT comprises tried and tested techniques, published in classic osteopathic publications and used internationally by the osteopathic community. These techniques were used in the pilot study that preceded the current clinical study (Bramati-Castellarin and Janossa, 2002).

The current study was set up as a patient centred, before and after intervention, study of effectiveness; using a set of VOT techniques. The consistency of the techniques was maintained by employing the same practitioner throughout the study. This study did not include an inter-rater reliability factor when executing the techniques since there was only one practitioner. The standard of techniques applied to different patients was consistent, as far as the subjects would allow.

Another issue of quality control and fidelity of the procedure is stability of the environment throughout the study period. Subjects tested, in this current study, have received VOT techniques in the same room (either private practice or school premises) and every effort has been made to keep the internal environment (same room, controlled temperature, same furniture, controlled noise levels, same personnel present) as constant as possible

during each session. The two internal environments where subjects were treated had the same environmental control throughout the study.

2.15 Collection of Faecal Samples and Materials Used

Samples were collected and placed in labelled disposable polystyrene screw cap tubes (Figure 2-14:). The tubes were given to the parents in batches for each of the three collection periods.

Figure 2-14: Disposable polystyrene screw cap tube used to collect samples

www.shutterstock.com

Period I (control/baseline); parents were given the appropriately numbered questionnaires, four collection tubes with numbered caps and four Safeboxes™ for sending the samples for analysis. Each Safebox™ was numbered sequentially from 1 to 4 (see table 2-4), making it straightforward to identify the appropriate box to be sent. Parents were asked only to date the sample and the form inside the Safebox™ Figure 2-15) which was coded for subject identification. The boxes were sent to King's College Hospital where the samples were stored at -20 ºC.

Table 2-4 Timeline for faecal sample collection

Study Period	Weeks	Faecal Sample	Lab Codes
Period I	1-6	1-4	1BTT -4BTT
Period II	7-12	5-8	1TT-4TT
Period III	18	9	1PTT

Legend to Table 2.4: Laboratory sample code BTT – before treatment, TT – Treatment period and PTT – post treatment

Figure 2-15: Safebox™

(Royal Mail, 2008)

Parents were trained in how to collect the stool samples, either from a potty or a nappy if the child was not yet potty trained. Calprotectin, M2-PK and lactoferrin remain stable at room temperature for up to seven days, allowing time for the sample to be posted. Parents were reminded by text message the day before the stool collection was due and again on the day of collection, to post the sample to King's College Hospital and to send the questionnaire to the researcher, thus ensuring that all the samples and questionnaires matched with the predetermined dates.

All laboratory analyses were undertaken by the Department of Clinical Biochemistry, King's College Hospital, Denmark Hill, London SE5 9RS. The stool samples were transported via Royal Mail, complying with the current postal regulations for the packaging of pathological specimens (Royal Mail, 2008). The law requires that packaging of pathological specimens must comply with packaging instruction P650 and the Royal Mail supplies Safeboxes™ that comply with all the regulations. The Safebox™ clearly indicated that it was transporting human material for diagnostic purposes and was designated biological hazard category B, and transported under the auspices of UN 3373. All provisions for the health and safety of specimen transportation conformed with the current regulations (Health and Safety Executive, 2005, Royal Mail, 2008, Department of Transport UK, 2009).

2.16 Analysis of Biochemical Markers

Sample analysis was undertaken at King's College Hospital, London, a centre of excellence in measuring faecal markers, and strictly followed the manufacturer's instructions (given below).

2.17 Sample Requirements for Calprotectin, M2-PK and Lactoferrin Analysis

Faeces are collected in a sterile screw cap container (Figure 2-14) and dispatched to the laboratory for analysis. Samples should not be kept at room temperature for more than 2 days (M2-PK) or 4 days (calprotectin and lactoferrin) and once received should be kept frozen at -18ºC or below until the time of analysis. One gram (calprotectin and lactoferrin) or 50-100 mg (M2-PK) of a random faecal sample is required for analysis. There are no dietary constraints for the subject prior to faecal collection for any of the markers.

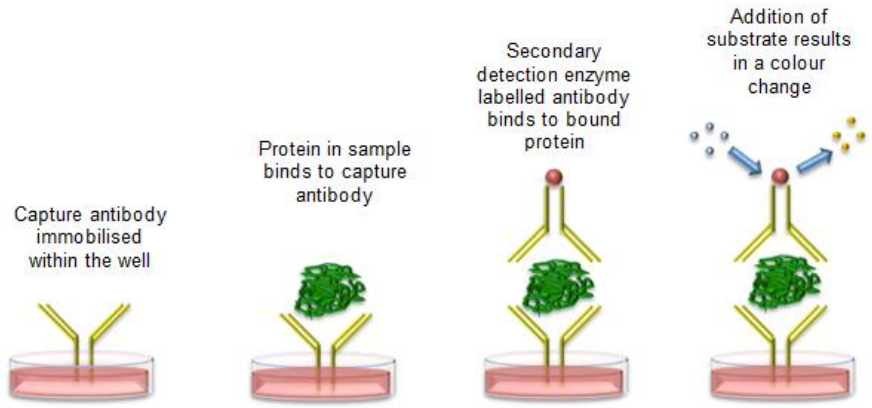
Faecal calprotectin, M2-PK and lactoferrin samples are stable for 6 months to 1year at -18ºC. After assay, samples are stored at -18ºC or below in appropriately labelled bags. The assay was performed on duplicate samples.

2.18 Sandwich ELISA (enzyme linked immunosorbent assay)

The concentrations of faecal calprotectin, M2-PK and lactoferrin were measured by a sandwich ELISA and a schematic showing the steps involved is shown in . A sandwich ELISA is typically performed in a 96-well plate, with an immobilised capture antibody specific for the antigen under analysis (in this case calprotectin/M2-PK/lactoferrin) coating the walls of each well. The sample being investigated is added to the well and any protein present will bind to the immobilised antibody. After washing to remove any non-bound material, an excess of a secondary detection antibody that is labelled with an enzyme is added. Addition of the enzyme substrate results in a colour change, proportional to the amount of protein present and this can be

enumerated on a plate reader set to the appropriate light absorbance. See Figure 2.16.

Figure 2-16: The steps involved in a sandwich ELISA



2.19 Calprotectin

Calprotectin EK-CAL©, from Buhlmann Laboratories, was used to detect calprotectin in the stool samples. Figure 2-17.

Figure 2-17: Calprotectin EK-CAL ©

(Buhlmann Laboratories, 2011)

The calprotectin assay is a quantitative ELISA using monoclonal antibodies for the detection of Calprotectin in the faeces. Initially, the calprotectin is extracted using a dissociating extraction buffer. The supernatant (after

centrifugation of the extraction buffer) is further diluted and used for the detection of calprotectin ELISA.

2.19.1 Calprotectin Preparation Procedure

40-100 mg of faeces, avoiding any undigested solid material like fibres 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 the 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 10,000 g for 20 minutes at +4ºC. 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 being 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.

2.19.2 Intra-assay precision

The intra-assay variance of Calprotectin EK-CAL © was calculated by a 20-fold determination of 6 samples (52.5 – 1246 μg/g calprotectin/mL stool); the mean coefficient of variance (CV) was 5.4% (2.7 – 8.1%).

2.19.3 Inter-assay precision

The inter-assay variance of Calprotectin EK-CAL © was calculated with 10 samples (18.1 – 1764 μg/g calprotectin/mL stool). The aliquots were tested according to the assay procedure in 10 different runs. The mean CV% was 10% (CV% 6.6 – 14.5).

2.19.4 Sensitivity

The detection limit of the assay is 10 μg/g of faeces.

2.19.5 Reference range

Faecal Calprotectin reference range 0.5 – 50 μg/g of faeces is considered normal.

2.20 M2-PK

M2-PK in stool samples was detected using the Schebo®•Tumor M2-PK™ ELISA Stool Test from ScheBo® Biotech, according to the manufacturer's instructions (Figure 2-18).

Figure 2-18: ©Schebo®·Tumor M2-PK™ ELISA Stool Test

(ScheBo® · Biotech AG, 2011)
Faecal M2-K kit is provided by ScheBo Biotech, Netanyastr. 3 (Europaviertel), 35394 Glessen (Germany)(ScheBo® · Biotech AG, 2011).

The ELISA plate is coated with a monoclonal antibody that only recognizes tumour from stool samples and standards. Tumour M2-PK binds to this antibody and a second, biotinylated, monoclonal antibody binds to tumour M2-PK during the next incubation. The assay is completed by adding a streptavidin, peroxidase conjugate, followed by TMB (3, 5,3',5'-tetra-methylbenzidine) substrate. TMB oxidises in the presence of peroxidase, giving a colour change that can be measured photometrically (ScheBo® · Biotech AG, 2011).

2.20.1 Intra-assay precision

The intra-assay variance of ©Schebo®·Tumor M2-PK™ was evaluated by a 20-fold determination of five samples (3.8-19.7 U Tumor M2-PK/ml stool). The mean coefficient of variance (CV) was 5.3% (3.0-7.9%).

2.20.2 Inter-assay precision

The inter-assay variance of ©Schebo®·Tumor M2-PK™ was calculated with five samples (1.9-18.2 U Tumor M2-PK/mL stool), that were tested on ten different days. The mean CV was 6.8% (4.4-9.4%).

2.20.3 Sensitivity

This test kit allows the quantification of Tumour M2-PK from 1 to 20 units/mL (U/mL). Values outside this range should be specified as <1 U/mL or >20 U/mL respectively.

2.20.4 Reference range

A reading of >4U/mL is considered to be a raised level of Tumour M2-PK in the stool and can be an indicator of colorectal polyps or colorectal cancer. Raised levels can also occur in acute and chronic inflammatory bowel disease and some other diseases of the digestive tract.

(ScheBo® · Biotech AG, 2011).

2.21 Lactoferrin

Lactoferrin was detected in stool samples using the Lactoferrin IBD-SCAN® from TechLab®, according to the manufacturer's instructions Figure 2-19.

Figure 2-19: Lactoferrin ELISA KIT IBD-SCAN®

(TechLab®, 2008)

The technical procedures were followed according to TechLab® (2008) IBD-SCAN catalogue specifications. Faecal Lactoferrin kit is provided by Tech Lab Blacksburg, VA24060 USA,(TechLab®, 2008).

The Lactoferrin test uses antibodies to human lactoferrin. The micro assay wells supplied with the kit contain an immobilised polyclonal antibody conjugated to horseradish peroxidase. In the assay, standards and serial dilutions of faecal samples are transferred to the micro assay wells. Lactoferrin present in the specimen, will bind to the immobilised antibody. After incubation and washing to remove any unbound protein, the antibody conjugate is added. The conjugate (red cap – Figure 2-19) will bind to a different receptor on the lactoferrin bound to the well. A second wash step is followed by addition of the substrate (blue cap – Figure 2-19) and the colour change measured is directly proportional to the concentration of lactoferrin in the sample.

2.21.1 Intra-assay precision

The intra-assay precision of the IBD-SCAN ™ test was determined by analysing reactions among nine faecal specimens. Five specimens had

elevated lactoferrin and four specimens with baseline lactoferrin. Each specimen was tested in quadruplicate. The mean CV was 12% (7.9-16.0%)

2.21.2 Inter-assay precision

The inter-assay precision was determined by analysing nine faecal specimens over a 3-day period. Each specimen was tested in quadruplicate. The mean CV was18.6% (0 - 47.7%).

2.21.3 Sensitivity

The detection limit of the assay is <6.25 ng/mL

2.21.4 Reference range

Faecal Lactoferrin reference range is 73 – 2370 ng/mL

(TechLab®, 2008).

A summary of technical information for each marker is presented in Table 2-5

Table 2-5 Summary of Technical Information for each Biochemical Marker

	Calprotectin	**M2-PK**	**Lactoferrin**
Intra-assay (CV)	5.4% (2.7 – 8.1%)	5.3% (3.0-7.9%).	12% (7.9-16.0%)
Inter-assay	10% (CV% 6.6 –14.5)	6.8% (4.4-9.4%).	18.6% (0 to 47.7%)
Sensitivity	10 μg/g	1 to 20 U/mL	<6.25 ng/mL
Reference range	<50 μg/g	>4 U/mL	73 – 2370 ng/mL

2.22 Questionnaire Structure, Design and Method of Use

A questionnaire was used to assess GI and behaviour changes during the three periods of the study. Parents completed the questionnaire according to their assessment of their child's GI and behavioural symptoms using a 24 parameter, ten point scale based on the Autism Research Institute Secretin Outcomes Survey Form, the 'S.O.S Form'; a validated and standardised questionnaire (Rimland, 1998, Brudnak et al., 2002, Unis et al., 2002, Esch

and Carr, 2004b, Erickson et al., 2005a, Sturmey, 2005, Williams et al., 2005). The different parameters used to assess symptoms are shown in Table 2-6.

According to Jerckings (1998) the use of questionnaires in quantitative research has several advantages in that a large amount of information can be collected in a relative short period of time, validity is not compromised by numbers of people involved in completing them, the data generated from the questionnaires can easily be quantified and so can be used scientifically, data can be compared and correlated with other research and also, questionnaires can be used to measure changes before/after intervention.

However, no scientific measuring tool is perfect and there are some known disadvantages of using a questionnaire to collect data for scientific research including reliability and accuracy of recall, level of subjectivity of perception when answering a question as well as omission from the questionnaire of questions that might have been important for the study.

The advantages and disadvantages of using the S.O.S questionnaire in this current study will be considered in the discussion section 4.3.4 (Period I – Study I (Part-D) – Discussion of sequential S.0.S Questionnaire.

Table 2-6: Questionnaire parameters used to assess behavioural and GI 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 utilisation
	8. Poor comprehension of verbal instructions
	9. Lack of eye contact
Ritual and Repetitive Activities	10. Abnormal repetitive gestures
	11. Need to maintain sameness
	12. Need of fixed routine
Digestive Signs	13. Diarrhoea
	14. Constipation
	15. Poor Appetite
	16. Bloating
	17. Flatulence
	18. Vomiting
General Signs	19. Unhappy
	20. Aggressive
	21. Destructive
	22. Spaced out/Non Interactive
	23. Agitated
	24. Disagreeable

Legend to Table 2-6: This questionnaire is a modified Autism Research Institute – S.O.S Form (Rimland, 1998).

A Likert Scale was used to assess severity of signs:

0 = never shows this particular sign or behaviour
1 = slight/unobtrusive
2-3 = mild
4-5 = moderate
6-7 = severe
8-9 = extreme/incapacitating

The questionnaire parameters were collated with the contemporaneous faecal marker results for calprotectin, M2-PK and lactoferrin.

The questionnaires were used during the three periods:

- Period I: The control/baseline period. Four questionnaires were completed during the first six weeks of the clinical trial. Questionnaires 1-4 were distributed at weeks 1, 3, 5 and 6 before the treatment period in order for parents to achieve familiarity with using the questionnaire and to act as a control baseline for responses.
- Period II: The treatment period. Four questionnaires were completed during the six week treatment period of the clinical trial. Questionnaires 5-8 were distributed at weeks 8, 9, 10 and 12 of the study.
- Period III: The post-treatment period. One questionnaire was completed six weeks after completion of treatment, at week 18 of the clinical trial.

A timetable for completion of questionnaires was agreed with the parents before commencement of the study (Table 2-7). Parents were provided with a stamped addressed envelope for each questionnaire and were reminded of the completion deadline by text message.

Table 2-7: Timeline for questionnaire completion

Study Period	Weeks	Questionnaires	Questionnaires Code
Period I	1-6	1-4	1BTT -4BTT
Period II	7-12	5-8	1TT-4TT
Period III	18	9	1PTT

Legend to Table 2.7: Questionnaires Code: BTT – before treatment, TT – Treatment period and PTT – post treatment

2.23 Measures of Outcome

2.23.1 S.O.S Questionnaire

To measure outcomes for the GI signs and symptoms and behaviour, the S.O.S questionnaires were completed, pre-treatment (control/baseline), during the treatment period and post treatment.

This questionnaire has been previously validated and standardised (Rimland, 1998, Brudnak et al., 2002, Unis et al., 2002, Esch and Carr, 2004b, Erickson et al., 2005a, Sturmey, 2005, Williams et al., 2005).

2.23.2 Biochemical Markers

To measure outcome for GI inflammation three faecal biochemical markers (calprotectin, M2-PK and lactoferrin) were measured in stool samples, pre-treatment, during the treatment period and post-treatment.

2.24 Schedule and Monitoring of Data Collection

Parents were provided with a specific timetable for posting the questionnaires and the faecal samples during each period (weeks 1, 3, 5 and 6 for the control/baseline period (Period I); weeks 8, 9, 10 and 12 for the treatment period (Period II) and week 18 for the post treatment period (Period III) (See section 2.22 and Table 2.7). They were also provided with instructions for filling in the numbered questionnaires and collecting the numbered faecal samples as well as the procedure for posting them.

The patient details were entered onto a secure practice management system and backed up in a secure remote system, conforming to the Data Protection Act. All of the questionnaires and the sample collection timetable per period were entered in the system as well as each individual appointment for each VOT.

An automated reminder text message advised the subject's parents of the specific date to post the questionnaires and samples. The same system was utilised to monitor the appointment dates, along with informing of any research dropouts or did not show (DNS) for any VOT session. Drop outs and DNS sessions are presented in the results section of this thesis.

Code numbered questionnaires from Periods I, II and III were entered in the TM2 system as received. Receipt of the corresponding Safeboxes™ was confirmed with the allocated laboratory assistant at King's College Hospital. The questionnaires, and their corresponding Safeboxes™, were individually coded by period i.e. questionnaire one before treatment was coded as BT1 and the Safeboxes™ before treatment one was coded BT1 (see tables 2-4 and Table 2-7).

During Period II, the parents received automated text messages to remind them both of appointments and to post the questionnaire, and to collect the faecal sample and to post the Safeboxes™. The text messages read as follows:

'This is a reminder that your next appointment for the Osteopathic Autism Research is tomorrow at.... time.'

And:

'This is a reminder for you to post questionnaire number X and sample number X by tomorrow.'

These procedures were put in place to monitor and record any missing questionnaires and samples.

2.25 Data Management and Avoidance of any Data Bias

Data management followed a strict procedure, outlined below, and aimed at avoidance of error and maintenance of data quality for analysis and interpretation.

All questionnaires submitted throughout the study period were stored securely, in a locked cabinet, until the study had been completed. After the end of the Period III, an independent researcher entered the raw data collected from the questionnaires into a spreadsheet. All the entries were rechecked by an independent statistician before the statistical analysis was performed. The researcher was not part of this process and all the data were analysed only after all the entries from the questionnaires were computed as raw data.

All of the samples received by Kings College Hospital were analysed by an independent laboratory assistant, who was not part of the study. The data generated from the three faecal biochemical markers (calprotectin, M2-PK and lactoferrin) was entered into a spreadsheet and emailed to the researcher. The researcher did not have any contact with the data prior to this, thus avoiding the risk of bias.

The researcher then cross referenced the data generated from the questionnaires with the data from the biochemical markers and an independent statistician checked the data and computed any missing data.

All the original data are archived, normally for five years.

The remainder of the faecal samples submitted will be kept, frozen, at King's College Hospital until June 2015.

2.26 Pre-Statistical Analysis Testing

A series of pre statistical testing was performed before any statistical analysis was completed.

2.26.1 Determination of the Sample Size

One of the pre-statistical analysis tests performed for this current study estimated sample population and the statistic power of the study. A monogram (Altman, 1982, Whitley and Ball, 2002) was used for calculating sample sizes (Cohen, 1977). In the current study, the sample size was estimated at n = 52 with Cohen's d = 0.78 with 80% power with a statistical significance 0.05 (p=0.05). All effort was taken to correctly identify the true effect size between the tested groups.

2.26.2 Test-Retest Analysis

The test-retest reliability is the most common measure of reliability and assesses the reliability between two scores using correlation analysis (Field, 2012; Tabachnick & Fidel, 2012). The resulting correlation between the scores is referred as the reliability coefficient. The reliability coefficient is graded from 0.9 or greater (excellent), 0.9 to 0.8 (good), 0.8 to 0.7 (acceptable), 0.7 to 0.6 (questionable), 0.6 to 0.5 (poor), and less than 0.5 (unacceptable) (Chronbach et al. 1972; Thorndike, 1947; Nunnally, 1978; Field 2012). This analysis was used for Study I.

2.26.3 Chronbach's Alpha Analysis

Chronbach's Alpha analysis, a coefficient of reliability (or internal consistency), was conducted on the S.O.S questionnaire to evaluate its reliability and the reliability of the four S.O.S questionnaire subscales (Appendix 7). Chronbach's Alpha scores above 0.7 are considered acceptable for reliability (DeVillis, 2003, Field 2009). The overall reliability of the S.O.S questionnaire was 0.881 on Chronbach's Alpha Scale suggesting a good internal consistency. Table 2-8 represents Chronbach's Alpha internal consistency levels. More details of the Chronbach's Alpha results for each questionnaire subscales are presented in the results section of this thesis. The evaluation of reliability of the S.O.S questionnaire was performed before any correlation between periods was completed. This was used for Study I.

Table 2-8 Chronbach's Alpha Internal Consistency Levels

Cronbach's Alpha	Internal Consistency
$\alpha \geq 0.9$	Excellent
$0.7 \leq \alpha < 0.9$	Good
$0.6 \leq \alpha < 0.7$	Acceptable
$0.5 \leq \alpha < 0.6$	Poor
$\alpha < 0.5$	Unacceptable

2.27 Statistical Analysis

The statistical analysis performed during Period I (control/baseline) established baseline levels for the three biochemical markers prior to the initiation of VOT. Baseline data were used to correlate the results between the three markers (calprotectin, M2-PK and lactoferrin) during Period I.

The data collected from the three biochemical markers and the questionnaires were analysed and correlated, taking the results of Period I, Period II and Period III.

The researcher had several meetings with Dr Bernardo Tura from the IDO'R Research Institute, Brazil and statisticians from the University of Westminster (Dr Clair Robertson) and from other institutions in the UK (Dr Stan Cohen). Moreover, Dr Andrew Dalby from Westminster University was consulted via the director of studies. The purpose of the meetings was to discuss the appropriate statistical analyses of the collected data and the specific statistical test used. The statisticians indicated multiple linear regression analysis as the most appropriate method. Multiple linear regressions were chosen due to the high sensitivity in measuring treatment effectiveness in each individual subject (Rosner, 2011). This type of analysis avoids the variability observed between subjects and which is often found in studies that recruit a separate set of individuals as controls (Stewart, 2010, Rosner, 2011).

Dr Tura suggested that the data generated from the set of 8 questionnaires could be correlated with the results of the 8 sets of the main biochemical marker - calprotectin. Each parameter from the questionnaire was analysed individually using a univariate model. Each parameter was analysed 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 for single outcome variables (Nakai, 2009). A mixed effect model was used to analyse the repeated observations for this study. In this model the analysis takes into consideration any missing data which could potentially have an effect on the end results, an inherent problem of longitudinal studies (Pan et al., 2012). The mixed effect model is used to correlate repetitive measures and it also measures the “within” and “between” subject error. This mixed effect model has been employed for the analysis of the data generated by longitudinal studies (Mallinckrodt et al., 2003, Pan et al., 2012) and Dr Tura has used these statistical analyses to test data from his own research studies (Aguiar et al., 2010, Benchimol-Barbosa et al., 2013).

Statistical tests performed on these data are outlined below.

2.27.1 Repeated Measure ANOVA

A repeated measure design is a sensitive model enabling predictors or trends to be uncovered. The advantage of this type of design is that it follows the same subject over a period of time enabling detection of variations in the tested parameters within the study population, even when the effect is small. (Rosner, 2011). The variability of the subjects is divided into ‘between subjects’ and ‘within subjects’.

Repeated measure ANOVA is a parametric statistical test that allows several groups to be simultaneously tested and indicates whether there are significant differences between them. ANOVA was performed on the data generated from the three biochemical markers collected during the three study periods. This type of analysis compared the differences between group means and the variation between and within groups. It also compares the same subject on a

continual variable across three or more periods of time (Period I, Period II and Period III in this case).

ANOVA produces an F value which is the ratio of the between-groups variance to the within-groups variance. See the Figure 2.19 for illustration of F-value.

Figure 2-19 illustration of ANOVA F Value

$$F = \frac{\text{Between-groups variance (variance calculated based on the entire sample)}}{\text{Within–groups variance (variance calculated separately for each group)}}$$

Repeated measure ANOVA assumes equal variances or homogeneity on a linear model. It assumes all errors are equal to each other, the variances are normally distributed and that the errors are independent. To test the equality of variance a Levene's test was performed on the raw data. Levene's test determines whether the variance in scores is the same across all groups. This means that the difference between the raw data across all groups is the same. The data does not violate homogeneity if Levene's test is 0.05 or greater. This analysis was used for Study I; II and III.

2.27.2 Wilk's Lambda

Multivariate Analysis of Variance (ANOVA) tests differences between the means of a group of subjects on a combination of dependent variables. The test is better known as Wilk's Lambda test. This statistical test is the most widely used to measure the proportion of variance in a combination of dependent variables indicating whether or not there is a relationship between the independent and dependent variables This analysis was used in study. This analysis was used for Studies I, II and III.

2.27.3 Bonferroni Adjustment

The Bonferroni adjustment is obtained by dividing the significance level by the number of pairwise comparisons; assuming a single test performance (See

Figure 2.20). For example, if the pairwise comparison is comparing 3 groups the single p value should be divided by 3 ; therefore the null hypothesis would only be rejected if p=0.017 (0.05/3=0.017) (Bland and Altman, 1995). This analysis was used in study IV.

Figure 2-20 Bonferroni Adjustment Calculation

$$\text{Stringent p value} = \frac{\text{Significance level assuming a single test (0.05)}}{\text{Number of pairwise comparisons}}$$

The advantage of Bonferroni adjustment is that it is simple to compute when performing multiple comparisons. It is not a statistical correction but a p value adjustment makes it more stringent. This type of adjustment is used to reduce the chances of false positive results (Type I error) (Bland and Altman, 1995). This analysis was used for Study I.

2.27.4 Pearson Correlation

Pearson's correlation is a parametric measure that indicates whether a statistically significant relationship exists between two variables. It also indicates how close the relationship is on a straight line as well as the direction of a linear relationship (increasing or decreasing). It is measured using the correlation coefficient (r) which can take any value between -1 and +1, the + indicating positive correlation whilst a – indicates a negative correlation. The perfect positive correlation is when r = +1 and perfect negative correlation when r = -1. Table 2-9 illustrates the value of the correlation co-efficient and the strength of the correlation. When the r value is zero, r =0, there is no linear correlation.

Prior to performing a Pearson's correlation, it is necessary to ascertain that the variables are continuous and normally distributed. There should be a linear relationship between variables and no significant outliers. This analysis was used in study I.

Table 2-9 The Pearson's Correlation Co- Efficient and the Correlation Strength

Value of correlation Co-Efficient	Internal Consistency
1	Perfect
0.8-0.9	Very Strong
0.5-0.8	Strong
0.3-.05	Moderate
0.1-0.3	Modest
>0.1	Weak
0	Zero/No correlation

Legend to Table 2-9: The Pearson's Correlation Co- Efficient and the Correlation Strength according to Evan (1996) – Straightforward statistics for the behavioural science.

2.27.5 Linear Mixed Effect Model

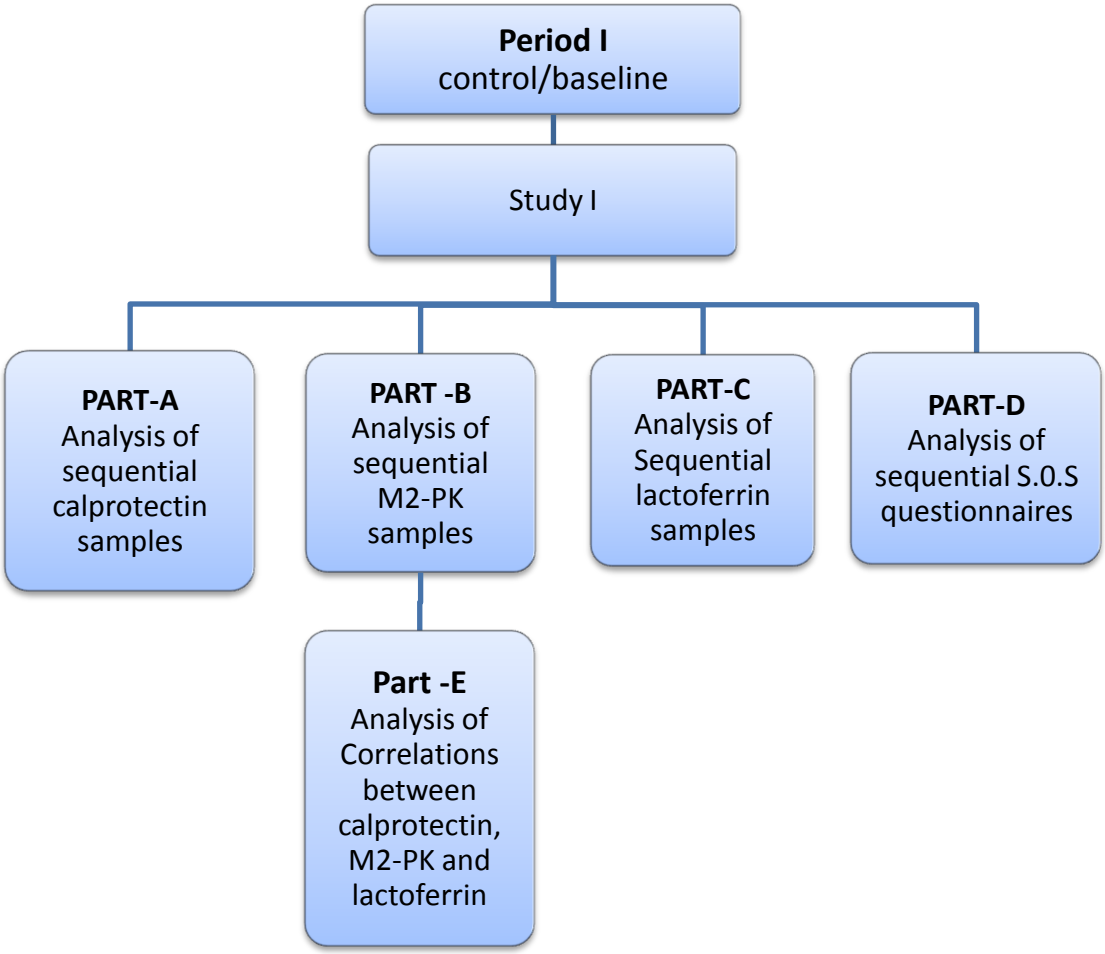
A linear mixed effect model incorporates fixed and random effects in the same model. It takes into consideration the fixed-effect i.e. behaviour of the entire population and random effects which are associated with the individual experimental units sampled from the population. The mixed effect model is used to correlate repetitive measures and it also measures the within and between subject error. It is used to analyse the repeated observations from longitudinal data. In this model the analysis takes into consideration missing data that could potentially have an effect on the outcome; an inherent problem of longitudinal studies (Pan et al., 2012). A mixed effect model has been employed for the analysis of the data generated by longitudinal studies (Mallinckrodt et al., 2003, Pan et al., 2012).

The mixed effect model has three parameters, specifically; standard deviation of the random effect, standard deviation of the residual effect 'per observation' and the fixed effects parameters that are labelled as intercepts. This analysis was used in study IV.

Chapter 3 - Results

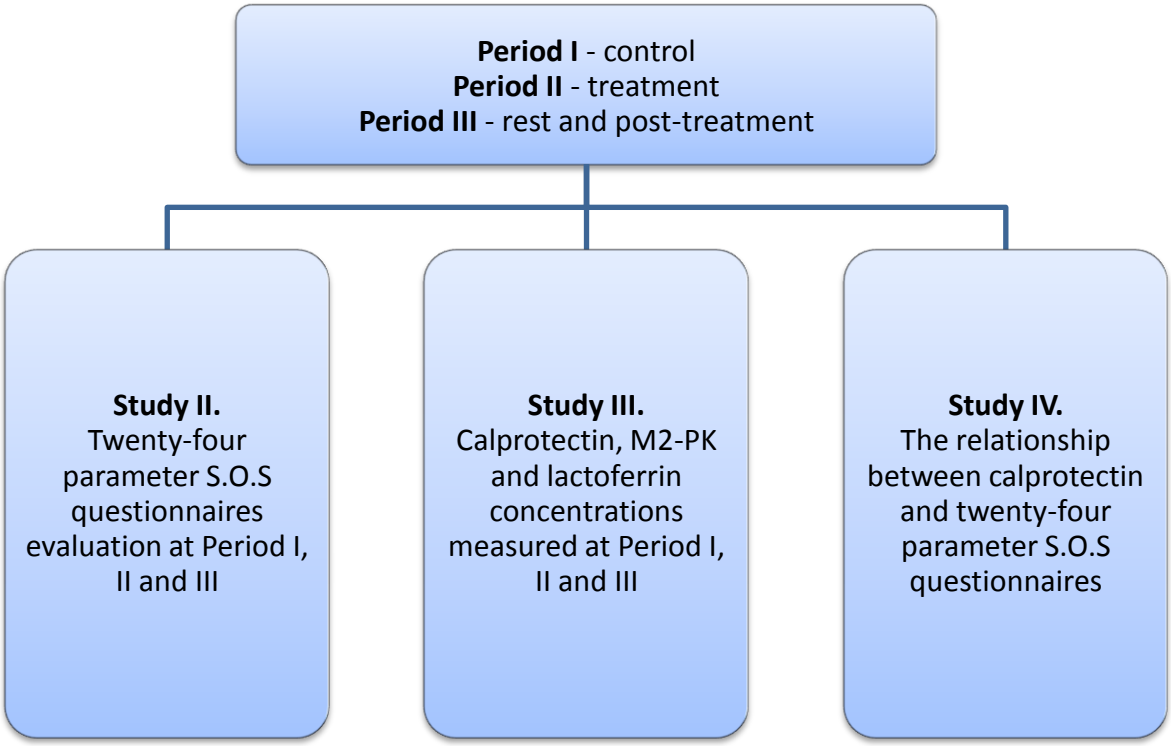
This chapter presents the detailed analysis of the data collected over the three periods of the research study. The results were analysed and divided into four studies. Figure 3.1 summarises Period I, with Study I divided into five parts, A, B, C, D and E. Figure 3.2 summarises Periods I, II and II for studies II, III and IV.

Figure 3-1 Sample groups from the control period



Data for the Study I part E is derived from parts A to C

Figure 3-2 Sample groups from the control, treatment and post-treatment period



3.1 Statistical Analysis Period I – Control/Baseline Period

Throughout this results section, the term “Control Period” should always be understood to mean the “Control/Baseline Period” since the pre-intervention samples act as the study controls and form a baseline for comparison with the post-intervention results.

The organisation of the data collected during the control period – Period I, is shown in Figure 3-1.

Period I. Control period (see methodology). At least three stool samples for biochemical (calprotectin, M2-PK and lactoferrin) analysis were collected during this period. Safeboxes™ were clearly labelled 1-4 to identify that they contained samples from this period.

3.1.1 Control Period Result Section

The control period section is presented in four parts to represent the four component parts of Study I. Study I is divided as follows:

Part A – Analysis of calprotectin in sequential stool samples.

Part B – Analysis of M2-PK in sequential stool samples.

Part C – Analysis of lactoferrin in sequential stool samples.

Part D – Analysis of sequential S.0.S Questionnaires.

Part E – Analysis of Correlations between calprotectin, M2-PK and lactoferrin from the sequential stool samples.

3.2 Raw Data

Raw data for collected faecal samples and questionnaires are presented in Appendix 7 and 8 respectively.

3.3 Results

Stool samples and Questionnaires were collected and the samples bio-analysed as described methods. The resultant data was statistically analysed using SPSS software package, IBM® SPSS® Statistics 21, and employing the appropriate statistical tests described in methods.

3.3.1 Period I – Study I (Part A) – Analysis of Calprotectin in Sequential Faecal Samples

The sample size for the study was 49. However, one patient failed to post samples 2, 3, and 4 and was withdrawn from this part of the study. The remaining 48 patients were included in the statistical analysis. All other participants had at least two results across the four pre-treatment measures, as required to calculate a mean and standard deviation (Table 3-1).

Table 3-1 Descriptive Statistics Calprotectin (mg/L) Concentration Scores

	M	SD	Range	N
Calpro 1	46.2	92.2	26.3-511	48
Calpro 2	76.8	240	28.6-1582	48
Calpro 3	54.2	99.3	44.7-630	48
Calpro 4	33.5	57.8	25.3-381	48

Legend to Table 3-1: This table represents the mean, standard deviation and range of the data set (n=48)

The range and the standard deviation have demonstrated between Calprotectin means 1 to 4 (Table 3-1) were variable, therefore a test-retest reliability analysis was performed. This test is the most common measure of reliability and uses correlation analysis. The reliability coefficient of calprotectin mean scores for the 48 subjects on four different occasions was calculated. The test-rest reliability, analyses the consistency of results over time. The test is applied to the same subject on two separate occasions (i.e. sample 1 collected during week one compared to sample 2 collected during week two) and the scores correlated to give the coefficient of reliability of that specific occasion. Results closest to 1 ($r = 1$) reflect excellent reliability, while results closest to 0 ($r = 0$) reflect unacceptable reliability between samples assessed on two separate occasions. (Field, 2012; Tabachnick & Fidel, 2012) (see methodology chapter for more information). The resulting correlation between the mean scores measured on two different occasions is referred to as the test-retest reliability coefficient for that specific point in time.

Correlations between samples 1 and 2 reflected poor reliability ($r = 0.60$, $p < 0.001$) while samples 1 and 3 ($r = 0.30$, $p < 0.001$), 2 and 3 ($r = 0.45$, $p = 0.001$), and 3 and 4 ($r = 0.30$, $p = 0.77$) had unacceptable reliability. Samples 1 and 4 had questionable reliability ($r = 0.65$, $p = 0.001$). Only samples 2 and 4 produced excellent reliability ($r = 0.90$, $p < 0.001$) (see Table 3-2).

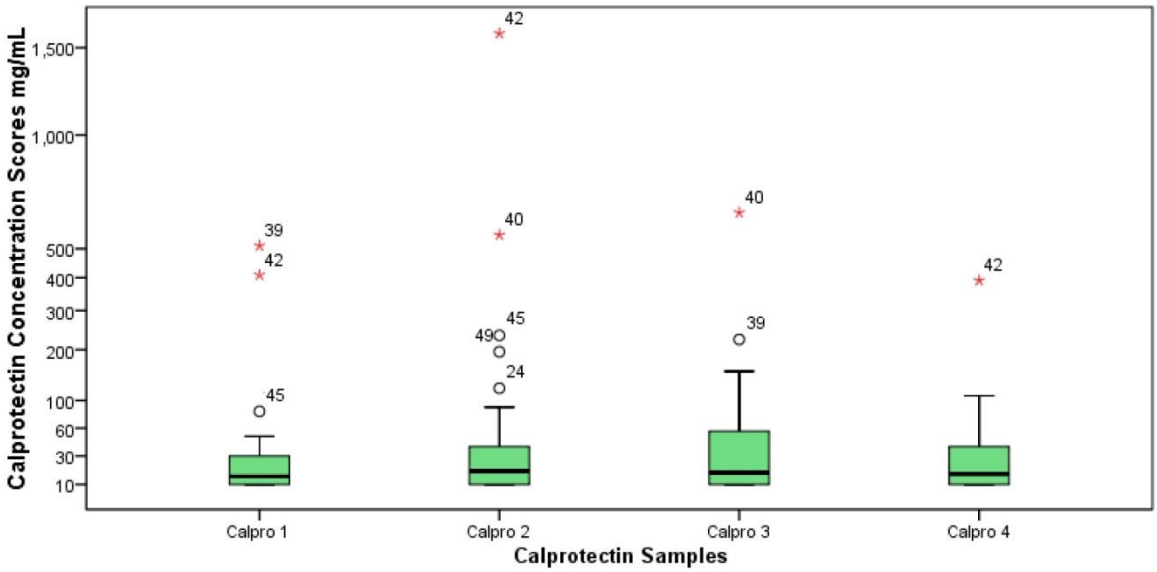
Table 3-2 Test-Retest Correlation Matrix of Calprotectin (mg/L) pre-treatment concentration scores (n = 48)

	Calpro 1	Calpro 2	Calpro 3
Calpro 2	0.60		
Calpro 3	0.30	0.45	
Calpro 4	0.65	0.90	0.30

As the test-retest reliability only tests the correlation between two samples over time it could not provide a baseline to be used here and further statistical analysis was necessary. Prior to determining the baseline, preliminary statistical analysis was necessary to test the general internal consistency of the marker using mean scores of the four samples collected over the six week period of the control/baseline period. To test the general reliability of the marker (or general internal consistency) a Cronbach's Alpha test was performed on the four mean measures of calprotectin pre-treatment. The result of the Cronbach's test suggests that the overall internal consistency of the baseline mean values was good with a score of 0.70 (Cronbach's Alpha = 0.70 or 70%) and so the mean scores for calprotectin are reliable for use as baseline measures for this work.

A repeated measure ANOVA was used to test the mean scores of the four pre-treatment samples. Since parametric analysis is highly sensitive to outliers, an outlier analysis was performed prior to the execution of the repeated measures ANOVA (see methodology information on ANOVA). For the analysis of the outliers a Box and Whisker plot analysis of all 48 subjects' individual samples was plotted. Box and Whisker plots, provide information on continuous variables including mean, interquartile range and outliers and are widely used for outlier detection (Pallant, 2006, Field, 2007). In this study, the outliers were marked with an asterisk and represented values that were three box lengths from the edge of the box. The results of the box and whisker plot indicated that there were three cases with outlier values. These three cases were withdrawn from the 48 subjects bringing the number of subjects to 45 (n=45) (see Figure 3-3).

Figure 3-3 Box & Whisker plot of calprotectin concentration (mg/L) over the control period for 4 sequential samples



Legend to Figure 3-3: Three subjects were classified as outliers and are indicated by asterisks. Initial sample size, n=48, was reduced to n=45 after exclusion of outliers (patients 39, 40 and 42)

A repeated measures ANOVA was conducted to determine whether there was a significant difference in calprotectin mean scores across the four pre-treatment concentration scores. The repeated measures ANOVA is used to compare the same subjects using a continuous variable (Field, 2012; Tabachnick & Fidel, 2012). There are three important indices that are produced in the results of the repeated measures ANOVA namely the Wilk's Lambda (W), the F ratio (F), and the p value. Wilk's Lambda is the product of the unexplained variance on each of the independent variables in the analysis (Field, 2012; Tabachnick & Fidel, 2012). The F ratio is where the variance in scores within the pre-treatment measures is compared with the variance in scores across the pre-treatment measures. If the p value is less than 0.05 then there is a significant difference in mean scores between at least two of the four pre-treatment measures (see methodology chapter for more information).

Only matching samples were included in the repeated measure ANOVA. The removal of the three outliers from the initial 48 (n=48) subjects resulted in 45 subjects for the analysis. Further review of the samples revealed that there

were another two subjects with only two samples, rather than the maximum four, for calprotectin analysis. These two subjects were withdrawn from the repeated measures ANOVA analysis bringing the sample size to 43 (n=43). Means and standard deviations of the 43 subjects included in the ANOVA analysis of calprotectin pre-treatment concentration scores are presented in Table 3-3.

Table 3-3 Descriptive Statistics Calprotectin (mg/L) Pre-treatment Concentration Scores (n=43)

	M	SD	Range	N
Calpro 1	28.7	34.8	10-164	43
Calpro 2	32.9	45.7	10-234	43
Calpro 3	34.6	36.4	10-153	43
Calpro 4	25.3	21.0	10-108	43

Legend to Table 3-3: This table represents the mean, standard deviation and the range after outlier removal.

Results of the repeated measure ANOVA indicated that there was no significant difference between the four pre-treatment concentration scores (see Table 3-4).

Table 3-4 Repeated Measure ANOVA for Calprotectin (mg/L) Pre-treatment Concentration Scores

			Hypothesis		
Effect	Value	F	df	Error df	p
Wilks' Lambda	0.91	1.30	3.00	40.00	0.285

As the rest-test reliability presented some differences when correlating two scores of the same subject on two different occasions (Table 3-2) it was necessary to assess the value for each calprotectin sample across the population (see Table 3-5). This analysis used the raw data presented in Appendix 7.This analysis included the 43 subjects who had all four pre-

treatment samples analysed plus the 3 subjects who were considered outliers but also had all four samples analysed. The total subject number for this analysis was 46 (n= 46).

Thirty subjects out of 46 (65.2%) had all 4 pre-treatment calproctectin concentration scores between 0 and 50mg/mL. Seven subjects (15.2%) had one concentration score above 50mg/mL, five subjects (11%) had two concentration scores above 50 mg/L and one (2.1%) had three concentration scores above 50 mg/mL. Three subjects out of the total of 46 (6.5%) had all four pre-treatment concentration scores above 50 mg/mL (see Table 3-5).

Table 3-5 Weekly Calprotectin Pattern of Individual Concentration Scores mg/mL

	N	%
All scores 50 or less	30	65.2
One score above 50	7	15.2
Two scores above 50	5	11
Three scores above 50	1	2.1
Four scores above 50	3	6.5
Total	46	100.0

Legend to Table 3-5: This table represents calprotectin value for 46 subjects who had all four samples analysed including the 3 outliers. (n=46)

Please see table Calprotectin reference range 2.19.5, in methods section.

3.3.2 Period I – Study I (Part B) – Analysis of M2-PK in Sequential Faecal Samples

The statistical analysis of M2-PK pre-treatment concentration scores followed the same structure as the calprotectin pre-treatment analysis. The sample size for M2-PK was the same as calprotectin pre-treatment number (see section 3.3.1). The sample size for this analysis was 48 (n=48) (see Table 3-6).

Table 3-6 Descriptive Statistics M2PK (U/mL) Pre-treatment Concentration Scores (n= 48)

	M	SD	Range	N
M2PK-1	2.6	4.2	1-19	48
M2PK-2	2.4	4.2	1-19	48
M2PK-3	3.6	5.1	1-19	48
M2PK-4	2.0	2.7	1-16	48

Legend to Table 3-6: This table represents the mean, standard deviation and range of the data set (n=48)

A test-retest reliability analysis was conducted to assess the reliability of M2-PK concentration scores within the 48 subjects. As indicated with the analysis of calprotectin, (see section 3.3.1) the test-rest reliability analyses the consistency of the results over time. The test is applied to the same subject on two separate occasions. The test-retest reliability is the most common measure of reliability and assesses the reliability of scores using the correlation analysis of an individual subjects (Field, 2012; Tabachnick & Fidel, 2012) (see methodology chapter for more information).

Results of the 48 subjects test retest reliability analysis of pre-treatment samples of M2-PK indicated that the correlations between samples 1 and 2 ($r = 0.41$, $p < 0.001$), 1 and 4 ($r = 0.46$, $p <= 0.001$), 2 and 4 ($r = 0.495$, $p < 0.001$) and 3 and 4 (0.393, $p = 0.01$) reflected unacceptable reliability. Samples 1 and 3 ($r = 0.59$, $p < 0.001$) and 2 and 3 ($r = 0.690$, $p < 0.001$) had questionable reliability (see Table 3-7).

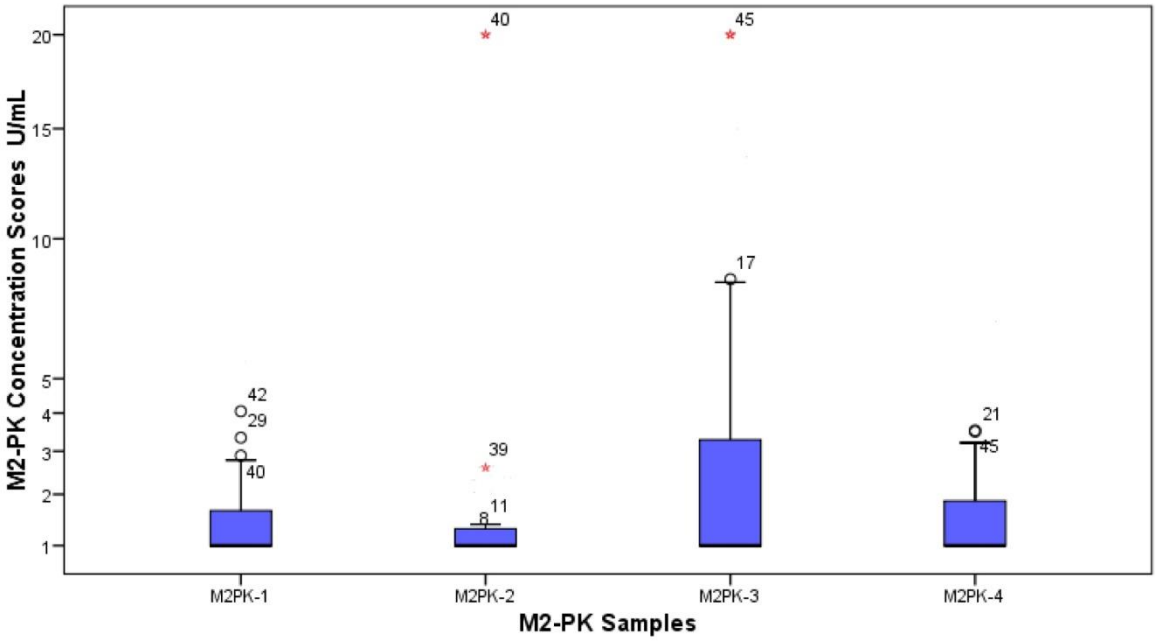
Table 3-7 Test-Retest Correlation Matrix of M2PK (U/mL) Pre-treatment Concentration Scores (n =48)

	M2-PK-1	M2-PK-2	M2-PK-3
M2-PK-2	0.41		
M2-PK-3	0.59	0.69	
M2-PK-4	0.46	0.49	0.39

As previously stated with the calprotectin analysis (section 3.3.1) the test-retest reliability only tests the correlation between two samples over time and it did not provide a baseline to be used here. Further statistical analysis was necessary. Prior to determining the baseline, preliminary statistical analysis was necessary to test the general internal consistency of the marker using the mean scores of the four samples collected over the six week period of the control/baseline period. To test the general reliability of the marker (or general internal consistency) a Cronbach's Alpha test was performed on the four M2-PK pre-treatment mean measures. The results of the Cronbach's test suggests that the overall internal consistency of the baseline for M2-PK pre-treatment mean scores is 0.8 (Cronbach Alpha=0.80 or 80%). This suggests that the mean scores for M2-PK measures are reliable to be used as baseline for this thesis.

Outlier analysis was performed on the pre-treatment M2-PK samples, as before (see section 3.3.1). Samples from 48 subjects were analysed and plotted before conducting repeated measures ANOVA. Results of the Box and Whisker plot analysis indicated that there were three outliers. These three outliers were excluded, reducing the sample size from 48 to 45 (see Figure 3-4).

Figure 3-4 Box & Whisker Plot of M2-PK concentration (U/mL) over the control period for four sequential samples)



Legend to Figure 3-4: Three subjects were classified as outliers and are indicated by asterisks. The initial sample size (n=48) was reduced to n=45 after excluding the outliers (subjects 39, 40, and 45).

A repeated measures ANOVA was conducted to determine whether there was a significant difference in M2-PK concentration mean scores across the four pre-treatment scores. Only matching samples were included on the repeated measures ANOVA for M2-PK. The removal of the three outliers from the initial 48 (n=48) subjects left 45 subjects. Further analysis of the samples revealed that there were two subjects with only two samples, rather than the maximum four, for M2-PK analysis. These two subjects were withdrawn from the repeated measures ANOVA analysis bringing the sample size to 43 (n=43). Means and standard deviation of the 43 subjects included in the ANOVA analysis of M2-PK pre-treatment scores are presented in Table 3-8.

Table 3-8 Descriptive Statistics M2PK (U/mL) Pre-treatment Concentration Scores (n= 43)

	M	SD	Range	N
M2PK-1	2.25	3.64	1-20	43
M2PK-2	2.10	3.50	1-20	43
M2PK-3	2.69	3.63	1-20	43
M2PK-4	2.02	2.85	1-17	43

Legend to Table 3-8: This table represents the mean, standard deviation and the range after removal of outliers.

Results from the repeated measures ANOVA indicated that there was no significant difference between the means of four pre-treatment concentration scores, W = 0.93, F (3, 40) = 0.91, p = 0.44 (see Table 3-9).

Table 3-9 Repeated Measure ANOVA for M2-PK (U/mL) Pre-treatment Concentration Scores

			Hypothesis		
Effect	Value	F	df	Error df	p
Wilks' Lambda	0.93	0.91	3.00	40.00	0.44

Similar to the calprotectin analysis the rest-test reliability presented some differences when correlating two M2-PK scores of the same subject on two different occasions (see Table 3-7) and it was necessary to analyse the value for each M2-PK sample across the population (see Table 3-10). This analysis used the raw data presented in Appendix 7.

This analysis included 43 subjects who had all four pre-treatment samples analysed plus the three subjects who were considered outliers but also had all four samples analysed. The total subject number for this analysis was 46 (n= 46).

Of the 46 subjects who had at least four valid concentration scores, there were 32 (70%) subjects who had M2-PK concentration scores of 4U/mL or

less. There were seven subjects out of 46 who had one M2-PK concentration score above 4U/mL (15%). Six out of 46 subjects (13%) had two M2-PK concentration scores above 4U/mL. There were no subjects with three concentration scores above 4U/mL, but there was one subject (2%) who had 4 M2-PK concentration scores above 4U/mL (see Table 3-10).

Table 3-10 Weekly M2-PK Pattern of individual Concentration Scores (U/mL)

	N	%
All scores 4 or less	32	70
One score above 4	7	15
Two scores above 4	6	13
Three scores above 4	0	0
Four scores above 4	1	2
Total	46	100.0

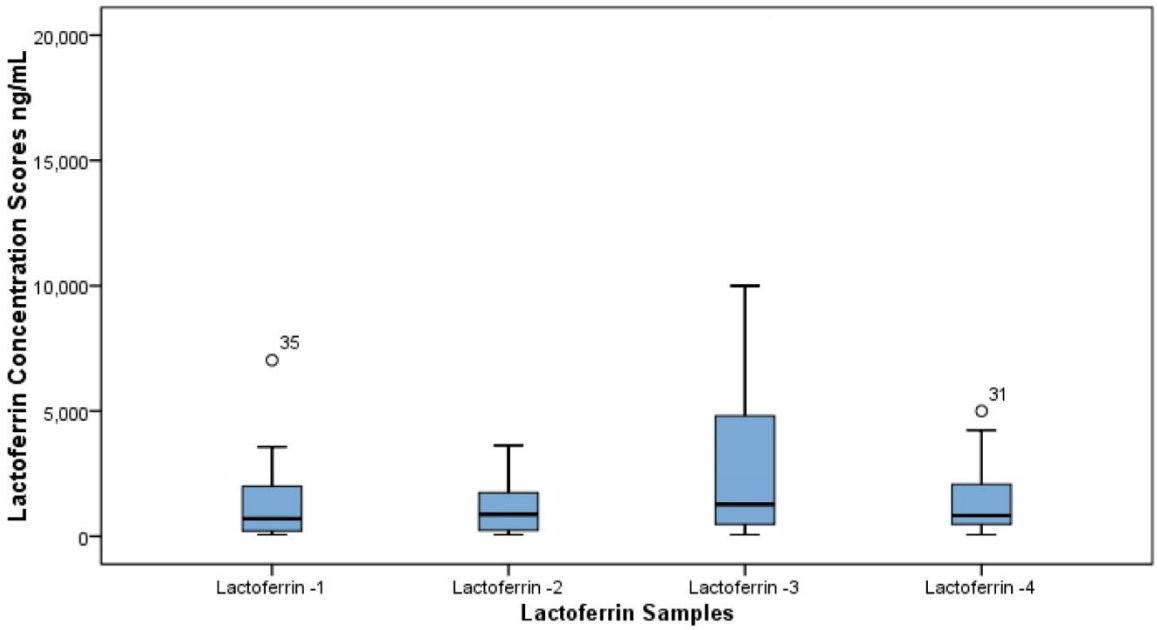
Legend to Table 3-10: This represents M2-PK value for 46 subjects who had all four samples analysed including the 3 outliers. (n=46)

Please see M2-PK reference range in methods, 2.20.4.

3.3.3 Period I – Study I (Part C) – Analysis of Lactoferrin in Sequential Faecal Samples

The final number of subjects who completed the study was 49 (n=49). Of these 49 subjects only 24 subjects had a complete set of four pre-treatment faecal samples analysed (n=24) for the biomarker lactoferrin. The loss of the subject numbers for this analysis was due to the accidental discarding of samples which was outside the control of the author (see Appendix 6). The box and whisker plot represents levels of lactoferrin samples for all the participants in the four sequential sample (see Figure 3-5).

Figure 3-5 Box & Whisker Plot of Lactoferrin concentration (ng/mL) over the control period sequential samples



Legend to Figure 3-5: There were no subjects marked with asterisks therefore there were no outliers in this analysis. The final subject number was 24 (n=24)

A test-retest reliability analysis was conducted to assess the reliability of lactoferrin scores for the 24 subjects. A correlation matrix was computed for each pair of scores. Results of the test-retest reliability analysis between pre-treatment samples of lactoferrin indicated that the correlations between all sample sets had unacceptable reliability samples 1 and 2 (r = 0.18, p =0.30), 1 and 3 (r = 0.33, p = 0.09), 1 and 4 (p= 0.25, p = 0.15), 2 and 3 (r = 0.29, p = 0.11), 2 and 4 (r = 0.37, p = 0.18) and 3 and 4 (r = 0.26, p = 0.19) (see Table 3-11). See methods for General Guidelines for interpreting Reliability Coefficient 2.26.2.

Table 3-11 Correlation Matrix of Lactoferrin (ng/mL) Pre-Treatment Concentration scores (n =24)

	Lactoferrin 1	Lactoferrin 2	Lactoferrin 3
Lactoferrin 2	0.15		
Lactoferrin 3	0.31	0.25	
Lactoferrin 4	0.24	0.12	0.26

Table 3-12 represents the means and standard deviation of 24 subjects for the lactoferrin pre-treatment period included in the ANOVA analysis.

Table 3-12 Descriptive statistics for lactoferrin (ng/ml) concentration in the sequential samples

	M	Std Dev	N
Sample 1	2100	3182	24
Sample 2	1470	2084	24
Sample 3	3313	4791	24
Sample 4	1366	1361	24

A repeated measures ANOVA was conducted to determine whether there was a significant difference between the means of lactoferrin concentration scores. Results of the repeated measures ANOVA indicated that there was no significant difference between the four lactoferrin pre-treatment concentration scores, W = 0.82, F (3, 21) = 1.45, p = 0.25 (see Table 3-13).

Table 3-13 Repeated Measure ANOVA for Lactoferrin Pre-Treatment Concentration Scores

			Hypothesis		
Effect	Value	F	df	Error df	p
Wilks' Lambda	0.82	1.45	3.0	21.00	0.25

Of the 24 subjects with data across all four pre-treatment measures, 18 (75%) subjects had all 4 pre-treatment concentration scores within the normal range (below 7.24 ng/mL). There were three subjects with one pre-treatment concentration score above the normal range (12.5%), and three subjects with two pre-treatment measures above the normal range (12.5%) (see Table 3-14).

Table 3-14 Weekly lactoferrin Pattern of individual Concentration Scores (ng/mL)

	N	%
All scores below 7.25	18	75.0
One Score7.25 or above	3	12.5
Two scores 7.25 or above	3	12.5
Total	24	100.0

Please see table lactoferrin reference range in 2.21.4, Methods section.

3.3.4 Period I – Study I (Part-D) – Analysis of sequential S.0.S Questionnaire

The statistical analyses of the four pre-treatment questionnaires were used to form a baseline for this thesis. The data collection was derived from four sequential, 24 parameters, ten point scale questionnaires based on the Autism Research Institute Secretin Outcomes Survey Form, the 'S.O.S Form'. The measurement was done via a Likert scale as outlined in the methods section.

The data analysis was performed on a total of 48 subjects (n=48). The statistical analyses were divided into descriptive statistics of each of the four sequential questionnaires and analysis of the means of the four questionnaire subscales that is; Social behaviour and communication, ritual and repetitive activities, digestive signs and general signs subscales. The results of the questionnaire baseline were later correlated with the results of the treatment and the post-treatment periods (Period II and III). Descriptive statistics for each questionnaire can be seen in Table 3-1.

Table 3-15 Descriptive Statistics Questionnaire 1 pre-treatment

Subscales	Parameters	Mean	SD
Social behaviour and communication	Lack of awareness and interaction with parent	2.70	2.19
	Abnormal greeting behaviour	3.48	2.62
	Abnormal comfort seeking	3.82	2.52
	Can't make friends	5.34	3.18
	Lack of awareness of social rules	5.55	3.07
	Lack of spontaneous speech	5.02	3.32
	Abnormal word utilisation	4.77	3.15
	Poor comprehension of verbal instructions	4.36	2.87
	Lack of eye contact	3.70	2.18
Ritual and Repetitive Activities	Abnormal repetitive gestures	4.65	2.96
	Need to maintain sameness	4.54	2.71
	Need of fixed routine	4.38	2.84
Digestive Signs	Diarrhoea	3.05	2.98
	Constipation	4.50	3.33
	Poor Appetite	3.23	2.85
	Bloating	3.55	2.89
	Flatulence	2.63	2.44
	Vomiting	1.04	1.96
General Signs	Unhappy	2.40	2.11
	Aggressive	2.78	2.61
	Destructive	3.04	3.01
	Spaced out/ Non Interactive	3.30	2.43
	Agitated	4.36	2.56
	Disagreeable	4.07	2.48

Legend to Table 3-15: This table represents the means and standard deviation for parameters of the pre-treatment questionnaire 1 using a measure on a 10 point Likert scale where 0 = never shows this particular sign or behaviour; 1 = slight/unobtrusive; 2-3 = mild; 4-5 = moderate; 6-7 = severe; 8-9 = shows extreme/incapacitating evidence ot this sign or behaviour.

Table 3-16 Descriptive Statistics Questionnaire 2 pre-treatment

Subscales	Parameters	Mean	SD
Social behaviour and communication	Lack of awareness and interaction with parent	3.00	2.42
	Abnormal greeting behaviour	3.98	2.90
	Abnormal comfort seeking	4.02	2.43
	Can't make friends	5.18	3.18
	Lack of awareness of social rules	5.41	2.92
	Lack of spontaneous speech	4.66	3.14
	Abnormal word utilisation	5.11	3.11
	Poor comprehension of verbal instructions	4.36	2.58
	Lack of eye contact	3.63	2.33
Ritual and Repetitive Activities	Abnormal repetitive gestures	4.33	2.55
	Need to maintain sameness	4.31	2.72
	Need of fixed routine	4.18	2.52
Digestive Signs	Diarrhoea	2.56	2.80
	Constipation	4.04	3.12
	Poor Appetite	2.49	2.76
	Bloating	3.27	2.66
	Flatulence	2.54	2.46
	Vomiting	.67	1.66
General Signs	Unhappy	2.54	2.23
	Aggressive	2.83	2.67
	Destructive	2.78	2.83
	Spaced out/ Non Interactive	3.35	2.82
	Agitated	3.77	2.54
	Disagreeable	3.78	2.69

Legend to Table 2-16: This table represents the means and standard deviation for parameters of the pre-treatment questionnaire 2 using a measure on a 10 point Likert scale where 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 3-17 Descriptive Statistics Questionnaire 3 pre-treatment

Subscales	Parameters	Mean	SD
Social behaviour and communication	Lack of awareness and interaction with parent	3.21	2.63
	Abnormal greeting behaviour	3.81	2.86
	Abnormal comfort seeking	3.62	2.61
	Can't make friends	5.40	2.96
	Lack of awareness of social rules	5.73	2.84
	Lack of spontaneous speech	4.81	3.40
	Abnormal word utilisation	4.18	3.24
	Poor comprehension of verbal instructions	4.29	2.52
	Lack of eye contact	3.52	2.36
Ritual and Repetitive Activities	Abnormal repetitive gestures	4.20	2.77
	Need to maintain sameness	4.07	2.63
	Need of fixed routine	3.78	2.64
Digestive Signs	Diarrhoea	1.63	2.30
	Constipation	3.95	3.27
	Poor Appetite	2.20	2.88
	Bloating	3.15	2.79
	Flatulence	2.74	2.43
	Vomiting	.51	1.32
General Signs	Unhappy	2.36	2.29
	Aggressive	2.68	2.61
	Destructive	2.83	2.68
	Spaced out/ Non Interactive	3.28	2.60
	Agitated	3.30	2.39
	Disagreeable	3.83	2.61

Legend to Table 2-17:This table represents the means and standard deviation for parameters of the pre-treatment questionnaire 3 using a measure on a 10 point Likert scale where 0 = never shows this particular sign or behaviour; 1 = slight/unobtrusive; 2-3 = mild; 4-5 = moderate; 6-7 = severe; 8-9 = extreme/incapacitating.

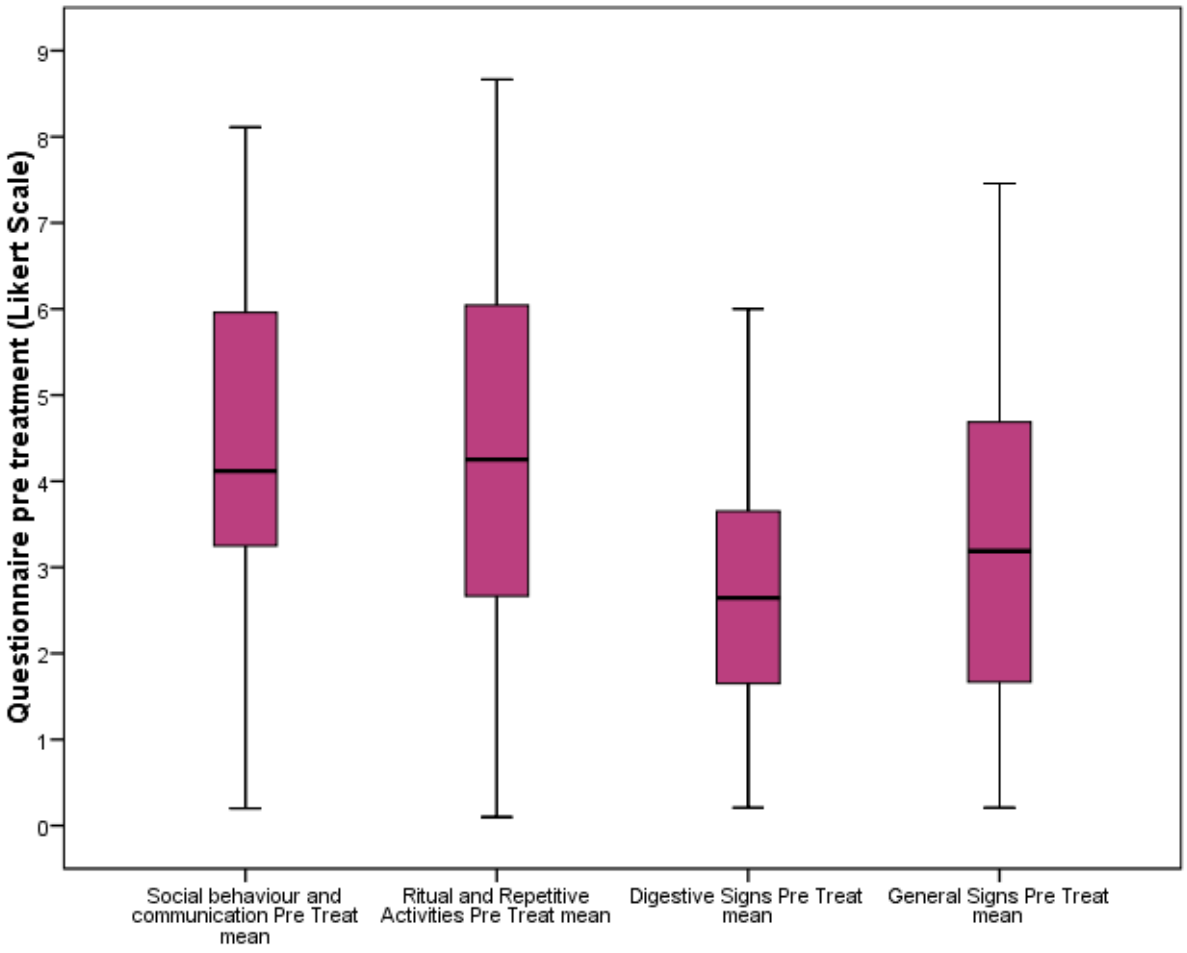
Table 3-18 Descriptive Statistics Questionnaire 4 pre-treatment

Subscales	Parameters	Mean	SD
Social behaviour and communication	Lack of awareness and interaction with parent	3.12	2.58
	Abnormal greeting behaviour	3.61	2.54
	Abnormal comfort seeking	3.85	2.77
	Can't make friends Q4	5.07	3.02
	Lack of awareness of social rules	5.69	3.00
	Lack of spontaneous speech	4.52	3.27
	Abnormal word utilisation	4.29	3.04
	Poor comprehension of verbal instructions	4.44	2.60
	Lack of eye contact	3.56	2.50
Ritual and Repetitive Activities	Abnormal repetitive gestures	3.98	2.63
	Need to maintain sameness	4.15	2.72
	Need of fixed routine	3.73	2.95
Digestive Signs	Diarrhoea	2.23	2.67
	Constipation	4.00	3.23
	Poor Appetite	3.19	3.35
	Bloating	2.95	2.85
	Flatulence	2.84	2.68
	Vomiting	.95	2.24
General Signs	Unhappy	2.20	2.25
	Aggressive	2.39	2.49
	Destructive	2.76	2.88
	Spaced out/ Non Interactive	3.60	2.83
	Agitated	3.85	2.36
	Disagreeable	3.71	2.92

Legend to Table 2-18: This table represents the means and standard deviation for parameters of the pre-treatment questionnaire 4 using a measure on a 10 point Likert scale where 0 = never shows this particular sign or behaviour; 1 = slight/unobtrusive; 2-3 = mild; 4-5 = moderate; 6-7 = severe; 8-9 = extreme/incapacitating.

The pre-treatment means subscales of the 24 parameters S.O.S questionnaire collected from the 48 subjects were plotted on a box and whiskers plot (see Figure 3-6).

Figure 3-6 Box & Whisker plot for categories of the pre-treatment questionnaire using a measure on a 10 point scale



Legend to Figure 3-6: 0 = never shows this particular sign or behaviour; 1 = slight/unobtrusive; 2-3 = mild; 4-5 = moderate; 6-7 = severe; 8-9 = extreme/incapacitating.

Cronbach's Alpha analysis was conducted to evaluate the reliability of the four pre- treatments S.O.S questionnaire and its subscales (S.O.S questionnaire subscales are listed in the methodology section). The results of the Cronbach's test suggest that the overall internal consistency of the mean scores for the pre-treatment questionnaires was 0.8 which is a good overall reliability (Cronbach's Alpha=0.8 or 80%). This suggests that the means scores for the pre-treatment questionnaire measures are reliable to be used as a baseline for this thesis. All of the 48 subjects subscales were assessed for means and standard deviation (see Table 3-19).

Table 3-19 Mean and Standard deviation for four categories of the pre-treatment questionnaire subscales

Subscales	M	SD	N
Social behaviour and communication	4.3	2.1	48
Ritual and Repetitive Activities	4.3	2.4	48
Digestive Signs	2.7	1.4	48
General Signs	3.1	1.9	48

3.3.5 Period I – Study I (Part-E) – Analysis of Correlations between calprotectin, M2-PK and lactoferrin

Statistical analysis was used to determine whether there was a correlation between calprotectin, M2-PK and lactoferrin concentration in the samples collected during the control/baseline period.

3.3.5.1 Pre-treatment Calprotectin Concentration Scores and Pre-Treatment M2-PK Concentration Scores

Five Pearson correlations were conducted to determine whether there was a significant linear relationship between pre-treatment calprotectin scores and M2PK scores in total and between each of the 4 pre-treatment measures. The Pearson r coefficient indicates to what extent the scores of two variables co-vary (Field, 2012; Tabachnick & Fidel, 2012). See methodology (Table 2-9) for Pearson correlation values according to Cohen (1983).

Results indicated that there was a significant, positive correlation that was moderate in size between the total calprotectin pre-treatment scores and the total M2PK scores, r = 0.40, n = 38, p = 0.01 (see Table 3-20 calprotectin total/ M2-PK total). There was no significant correlation between pre-treatment 1 calprotectin and M2PK scores, r = 0.18, n = 38, p = 0.27, pre-treatment 2 calprotectin and M2PK scores, r = 0.23, n = 37, p = 15, or pre-treatment 4 calprotectin and M2PK scores, r = 0.06, n = 35, p = 0.73 (see Table 3-20). However, there was a significant correlation between pre-

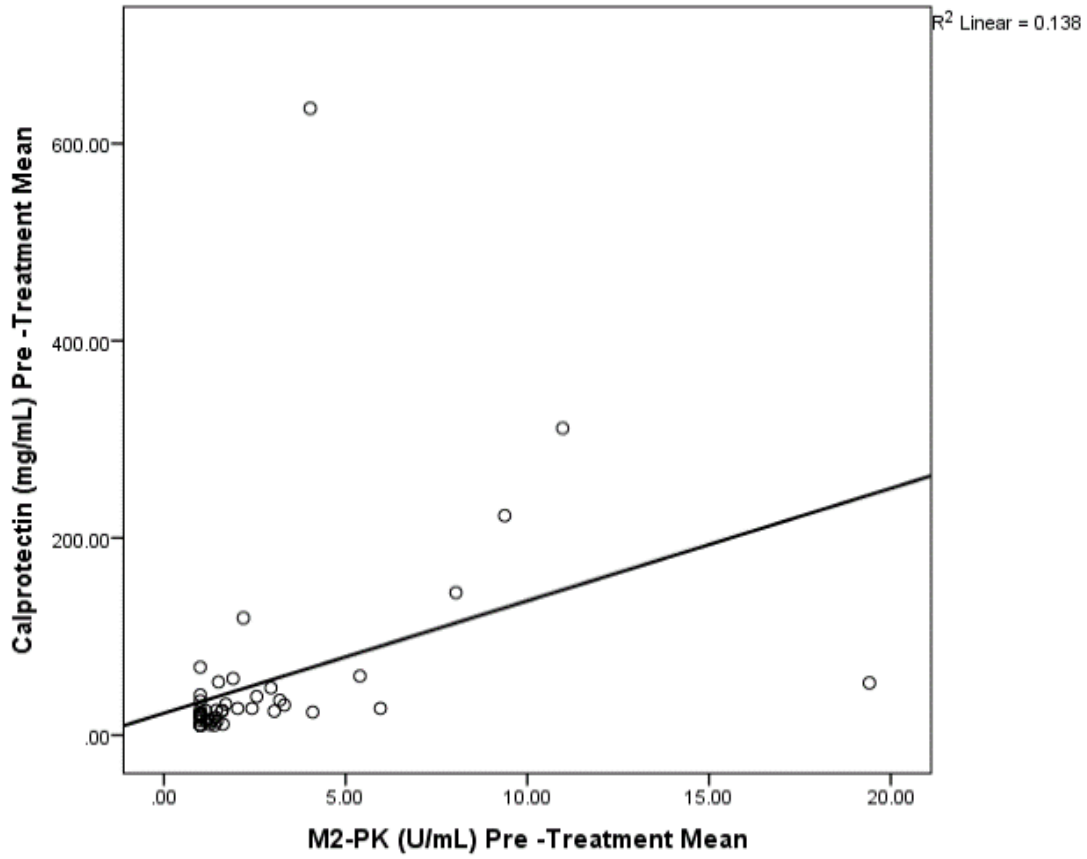
treatment 3 calprotectin and M2PK concentration scores that was moderately strong, r = 0.43, n = 36, p = 0.007 (see Table 3-20 and Figure 3-7).

Table 3-20 Correlation Matrix of Calprotectin and M2PK pre-treatment Concentration Scores

	Calpro 1	Calpro 2	Calpro 3	Calpro 4	Calpro Total
M2PK 1	0.18				
M2PK 2		0.23			
M2PK 3			0.43*		
M2PK 4				0.06	
M2PK Total					0.40*

*Significant correlation p<0.05

Figure 3-7 Scatterplot of pre-treatment Calprotectin and M2PK Concentration mean scores.



3.3.5.2 Pre-treatment Calprotectin and Lactoferrin Concentration Scores

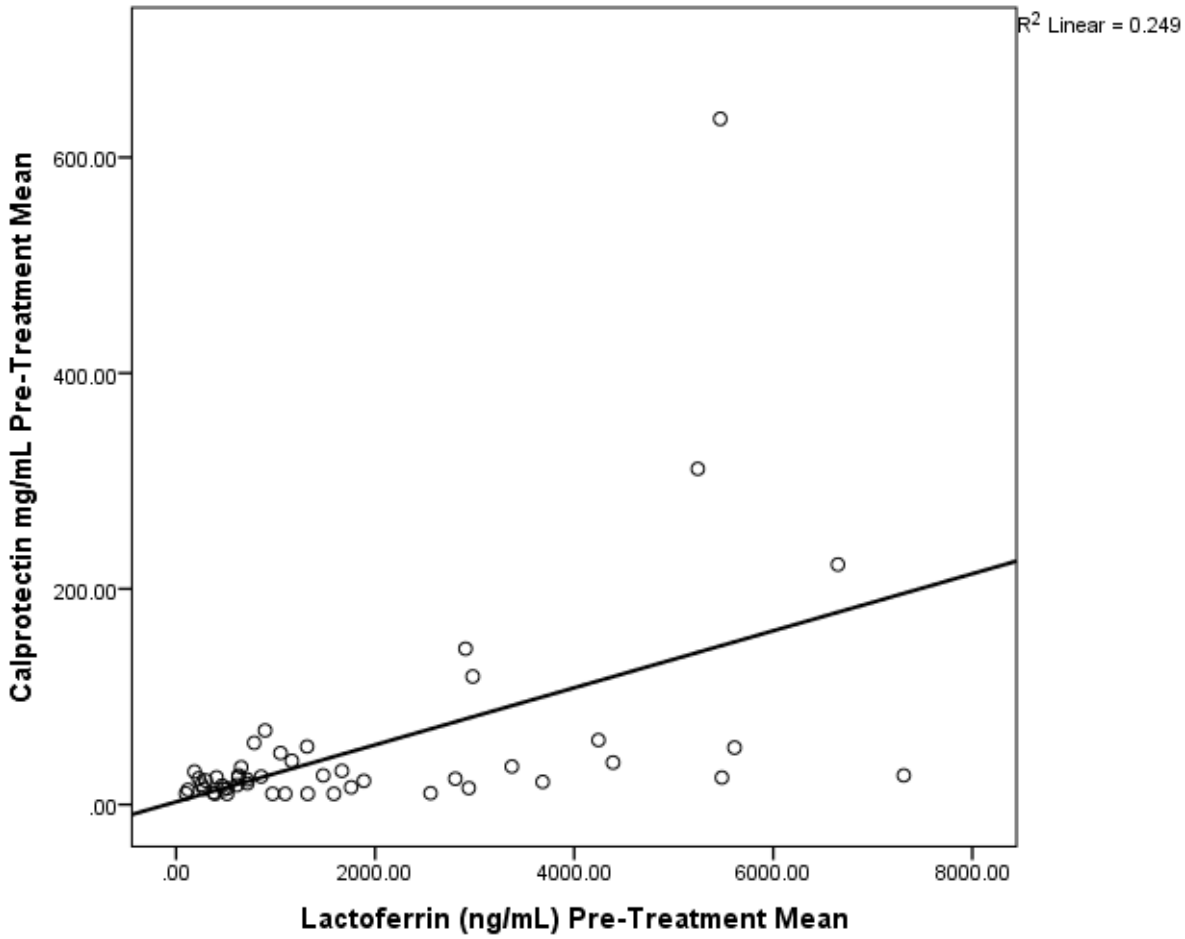
Bivariate correlations were performed to determine if there was a significant linear relationship between total pre-treatment calprotectin and lactoferrin mean concentration scores, as well as for individual pre-treatment concentration scores. Results indicated that there was a significant, strong, positive relationship between the total calprotectin scores and the total lactoferrin scores, r = 0.65, n = 24, p < 0.001, (based on Cohen's guidelines, Cohen 1983) (see methodology, 2.26.1) (see Table 3-21 calprotectin total/lactoferrin total). Results also indicated that there was a significant strong positive correlation between pre-treatment 1 of calprotectin and lactoferrin scores, r = 0.75, n = 37, p = < 0.001, and a significant, moderate, correlation between pre-treatment 3 of calprotectin and lactoferrin scores, r = 0.46, n = 30, p <= 0.01 (see Table 3-21). There was no significant correlation between pre-treatment 2, (r = 0.26, n = 46, p = 0.08), or pre-treatment 4 (r = 0.14, n = 42, p = 0.34) concentration scores (see Table 3-21). The total mean concentration scores of calprotectin and lactoferrin showed a strong linear relationship (see Figure 3-8).

Table 3-21 Correlation Matrix of Calprotectin and lactoferrin pre-treatment concentration scores

	Calpro 1	Calpro 2	Calpro 3	Calpro 4	Calpro Total
Lactoferrin 1	0.75*				
Lactoferrin 2		0.25			
Lactoferrin 3			0.46		
Lactoferrin 4				0.14	
Lactoferrin Total					0.65*

*Significant correlation p <0.05

Figure 3-8 Scatterplot of pre-treatment Calprotectin & Lactoferrin mean concentration scores.



3.4 Statistical Analysis Period I, Period II and Period III

This section correlates the data collected during the Control/Baseline Period (Period I) (see section 3.1) with the data collected during and post treatment periods (Periods II & III).

Data collected during Period II consisted of four questionnaires completed during the six week treatment period of the clinical trial. At least three stool samples for calprotectin/M2-PK/lactoferrin analysis were also collected. Data collected from Period III was generated from one questionnaire and one faecal sample for calprotectin/M2-PK/lactoferrin analysis collected six weeks after completion of the treatment period (see section 2.9 in methods for detailed information of each period).

3.4.1 Period I, Period II and Period III results section

In this section of the results chapter the data are presented as three studies which correspond to the analysis of data from the periods I, II and III. This is discussed in the discussion chapter of this thesis. The organisation of the studies – Period I, Period II and Period III is shown in Figure 3-2.

Study II: Twenty-four parameter S.O.S questionnaires evaluation at Period I, Period II and Period III.

Study III: Calprotectin, M2-PK and lactoferrin concentrations measured at Period I, II and III.

Study IV: The relationship between calprotectin and twenty-four parameter S.O.S questionnaires.

3.5 Results Study II - Twenty-four parameter S.O.S questionnaires evaluation at Period I, Period II and Period III

All results from the control, treatment and post-treatment periods of the study were recorded and analysed. The data generated from the questionnaires collected from the control period were used as a baseline which was then compared to the data generated from the treatment and post-treatment periods.

The S.O.S questionnaire was used to assess behavioural and GI symptoms and is structured as a 24 parameter grid, with four sub-scales for social behaviour, ritual and repetitive activities, digestive symptoms, and general symptoms (Rimland, 1998, Brudnak et al., 2002, Esch and Carr, 2004b, Erickson et al., 2005a, Sturmey, 2005, Williams et al., 2005) (see Appendix 5).

3.5.1 Repeated Measure ANOVA Period I, II and III – S.O.S Questionnaire

A repeated measure ANOVA was conducted to determine whether there was a significant difference between the means for the control, treatment and post-treatment scores accessed via the modified S.O.S questionnaire. The

independent variable was time (periods I, II, and III) and the dependent variable was the mean scores across the questionnaires administered during the control, treatment, and post-treatment periods. Wilk's Lambda was the multivariate statistic used to determine whether there was a significant main effect. A significant main effect, indicated by a p value of less than 0.05, was followed up by post comparison tests between each of the three mean scores.

Results of the repeated measures ANOVA indicated that there was a significant difference between the mean scores, Wilk's Lamba (W) = 0.76, F (2, 35) = 5.27, p = 0.01.

To determine which groups were significantly different from each other, post-hoc tests employing the Bonferroni adjustment were conducted. Results of the post-hoc test revealed that the control mean score (M = 3.55, SD = 1.30) was significantly higher than both the treatment mean score (M = 3.14, SD = 1.31) and the post-treatment mean score (M = 3.2, SD = 1.23). However, there was no significant difference between the treatment mean score and the post-treatment mean score.

3.5.2 Descriptive statistics performed in each Questionnaire Subscale

Descriptive statistics were performed on each individual sub-scale (see Table 3-22; Table 3-23; Table 3-24 and Table 3-25).

Table 3-22 Descriptive statistics for social behaviour and communication subscale

Study Period	N	Mean	SD	Min	Max
Control	48	4.34	2.14	0.20	8.11
Treatment	45	4.09	2.00	0.72	8.00
Post-treatment	29	3.76	2.18	0.44	8.71

Legend to Table 3-22: This table represents the means and standard deviations for parameters social behaviour and communication of the pre-treatment, treatment and post-treatment questionnaire using a measure on a 10 point Likert scale where 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 3-23 Descriptive statistics for ritual and repetitive activities subscale

Study Period	N	Mean	SD	Min	Max
Control	48	4.34	2.14	0.10	8.67
Treatment	44	3.89	2.05	0.00	8.17
Post-treatment	29	3.49	2.10	0.00	7.00

Legend to Table 3-23: This table represents the means and standard deviations for parameters ritual and repetitive activities of the pre-treatment, treatment and post-treatment questionnaire using a measure on a 10 point Likert scale where 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 3-24 Descriptive statistics for digestive signs subscale

Study Period	N	Mean	SD	Min	Max
Control	48	2.75	1.49	0.21	6.00
Treatment	45	2.21	1.34	0.08	5.40
Post-treatment	29	2.10	1.70	0.00	6.20

Legend to Table 3-24: This table represents the means and standard deviations for parameters digestive signs of the pre-treatment, treatment and post-treatment questionnaire using a measure on a 10 point Likert scale where 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 3-25 Descriptive statistics for general signs subscale

Study Period	N	Mean	SD	Min	Max
Control	48	3.18	1.90	0.21	7.46
Treatment	45	2.99	1.91	0.13	7.50
Post-treatment	29	2.76	1.85	0.17	7.83

Legend to Table 3-25: This table represents the means and standard deviations for parameters general signs of the pre-treatment, treatment and post-treatment questionnaire using a measure on a 10 point Likert scale where 0 = never shows this particular sign or behaviour; 1 = slight/unobtrusive; 2-3 = mild; 4-5 = moderate; 6-7 = severe; 8-9 = extreme/incapacitating.

3.5.3 Repeated Measure ANOVA performed in each questionnaire subscale

A series of repeated measures ANOVAs were conducted to determine whether there were significant differences across pre-treatment, treatment, and post-treatment time frames (Period I,II and III) on subscale questionnaire scores. The subscales in question were social behaviour and communication, ritual and repetitive activities, digestive signs, and general signs. Pos hoc test (Bonferroni test) was used if the ANOVA idetifed any significance within the means of the periods. Bonferroni tested the three period means and explored the differences between them, providing specific information on each mean and therefore identifying any significant difference from each period tested.

3.5.3.1 Repeated measure ANOVA for subscale – social behaviour and communication

Repeated measure ANOVA analysis indicated that there was a significant difference within the three time periods for the subscale social behaviour and communication, $W = 0.39$, $F(1, 27) = 104.55$, $p < 0.001$. The Bonferroni adjustment was used to maintain the p value at 0.05. Post hoc tests indicated that social behaviour and communication pre-treatment scores ($M = 4.41$, $SD = 2.12$) were significantly higher than treatment ($M = 3.93$, $SD = 2.13$) and post-treatment scores ($M = 3.76$, $SD = 2.18$). There were no significant differences beween the treatment and post-treatment social scores.

3.5.3.2 Repeated measure ANOVA for subscale – ritual and repetitive activities

Results of the ritual and repetitive activities subscale repeated measures ANOVA revealed that there was a significant difference between test scores, $W = 0.31$, $F(2, 26) = 6.04$, $p = 0.007$. Post hoc tests indicated that the ritual and repetitive activities pre-treatment ($M = 4.25$, $SD = 2.43$) was significantly higher ($p = 0.05$) than the post-treatment scores ($M = 3.49$, $SD = 2.10$), but not significantly different ($p = 0.05$) from the treatment test ($M = 3.79$, $SD = 2.21$). There was no significant difference between ritual and repetitive activities treatment and post-treatment scores.

3.5.3.3 Repeated measure ANOVA for subscale – digestive signs

The repeated measure ANOVA for the digestive signs produced a significant main effect, W = 0.75, F (2, 27) = 4.42, p = 0.02. Post hoc results revealed pre treatment scores (M = 2.72, SD = 1.48) that were significantly higher (p=0.05) than both treatment scores (M = 2.21, SD = 1.36) and post-treatment scores (M = 2.10, SD = 1.74). There was no significant difference between treatment and post-treatment scores.

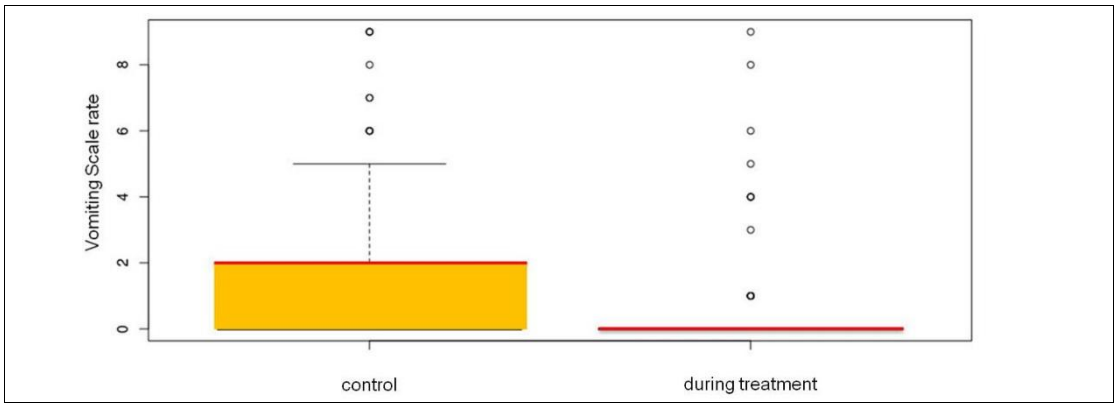
3.5.3.4 Repeated measure ANOVA for subscale – general signs

The analysis of variance for the general signs subscale produced a non significant main effect, indicating that there was no difference between pre-treatment (M = 3.2, SD = 1.79), treatment (M = 3.10, SD = 2.76), and post-treatment scores (M = 2.76, SD = 1.85), W = 0.88, F(2, 26) = 1.64, p = 0.21.

The 24 parameter questionnaires were analysed individually using Wilcoxon signed-rank test. Data from the four questionnaires collected during the control period were analysed and compared with the four completed during the treatment period. Statistical analysis indicated an improvement in three of the 24 parameters tested. The GI parameters that demonstrated a significant improvement after treatment with VOTs were ‘vomiting’ (p = 0.00029) (see Figure 3-9) ‘poor appetite’ (p= 0.039) (see Figure 3-10), and ‘eye contact’ (p = 0.035) (see Figure 3-11), the latter being one of the most characteristic social behavioural signs of autism.

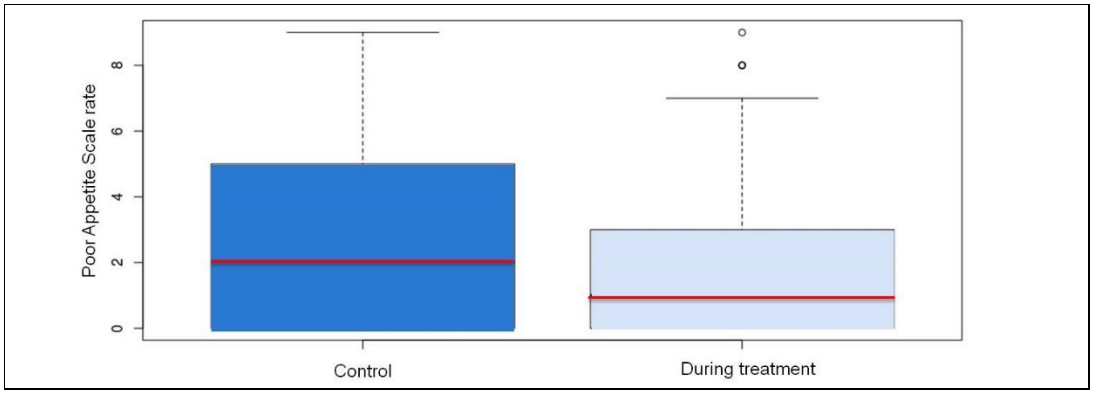
The data for the box and whisker plots was divided into four equal groups in order to generate three cut-off points or quartiles (Q1,Q2 and Q3). The quartiles split the data into four equal groups of 25% (25%(Q1), 50% (Q2) and 75% (Q3).

Figure 3-9: Box & Whisker plot for vomiting scores during the control and treatment periods



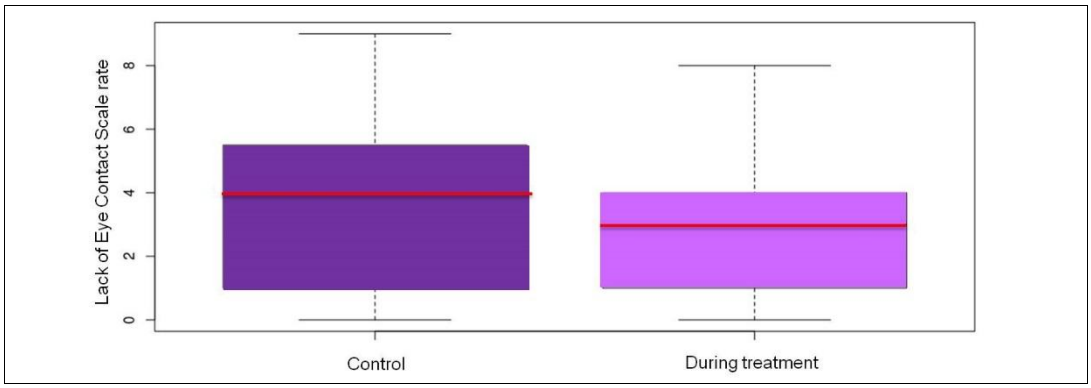
Legend to Figure 3-9: Children with autism demonstrated a significant decrease in vomiting scores after six weeks of VOT treatment (p = 0.00029). The data indicate that 75% (Q3) of the subjects showed a decrease in their symptom score of 2 points on a 10 point scale from the control period to the treatment period.

Figure 3-10: Box & Whisker plot for poor appetite scores during the control and treatment periods



Legend to Figure 3-10: Children with autism demonstrated a significant decrease in poor appetite scores after six weeks of VOT treatment (p = 0.039). The data indicates that 50% (Q2) of the subjects showed a decrease in their symptom score of 2 points on a 10 point scale from the control period to the treatment period.

Figure 3-11: Box & Whisker plot for lack of eye contact scores during the control and treatment periods



Legend to Figure 3-11: Children with autism demonstrated significant decrease in lack of eye contact scores after six weeks of VOT treatment (p =0.035). The data indicates that 75% (Q3) of the subjects showed a decrease in their symptom score of 1 point on a 10 point scale from the control period to the treatment period.

3.6 Results Study III - Calprotectin, M2-PK and lactoferrin concentrations measured at Period I, II and III

The statistical analysis of the study was performed using the SPSS software package, IBM® SPSS® Statistics 21, for each individual marker separately during the three distinct periods of the study (Period I, Period II and Period III).

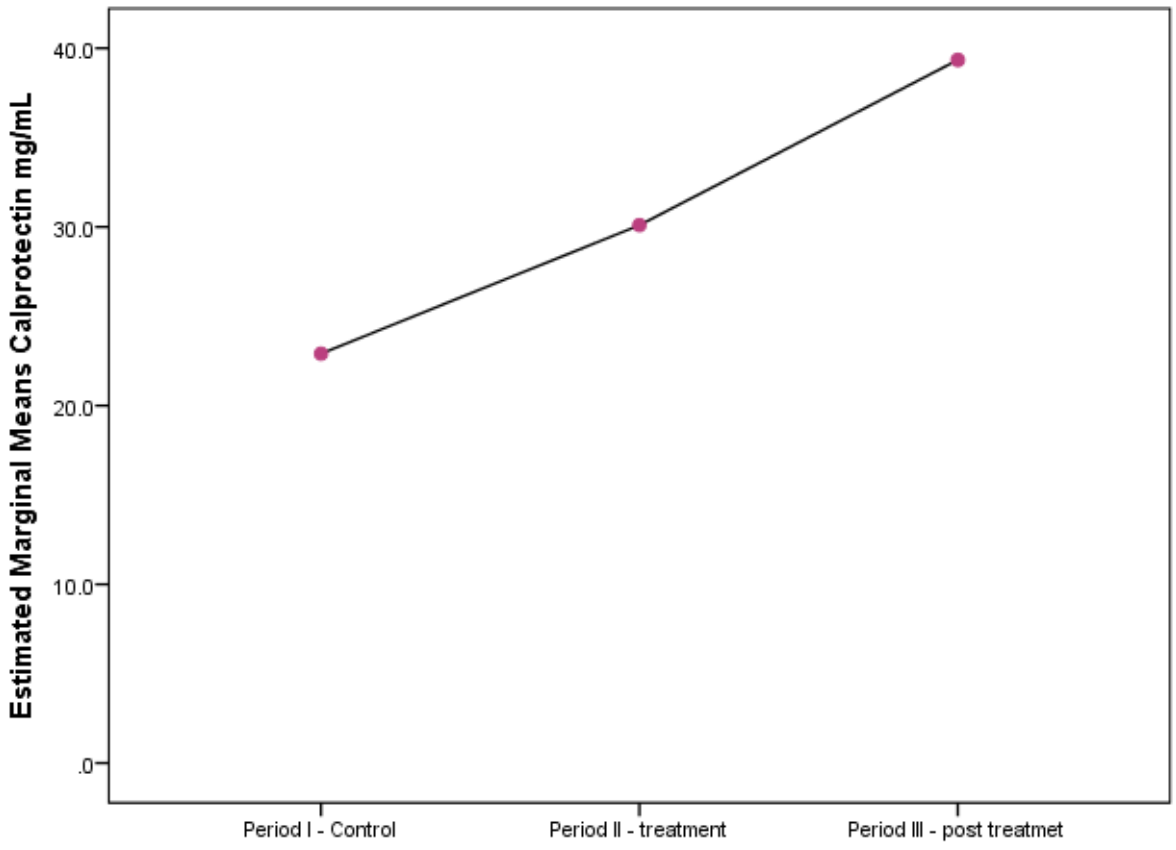
3.6.1 Calprotectin

A repeated measure ANOVA was conducted to evaluate the differences in calprotectin levels in samples from Period I - control, Period II - treatment and Period III - post-treatment (one subject was excluded as this subject had only one sample for the pre-treatment period – see section 3.3.1). Three mean concentrations were calculated using the calprotectin levels corresponding to the three distinct periods (see Table 3-26). Only subjects with no missing calprotectin values across the three periods, were included in the analysis; therefore, the final number of subjects was 29 (n=29).

Table 3-26 Descriptive statistics for calprotectin concentrations

Calprotectin	M	SD	N
Control Mean	22.9	13.2	29
Treatment Mean	30.1	29.4	29
Post-treatment Mean	39.3	57.2	29

Figure 3-12 Mean concentrations of calprotectin during Period I, Period II and Period III



Legend to Figure 3-12: This figure represents the mean scores (mg/mL) for calprotectin samples collected during the three distinct periods of the study.

The results of the repeated measures ANOVA indicated that there was no significant difference between the three mean scores (Wilk's Lambda = 0.89, F (2, 27) = 1.61, p = 0.21 (see Table 3-27).

Table 3-27: Repeated measures ANOVA – Wilk's Lambda for calprotectin concentrations in Period I, Period II and Period III

Wilks' Lambda	F	Hypothesis df	Error df	p
0.893	1.61	2.00	27.00	0.21

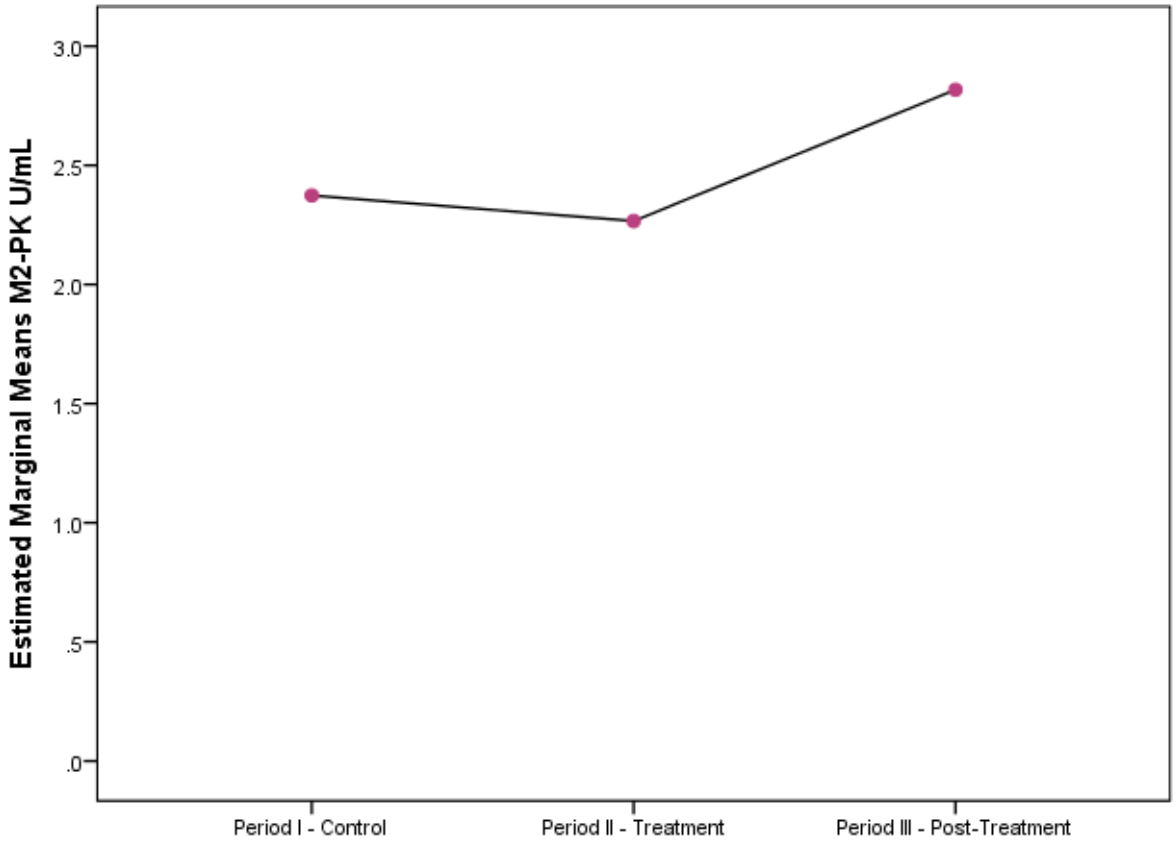
3.6.2 M2-PK

A repeated measure ANOVA was conducted to evaluate the differences in the M2-PK concentrations in the samples from Period I - control, Period II - treatment and Period III - post-treatment. Only subjects with no missing M2-PK values across the three periods, were included in the analysis therefore the final number of subjects was 29 (n=29). Three mean concentrations were calculated using the M2-PK levels corresponding to the three distinct periods (see Table 3-25). The Box & Whisker plot represents the estimated marginal means of M2-PK (U/mL) across the three periods (see Table 3-28).

Table 3-28: Descriptive statistics for M2PK concentrations (U/mL)

M2-PK	M	SD	N
Control Mean	2.37	3.55	29
Treatment Mean	2.27	2.89	29
Post-treatment Mean	2.82	3.91	29

Figure 3-13: Mean concentrations of M2-PK during Period I, Period II and Period III



Legend to the figure 3-13: This figure represents the mean scores (U/mL) for M2-PK samples collected during the three distinct periods of the study.

The results of the repeated measures ANOVA indicated that there was no significant difference between the three mean scores (Wilks' Lambda = 0.947, F (2, 32) = 0.900, p = 0.417 (see Table 3-29).

Table 3-29: Repeated measures ANOVA – Wilk's Lambda for M2PK concentrations in Period I, Period II and Period III

Wilks' Lambda	F	Hypothesis df	Error df	p
0.947	0.900	2.000	32.000	0.417

3.6.3 Lactoferrin

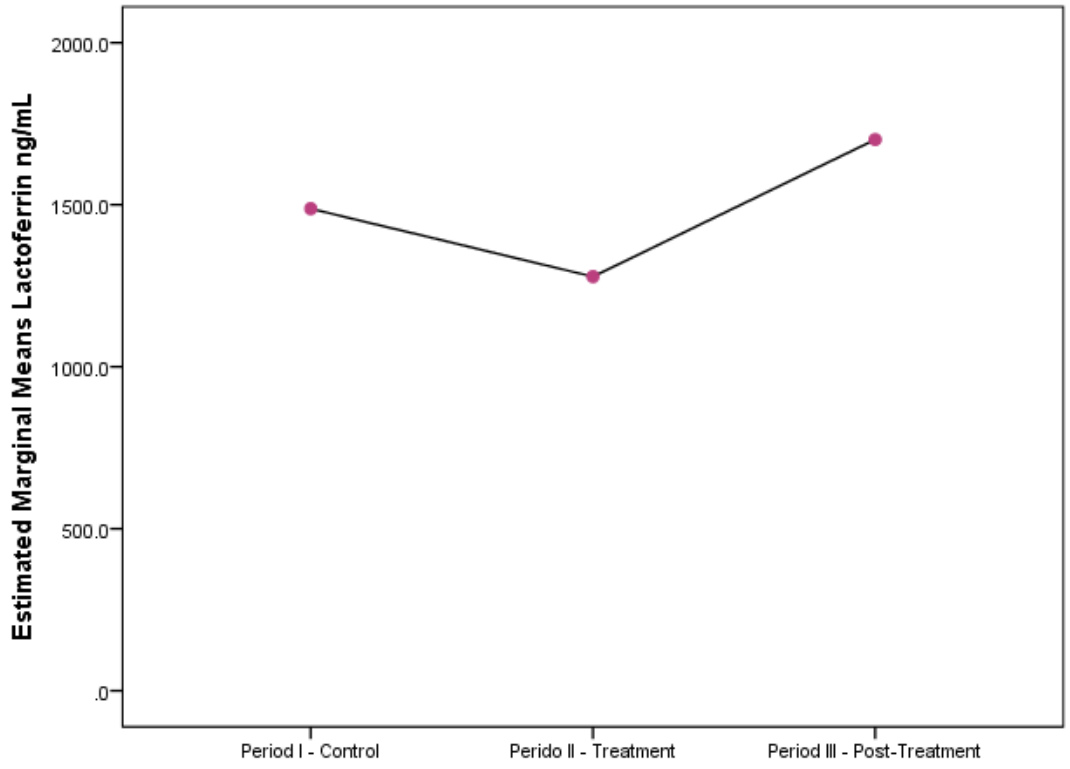
A repeated measure ANOVA was conducted to evaluate the differences in the lactoferrin concentrations in the samples from Period I - control, Period II -

treatment and Period III - post-treatment. Three mean concentrations were calculated using lactoferrin scores corresponding to the three distinct periods (see Table 3-30). Only subjects with no missing lactoferrin values were included in the analysis; therefore, the final number of subjects was 24 (N=24) (please see Appendix 10 for King's College Hospital letter on the accidental discard of samples). The Boxplot represents the estimated marginal means of lactoferrin (ng/mL) across the three periods (see Figure 3-14).

Table 3-30: Descriptive statistics for lactoferrin concentrations

Lactoferrin	M	SD	N
Control Mean	1488	1621	24
Treatment Mean	1278	1123	24
Post-treatment Mean	1701	1912	24

Figure 3-14: Lactoferrin mean scores over Period I, Period II and Period III



Legend to the Figure 3-14 figure represents the mean scores (ng/mL) for lactoferrin samples collected during the three distinct periods of the study.

The results of the repeated measures ANOVA indicated that there was no significant difference between the three mean scores (Wilks' Lambda = 0.94, F (2, 20) = 0.64, p = 0.54 (see Table 3-31).

Table 3-31: Repeated measures ANOVA – Wilk's Lambda for lactoferrin concentrations in Period I, Period II and Period III

Wilks' Lambda	F	Hypothesis df	Error df	p
0.94	0.61	2.000	20.000	0.54

3.7 Results Study IV - The relationship between calprotectin and twenty-four parameter S.O.S questionnaires.

3.7.1 Data Collection Procedure

The data collected was analysed using the R Project for Statistical Computing version 2.15 (R Development Core Team, 2012). GI inflammation was assessed using faecal calprotectin levels while GI and behavioural symptoms were assessed using the completed questionnaires (See section 2.22 for S.O.S questionnaire parameters and methods of use).

3.7.2 Association between questionnaire data and calprotectin adjusted by patient and sample

The data generated from the set of eight questionnaires were correlated with the results of the eight sets of faecal calprotectin concentrations. The twenty-four parameters from the questionnaires were matched with the faecal sample collecting times and calprotectin results. Each parameter from the questionnaire was analysed individually using a univariate model.

Table 3-32 shows the results of the univariate analysis, which is a method commonly used in prediction research as it includes all the univariate variables tested. The variable where p <0.15 were manually deleted and not included in the multivariate model (see raw data appendix 7). Each parameter was analysed repeatedly at several time points, corrected by patient and by sample, and correlated to the levels of calprotectin detected. Univariate analysis is an established strategy to measure longitudinal data for single outcome variables (Nakai, 2009). Aiming to keep variables within the regression model, a more liberal p = 0.15-0.25 was employed, which has been established as a common procedure within predictor research (Harrell et al., 1996, Kalkman et al., 2003). A p value of <0.15 was set as an initial filter for the univariate model of analysis (Bursac et al., 2008, Heinze, 2008). 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) were found to be significant. The parameters ‘constipation’ (p = 0.108) and ‘bloating’ (p = 0.086) were not significant at p <0.05, but both parameters fit the required filter criteria with p <0.15 and were incorporated in the multivariate analysis.

Table 3-32: 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*

Legend to the Table 3-32:This table includes all the univariate variables tested. As is common in prediction research those variables where p >0.15 were manually deleted and not included in the multivariate model. The asterisks indicate that the variable was kept in the multivariate model.

The six variables in Table 3-32 were retested using the multivariate model, and the variables presenting with the least significant p values were successively eliminated from the mixed effect model. This resulted in a more concise model of variables as represented in Table 3-33.

Table 3-33: Final univariate model

Variable	p set at <0.15 used as an initial filter
1. Lack of awareness and interaction with parent	0.02*
2. Abnormal repetitive gestures	0.0009*
3. Need to maintain sameness	0.0093*
4. Need of a fixed routine	0.0003*
5. Constipation	0.10
6. Bloating	0.08

Legend to the Table 3-33: This includes all of the variables that satisfied the initial filter of p <0.15 in the univariate analysis of the mixed effect model. Parameters 1-4 demonstrated a significant correlation with the inflammatory marker calprotectin at p <0.05. The parameters 'constipation' and 'bloating', although not significant in the univariate model were when p <0.15, which satisfies the filter value required to be included in a multivariate model.

The six parameters from the univariate model were then included on the multivariate model and retested. The data indicates a strong association between several parameters from the questionnaire and levels of faecal calprotectin. The parameter 'need for a fixed routine' was found to be highly significant (p<0.00009), and the multivariate coefficient of 3.227 suggests that autistic children who display an increase in a 'need for a fixed routine' also show elevated calprotectin levels which indicates GI inflammation. Paradoxically, the parameter 'constipation', although demonstrating a significant change (p <0.02) gave a negative multivariate coefficient of -1.584, corresponding to a decrease in calprotectin levels, possibly indicating decreased GI inflammation (see Table 3-34). This negative value for the parameter 'constipation' is directly associated with the positive value for 'need for a fixed routine', which may be considered as independent but reciprocal predictors of GI inflammation in these autistic children.

Table 3-34: Final Multivariate Model

Variable	Multivariate Coefficient	SE	p-value
(Intercept)*	22.8	4.0	p set at < 0.05
Need of a fixed routine	3.2	0.8	0.00009
Constipation	-1.5	0.6	0.02269

Legend to Table 3-34: This represents the final multivariate model results for the 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 by levels of calprotectin. *Intercept is a baseline for the calprotectin concentration in this multivariate model.

3.8 Case history anecdotal Observations

The following information is anecdotal and is not intended to be correlated or compared with the outcome measures of this thesis. However, it was included in the results section as case history anecdotal observations as it demonstrated value in illustrating the behaviour and GI signs and symptoms of autistic children during treatment.

Following acceptance of inclusion criteria, the osteopathic sessions were organised once a week for six weeks. All subjects were treated using the same set of VOTs described in the methodology section of this thesis. Due to the nature of autism, the approach to the subject was accommodated according to the subject's behaviour.

Generally, all of the subjects had to spend some time getting acquainted with the osteopath. This was done by giving them the time to explore the room and to become comfortable with the environment. Normally the osteopath used some toys and verbal communication to be able to approach the child. Once the verbal contact was made and a little interaction between the subject/osteopath occurred, the osteopath started the treatment.

Usually an osteopathic session was undertaken on a plinth, however, that was seen as a big imposition on the subjects. Therefore, the subjects were shown to the plinth or to a floor mat (option from the plinth), allowing them to decide

where they felt most comfortable to lie down. The VOT sessions were patient centred and it was the osteopath who had to adapt the treatment position to be able to treat the subjects. This means that the treatment may have occurred with some variation, with the subject sitting on a chair or standing in the corner of the room facing the wall. All these places within the room were chosen by the subject.

After the first or second session, some subjects displayed a clear understanding of the treatment. This was demonstrated by running to the treatment room and lying on the plinth or on the floor mat. They did not appear to be fazed or worried that they were about to be treated. More often than not, the subjects would place their hands on their abdominal area and would lift their top in an attempt to indicate that they were about to be treated or, apparently, that they wanted to be treated. More than one subject would place their hands on the osteopath's hand in an attempt to help with the techniques or to indicate they were content to be treated.

The subjects had some interesting responses that were quite remarkable. One specific subject who was non-verbal and very low on the ASD spectrum managed to verbalise 'butterfly' after the 4th session. This was by making eye contact with the osteopath and pointing to a butterfly mobile hanging in the corner of the treatment room. The parent, who witnessed the behaviour of the child, was clearly surprised and emotionally affected by the response. It was observed that this particular subject continued to improve over the next few sessions, demonstrating more calmness and the ability to make more eye contact with the practitioner. As anecdotally reported by the parents, eye contact and verbalisation was not a usual occurrence of their child.

Another interesting case was of a boy that displayed signs of faecal impaction on the lower quadrant of the abdomen and was constipated for 2 weeks prior to the initiation of the VOTs. The response that the subject had was remarkable; during the session he stood up and ran to the toilet (next door to the treatment room) and was able to pass a motion. This occurred at every VOT session he had during the study period. These observations may possibly imply that the treatment perhaps influenced the peristaltic motion.

There were definitely some difficulties when treating the children due the nature of the behaviour that some were displaying. Some were screaming and pushing, punching, spitting and hair pulling. However, the remarkable observation was that not a single child of the 49 treated had to be excluded due to not being able to cope with the session. Usually, the very ‘bad’ behaviour faded in less than 10 minutes into the session, which in itself is a remarkable response.

Chapter 4 - Discussion

Patients with an autistic disorder may present with GI symptoms, such as abdominal distension and pain, constipation, chronic diarrhoea, foul-smelling stools and/or flatulence (Shattock and Savery, 1996, Lewis, 1998, Jyonouchi et al., 2005a, Jyonouchi et al., 2011) and a link has been suggested between these GI symptoms and the cognitive deficit and abnormal behaviour of autistic children (D'Eufemia et al., 1996, Horvath et al., 1999, Horvath, 2000, Horvath and Perman, 2002, Koves et al., 2004, Valicenti-McDermott et al., 2008, Ibrahim et al., 2009, Nikolov et al., 2009, Chaidez et al., 2013).

Carr and Owen-Deschryver (2007) proposed that the intensity and frequency of pain in autistic individuals could lead to problem behaviour and low spectrum ASD individuals with impaired language skills, may express GI pain and discomfort through body mannerisms and self-harm (Carr and Owen-Deschryver, 2007). According to Breau et al. (2003), the lower the spectrum of behavioural or mental impairment the greater their pain and discomfort. A comparative study of 960 children including children developing normally, children with developmental delays and autistic children, suggested that the frequency of GI symptoms in ASD children is three times higher than in children developing normally (Chaidez et al., 2013). Chaidez et al. (2013) also suggested that the co-morbidity between behaviour and GI symptoms in autistic subjects may suggest possible treatment approaches.

A report analysing statements based on expert opinion has attempted to establish the importance of assessing the GI function of children diagnosed as autistic and suggests that these autistic children present with atypical GI symptoms in and may go undiagnosed. Such children should not only be investigated and treated for their behavioural problems but also for their GI problems (Buie et al., 2010). A gut-brain axis has been proposed, linking autistic behavioural dysfunction and GI symptoms, whereby the worsening of behavioural symptoms may possibly be due to inflammatory gut reactions meditated by immunological signals (Jyonouchi et al., 2005a, Reichelt and Knivsberg, 2009, Forsythe et al., 2010).

Kearney and Brown-Chang (2008)Kearney and Brown-Chang (2008)Kearney and Brown-Chang (2008)Kearney and Brown-Chang (2008)Kearney and Brown-Chang (2008)Kearney and Brown-Chang (2008)Complementary and alternative methods of treatment have a role in IBS in adults due to the psychological benefits of combined 'body and mind' methods, Kearney and Brown-Chang (2008). However, the use of complementary medicine in the management of autistic children suffering from GI symptoms is in its infancy. This thesis has attempted to investigate the use of visceral osteopathy in autistic children, suffering from the classical behavioural and GI problems, by using objective measures to assess potential changes.

4.1 Overview of the Studies Conducted

Study I (see results chapter section 3.3) analysed the data collected during Period I – control, and created a baseline for the statistical analysis during the latter studies. Studies II, III and IV reported on the analysis of the data collected during the distinct study periods: Period I - control, Period II - treatment and Period III - post-treatment, and compared the baseline data collected during Period I with that from Periods II and III.

In this chapter the results of all four studies are integrated and there is also a discussion of the incidental findings resulting from the analysis of the research baseline (Period I) and a comment on the anecdotal observations from the case notes (see also Results 3.8, Case History Anecdotal Observations).

4.2 Sequential Calprotectin, M2-PK and Lactoferrin Sampling

The three markers used for sample analysis in this thesis have been selected due to their high specificity and sensitivity in diagnosing IBDs as well as their reported positive correlations (Bunn et al., 2001, Summerton et al., 2002, Fagerberg et al., 2003, Sherwood et al., 2005, Walker et al., 2007, Sidhu et al., 2010b, Yamamoto et al., 2013). Study I generated from the control period is presented in five parts (Period I – Part A, B, C, D and E). Period I - parts A, B, and C are the results of the baseline for the three faecal biochemical markers. Part D is the result of the analysis of the sequential S.O.S

questionnaires and Part E is the correlation of the three biochemical markers during Period I.

Laboratory analysis of the three selected markers followed strict manufacturer's guidelines stated in the methodology section of this thesis (see methods 2.19 – 2.21). Each of the samples were analysed by a laboratory technician who was blinded to the study to avoid bias of the results.

4.3 Discussion of studies

4.3.1 Period I – Study I (Part A) – Calprotectin in Sequential Faecal Samples

Faecal calprotectin is a stable marker of GI inflammation that has been used as a pre-screening tool for UC and CD (Fagerberg et al., 2005). Faecal calprotectin also has very high levels of specificity (100%) and sensitivity (80%) for GI diseases in children with IBD (Bunn et al., 2001). This non-invasive test is proven to be valuable both in distinguishing between IBD and IBS in children, and as a pre-test for more invasive examinations such as colonoscopy (Aadland and Fagerhol, 2002, Summerton et al., 2002, Szarszewski et al., 2003, Yui et al., 2003, Fagerberg et al., 2005, Manz et al., 2012). Elevated faecal calprotectin is a more specific and precise identifier of GI inflammation than serum markers, such as, serum tumour necrosis factor (TNF), TNF-α receptor levels, and various interleukins (IL-1, IL-6, IL-8), due to its direct contact with the intestinal mucosa (Pardi and Sandborn, 2005). Calprotectin detection could therefore be used as a non-invasive screening tool for identifying organic diseases of the small intestine or large bowel (Striz and Trebichavsky, 2004, Fagerberg et al., 2005, Lundberg et al., 2005, Stroncek et al., 2005). The calprotectin diagnostic ELISA is a good tool, since high calprotectin levels correlate well with the severity of UC and CD in children (Bunn et al., 2001, Fagerberg et al., 2005).

Calprotectin was selected as a faecal marker for the current study owing to its stability and high levels of specificity and sensitivity for IBDs. The use of a non-invasive marker minimised disruption to the child's day to day environment.

The objective of this study (Period I - Part A) was to determine the baseline for calprotectin using the four faecal samples collected. The baseline data for these sequential calprotectin samples (1- 4) was analysed in 48 subjects using a series of statistical tests presented in the result section (Period I – Part A).

Continuous variability of calprotectin was represented via a Box and Whisker plot (see Figure 3-3). The presence of outliers across the Box and Whisker plot influenced the SD and the mean across the four pre-treatment calprotectin samples (see Table 3-2) resulting in a wide spread of the data.

As previously stated, it has been shown that calproctectin is a remarkably stable and reliable marker to assess IBDs using a faecal sample (Bunn et al., 2001, Aadland and Fagerhol, 2002, Summerton et al., 2002, Fagerberg et al., 2003, Szarszewski et al., 2003, Striz and Trebichavsky, 2004, Lundberg et al., 2005, Langhorst et al., 2008, Manz et al., 2012). The marker has proven to be highly reliable (Ton et al., 2000, Fagerberg et al., 2003) regarding stability, sensitivity and specificity but these studies were based on single samples and therefore cannot be relied upon to shed light on the intra-individual biological variability of the marker. These are two distinct and important features of bio-analysis. The measurement of the marker itself is stable, sensitive, specific and reproducible but the production/secretion from the GI tract varies according to the patho-physiological state of the subject and thus there is intra-individual biological variability . Gilbert et al. (1996) and Husebye et al. (2001) analysed a series of faecal samples from “normal” subjects and found that they displayed biological variability. The authors suggest that day to day physiological change contributes to significant intra-individual biological variation. The published data on sequential calprotectin samples are sparse, however, giving little evidence to determine reliability of baseline intra-individual biological variability . Therefore, it was necessary to attempt to create a biological/physiological baseline over time for comparison with a post-intervention calprotectin assessment. The initial analysis performed on the results was a Cronbach’s Alpha test (see methodology 2.26.3 for information on the test). According to Cronbach’s Alpha test the baseline

results indicated that the four calprotectin samples showed internal reliability of 0.70 or 70%. According to the literature (Bland and Altman, 1997) a Cronbach's Alpha result of 0.70 indicates a satisfactory reliable measure (Bland and Altman, 1997, Tavakol and Dennick, 2011).

A test-retest reliability analysis was performed on matching samples during the six weeks of the pre-treatment period to determine how calprotectin was excreted in stool samples over time. The individual results of the test-retest reliability (see table 3-2) suggested that the three correlations, from the four collected pre-treatment calprotectin samples, ranged from unacceptable to poor reliability with only one correlation suggesting an excellent reliability. The results from the test-retest reliability suggest that the results of the sequential calprotectin pre-treatment samples were influenced by biological variation over time.

It is important to highlight the results from the Cronbach's Alpha (Cronbach's Alpha = 0.7) analysis that suggest a satisfactory overall reliability for the mean values baseline data. This interestingly contrasts with the individual subject sequential results (Table 3-6). The reliability (Cronbach's Alpha = 0.7) of the mean baseline results, however, suggests that the mean results collected from the pre-treatment calprotectin samples may be compared with the treatment period and post treatment periods with confidence.

According to Rosner (2011) the normal procedure in analyses of variance ANOVA is to eliminate outliers, three box lengths from the edge of the box, as they usually skew the results. Outliers were, therefore, withdrawn from the data set before the ANOVA was performed. It was observed that withdrawing the outliers from the data set decreased the SD spread. (see Table 3-3). The ANOVA analysis was performed on a total of 172 matching samples from the 43 subjects. The results from the ANOVA show no significant difference in the means across the four pre-treatment scores, suggesting that the data is stable with minimal variance. These mean data were then used as a baseline throughout the thesis.

As previously stated, outliers may reflect and potentially reveal an important occurrence. A decision was made, therefore, to reintroduce the outliers on the final descriptive table of this study (see Table 3-5). The analysis of the four sequential calprotectin samples, including the outliers (n=46) (See Table 3-5) suggests that 65% (30/46) of this autistic population displayed relatively stable calprotectin levels over time, and these were mostly within the normal range. However, further, individual, analysis suggests that 35% (16/46) of this population showed a wider calprotectin reference range over the six week period.

Levels of calprotectin determined across the four sequential samples showed that 15% (7/46) of the total population included at least one of the scores entering the IBS clinical range of 51-121 mg/mL, and each of these individuals exhibited a 25% chance of inflammation. Five subjects (11%) presented with two of the four scores between 56-640 mg/mL, and therefore displayed a 50% chance of active/non-active IBD. One subject (2%) had three scores above 50 mg/mL, between 121-195 mg/mL, and consequently this subject had a 75% chance of IBS. Finally, there were three subjects where all four of their calprotectin scores were above 50 mg/mL, and these were between 66–1593 mg/mL. These subjects had a 100% chance of their calprotectin results indicating non-active/active IBD.

It is possible that individual physiological changes may have affected the results of test-retest reliability. The observation that changes occurred between individual calprotectin samples over a relatively short period of time does not explain why results varied, only that they did vary. These changes may be influenced by the day to day physiological changes that occur in the human living organism, potentially being the result of biological variation. Biological variation is the result of a continuous fluctuation of internal biological components. These variations may occur as a cyclical variation hourly, daily, monthly or randomly over any timeframe (Ricós et al., 2009, Perich et al., 2014). Despite there being a large amount of literature on the reliability of calprotectin as a marker of GI inflammation there is very little literature on the intra-individual biological variability or biological variation of

the marker over time (Husebye et al., 2001). These authors have, however, raised the question of the day to day variability of the marker. Their study tested eight consecutive calprotectin samples in fourteen subjects (Husebye et al., 2001). Results revealed that in healthy controls 63% of subjects displayed all eight calprotectin values <30 mg/mL which is normal range for calprotectin levels. Seven of the fourteen subjects (37%) had high or abnormal levels of calprotectin in at least one measurement. The results suggested by Husebye et al. (2001) are confirmed by this current study. Currently, This variation of calprotectin over time cannot be explained.

The data from this study suggest that one random stool sample is not sufficient to reliably indicate inflammation or to reliably assess, either positively or negatively, autistic children for organic diseases such as IBDs or IBS. Thus, a single measurement of calprotectin levels may be insufficient, either to corroborate the reported clinical symptoms or to establish a diagnosis. In fact, biological variation could create an artefactually higher chance of the calprotectin result not correlating with, or reflecting, the reported clinical symptoms. Autistic children with clear clinical symptoms of IBS or IBD should therefore have more than one, preferably three, sequential faecal samples taken and analysed to determine calprotectin concentration more confidently.

There are to date two studies that have analysed sequential faecal calprotectin samples, both with smaller population samples than this study and with normal, symptom free subjects (Gilbert et al., 1996, Husebye et al., 2001). This current study has analysed sequential samples from autistic children who also display GI symptoms. The results of this study suggest that intra-individual biological variability of calprotectin in sequential samples needs more research for complete understanding.

4.3.2 Period I – Study I (Part B) – Discussion of M2-PK in Sequential Faecal Samples

Recently, the dimeric isoform of pyruvate kinase, M2-PK, has been identified as a novel metabolic marker for various tumours, including colorectal (Sherwood et al., 2005, Ewald et al., 2007), pancreatic, lung, ovarian (Ahmed

et al., 2007), and breast (Sherwood et al., 2005, Ahmed et al., 2007, Lazarev et al., 2010). M2-PK reflects tumour metabolic activity and provides useful information on follow-up screening after surgery or during chemotherapy (Eigenbrodt, 2001). It is also considered to be a stable marker for the detection of IBD (Chung-Faye et al., 2007, Day et al., 2012).

A recent study reported that the faecal M2-PK marker has a sensitivity of 92% for the detection of colorectal cancer, 60% for the detection of large polyps and 25% for the detection of small polyps, and specificity was also 92% (Koss et al., 2005).

Chung-Faye et al. (2007) demonstrated a possible linear correlation between levels of calprotectin and the levels of M2-PK and suggested M2-PK to be a novel marker to differentiate IBD from functional bowel disorders. Another study demonstrated that M2-PK is an effective marker for detecting GI mucosal inflammation in children suffering from CD when compared to healthy controls. This suggests that M2-PK is a possible non-invasive marker for detecting active IBD in a paediatric population (Day et al., 2012).

M2-PK was selected as a faecal marker for assessing GI inflammation in autistic subjects because of its linear correlation with calprotectin levels (Fengming and Jianbing, 2014) and its non-invasive quality. Moreover, both markers could be assessed from the same stool sample, facilitating sample control. In addition, M2-PK is suggested to be a useful non-invasive marker of cell turnover, helpful in assessment and differentiation of IBD and IBS in children (Czub et al., 2007).

The sequential analysis of M2-PK (Period I – Part B) matches with the analysis of calprotectin sequential samples. The marker is stable and displays high levels of specificity and sensitivity in detecting IBDs. To date no literature has been found on sequential sampling for M2-PK in either adults or children. Therefore, it was necessary to create a baseline for M2-PK samples to be used to correlate with the periods II and III of the current thesis.

The same stool specimen collected from 48 subjects (n=48) used for calprotectin (Period I –Part A) assessment was utilised for the analysis of M2-PK. The statistical analysis of the initial 48 subjects presented a wide standard deviation for the collected sequential faecal M2-PK samples (Period I – Part B). The SD ranged from 2.7 to 5.1 for a mean range of 1 - 19 (see Table 3-6). The results from the sequential M2-PK samples show a similar trend and tend to corroborate the results from the analysis of calprotectin pre-treatment therefore also raising questions on the intra-individual biological variability of the marker.

Intra-individual biological variability of M2-PK may be suggested by the results from the test re-test reliability analysis that showed unacceptable to questionable reliability when comparing individual samples over time (see Table 3-7). However, the analysis performed for the Cronbach's Alpha suggests a very strong overall internal consistency (Cronbach's Alpha = 0.8 or 80%) of the pre-treatment mean result s. Therefore, the mean levels from pre-treatment M2-PK samples s can be used as a baseline to compare the treatment period and post treatment period means.

Outliers were also present in the analysis of M2-PK weekly samples. These were excluded before the ANOVA analysis was performed bringing the sample numbers to 45 (outliers - subjects 39, 40 and 45). Two of the outliers that were detected on the M2-PK analysis were also detected in the calprotectin outlier analysis (subjects 39 and 40) (Figure 3-3 and 3-4).

The repeated ANOVA (see Table 3-9) demonstrated a significant lack of variation between the four M2-PK sequential scores. These results together with the results of the Cronbach's Alpha confirm that the mean data can be used as a baseline to correlate with the means of the periods II and III.

However, the analysis in table 3-10 demonstrates differences in the weekly M2-PK value levels ranging from 1U/mL to 20 U/mL (normal to abnormal). This variation in the sequential values for M2-PK for individual subjects may indicate that, as with calprotectin analysis (Period I – Part A), the weekly samples for M2-PK may be influenced by physiological intra-individual

biological variability. According to the data obtained, a single sample analysis gives a 70% of chance of showing a consistent level of M2-PK. However, there is a 30% chance that any of the four samples could display an inconsistent result. Therefore, subjects relying on diagnosis via collection of a single sample have a 30% chance of being incorrectly diagnosed.

Results from M2-PK statistical analysis of sequential samples are similar to the analysis of calprotectin. To assess M2-PK levels reliably, either for a normal or for an abnormal range in autistic children, a single sample faecal M2-PK analysis is insufficient to confidently corroborate the clinical signs/symptoms or to establish a biochemical diagnosis. In the absence of publications regarding sequential M2-PK faecal markers, corroborative evidence from the literature was not possible.

The novelty of this study lies in the analysis of four sequential M2-PK faecal samples from autistic children suffering from GI symptoms. The result of this study is similar to the calprotectin sequential analysis, both markers may potentially be influenced by intra-individual biological variability and therefore more research on sequential samples is advised for both markers.

4.3.3 Period I – Study I (Part C) – Discussion of Lactoferrin in Sequential Faecal Samples

Lactoferrin is a 703 amino acid iron binding glycoprotein present in mucosal secretions and in the secondary granules of neutrophils (Levay and Viljoen, 1995, Walker et al., 2007). Is has been suggested that lactoferrin is a possible marker for assessing IBD activity and, therefore, a potential non-invasive marker of inflammatory bowel diseases such as CD , UC and a discriminative marker of IBD and IBS (Sidhu et al., 2010a, Sidhu et al., 2010b). Lactoferrin has the ability to respond to several changes in homeostatic balance, as its' biological functions range from anti-bacterial, anti-viral, anti-parasitic, anti-fungal to anti-inflammatory (Gonzalez-Chavez et al., 2009, Jenssen and Hancock, 2009). A recent study evaluated lactoferrin levels in faecal samples from 170 children with a mean age of 13.4 years, to assess IBD disease activity. The study suggested that faecal lactoferrin levels

are a reliable non-invasive marker within the paediatric population (Walker et al., 2007).

In the last decade studies have shown that lactoferrin has very high specificity and sensitivity levels for identifying IBDs and IBS (Kane et al., 2003, Lundberg et al., 2005, Sidhu et al., 2010b). A study by Kane et al. (2003) suggested that faecal lactoferrin was 90% specific for diagnosing active IBD and 100% specific in discarding IBS as a potential diagnosis, whilst Langhorst et al. (2008) suggested that lactoferrin levels were 85% sensitive and 77% specific diagnosis of IBS.

Sidhu et al. (2010a) analysed the specificity and sensitivity of lactoferrin in CD, compared with the use of capsule endoscopy; a novel wireless imaging device, as a means for diagnosing IBD. The study suggested a correlation between faecal lactoferrin levels and capsule endoscopy ($p = 0.03$). Lactoferrin sensitivity and specificity was reported to be 71% and 100%, respectively; however, only seventeen patients were recruited to this study (Sidhu et al., 2010a). Another study by Sidhu et al. (2010b) analysed single samples from subjects to determine the sensitivity and specificity of faecal lactoferrin in differentiating between IBD and IBS. This study recruited a total of 465 subjects, 137 with IBS, 126 with UC, 104 with CD and 98 healthy volunteers and suggested that lactoferrin has a higher discrimination for IBD compared with IBS; with a sensitivity of 67% and a specificity of 96%.

The rationale for the use of lactoferrin as a marker of inflammation was due to its non-invasive nature as well as a high correlation with faecal calprotectin (Period I -Part I) in diagnosing IBDs. Therefore, both markers act well as surrogate markers for disease activity.

The sample size for this analysis differed from the analysis of calprotectin and M2-PK due to an accidental discard of samples by the laboratory, and was, therefore, outside of the author's control (see Appendix 10). The total sample size for this study was 24 (n=24).

A test-retest reliability analysis revealed that individual lactoferrin samples had unacceptable reliability (see table 3-11). However, further analysis was performed to assess the variation between each sample mean. A repeated measure ANOVA (n=24) was conducted which revealed no significant variation between the four lactoferrin sample mean values, W = 0.82, F (3, 21) = 1.45, p = 0.25 (see Table 3-13). These results confirm that the mean data can be used as a baseline to correlate with the periods II and III of the current thesis.

According to Lundberg et al. (2005) and TechLab® (2008) (see methodology table 2.21.4) lactoferrin faecal sample results below 7.25 µg/mL are considered negative for IBD and IBS. In this study, 18 subjects (75%) (see Table 3-14) presented with all four scores below the cut-off level (7.25 µg/mL), thereby indicating a negative test result for either IBD or IBS. The remaining six subjects, (25%) (see Table 3-14), presented with one or two scores above the cut-off level (7.25 µg/mL), and were therefore consistent with a positive result for either IBD or IBS. The data suggest that 75% showed consistent results across the sequential samples. However, the remaining 25% of the weekly sample results were not consistent. Therefore, in common with calprotectin and M2-PK sample analysis, it is possible that the variation of the samples was due to individual intra-individual biological variability.

The novelty of this study was the analysis of four sequential lactoferrin faecal samples from autistic children suffering from GI symptoms. All previous studies found have been based on single faecal lactoferrin samples and, it has not been possible to produce corroborative evidence from the literature.

Although the data suggest that a single sample analysis gives a 75% chance of being consistent for levels of lactoferrin, further analysis revealed a wide variation of the marker across the weekly individual results. To reliably assess, either for normal or for abnormal lactoferrin ranges in autistic children, and for organic diseases such as IBD or IBS, a single lactoferrin faecal analysis may not be sufficient to either corroborate the clinical symptoms or to establish a biochemical diagnosis.

A limitation of this study was the small number of subjects tested (n=24). Even though the sample size was smaller compared with the other two markers tested in this thesis, lactoferrin results were similar in trend to the other markers tested. It is concluded that potentially all three markers may be influenced by individual day to day intra-individual biological variability and therefore more research on sequential faecal samples markers is necessary.

4.3.4 Period I – Study I (Part-D) – Discussion of sequential S.0.S Questionnaire

As stated previously in the methodology section of this thesis, an outcome measure used to assess behaviour and GI signs was a ten point scale questionnaire based on the Autism Research Institute Secretin Outcomes Survey Form (the 'S.O.S Form'), which is a validated and standardised questionnaire (Rimland, 1998, Brudnak et al., 2002, Unis et al., 2002, Esch and Carr, 2004, Erickson et al., 2005, Sturmey, 2005, Williams et al., 2005). To create a baseline, parents completed the questionnaire four times during the pre-treatment period (Period I/baseline control period) according to their assessment of their child's GI and behavioural signs and symptoms (see questionnaire appendix 5)

Statistical analyses of the four S.O.S pre-treatment questionnaires of the 48 subjects (n=48) are presented in the results section (see results chapter section 3.3.4). Descriptive analysis of the 24 parameter questionnaires were divided into four tables (see Table 3-15, Table 3-16, Table 3-17 and Table 3-18). Each of the parameters was individually assessed for the means and standard deviation which varied according to each parameter.

The results from the Cronbach's Alpha (Cronbach's Alpha = 0.8 or 80% of reliability) analysis revealed that the S.O.S questionnaire displayed good overall reliability as a tool for measuring behaviour and digestive signs in children suffering from ASD. This suggests that the tool can be used reliably to compare data across the different periods of this thesis.

The S.O.S questionnaire parameters were subdivided into four sections called the subscales. Each subscale recorded one specific group of signs. The

results of the statistical analysis of the questionnaire per subscale can be seen in the Box & Whisker Plot, Figure 3-6. The subscale, 'Social Behaviour and Communication', (see questionnaire appendix 5) demonstrated a similar mean and standard deviation to the subscale 'Ritual and Repetitive Activities'. In comparison the subscale 'Digestive Signs' and 'General Signs' showed much lower means and SDs indicating that the subjects were more affected using these parameters as assessment criteria.

According to Jerckings (1998), the advantages of using questionnaires in quantitative research include that a large amount of information can be collected in a relative short period of time, information can be collected by a number of people with limited effect on the validity, the data generated from the questionnaires can be quantified easily and therefore used scientifically,; data can be compared and correlated with other researches, and data can be used to measure changes before and after intervention.

However, no scientific measuring tool is perfect and there are some known disadvantages of using a questionnaire to collect data for scientific research. These are: reliability and accuracy of recall, the level of subjectivity of people's perception when answering a question, and the possibility that important questions for the study may not have been answered (Rosner, 2011).

All of the questionnaires used for the baseline and for later stages of this research were based on parental perception of their child's behaviour and digestive signs. Using parent's perceptions may be seen as a limitation to the study potentially skewing the data because parents might underestimate or overestimate the scoring system of the questionnaire erroneously. This may be dependent on several factors; including; proper understanding of what was asked, lack of commitment when answering the questionnaire or, for example, a subconscious desire to please the researcher. This may generate a bias that could potentially skew the data. In an attempt to decrease the possibility of the data being influenced by bias, parents were asked to answer the same questionnaire at four different instances during a six weeks period. Splitting

the questionnaires into different times may potentially create a random answering effect and potentially decrease the possibility of the bias.

Parents and guardians of autistic children are constantly affected by the stresses of dealing with the day to day aspects of raising a child with ASD. In a report by Nock and Kazdin (2001), the authors suggested that parents of autistic children have low expectations of any type of treatment for their child. This could result in the possibility that parents over-grade the symptoms and under-grade any positive effect of the treatment when responding to a questionnaire (see discussion in Period II of this thesis). This effect, if it occurred, would result in underestimation of any positive post-treatment response.

Even though each parent's perception was important for this study, parents themselves were not assessed as it was beyond the scope of this thesis. Hence, no assumptions can be made in terms of their wellbeing and how that may have affected the questionnaire scores. Acknowledging this, it cannot be denied that their own wellbeing may have affected their answers to the questionnaires.

At this stage there is no conclusion to be drawn from the baseline results of the four sequential S.O.S questionnaires. The questionnaire parameters were re-tested during the treatment period and collated with the contemporaneous faecal marker results for calprotectin, M2-PK and lactoferrin in a later stage of the research.

4.3.5 Period I – Study I (Part-E) – Discussion of Correlations between calprotectin, M2-PK and lactoferrin

The final statistical analysis performed on the baseline data was a correlation analysis between calprotectin/M2-PK and calprotectin/lactoferrin. The correlation utilised the results from calprotectin baseline on matched samples for M2-PK and lactoferrin. The rationale for utilising calprotectin as the main marker for this thesis was its remarkable stability and its high specificity (100%) and sensitivity (80%) for diagnosing IBDs and IBS. M2-PK and lactoferrin were used as secondary markers. Therefore, this study attempted

to assess whether a correlation existed between biochemical markers in sequential faecal stool samples taken from autistic children suffering from GI symptoms.

A Pearson correlation (see methodology 2.27.4 for statistical test explanation) was conducted to determine whether there was a significant linear relationship between the means of the calprotectin and M2-PK sample concentrations. The results indicated that there was a significant moderate positive correlation ($r = 0.40$) between the calprotectin and M2-PK means ($r = 0.40$ $n = 38$ 2-tailed $p = 0.01$), significant at $p < 0.05$, (see table 3-20) (see section 3.3.5.1). Increases in calprotectin faecal concentrations were weakly associated with increases in M2-PK faecal concentrations (see

Figure 3-7). A correlation between calproctectin and M2-PK was previously suggested by Chung-Faye et al. (2007), however these authors utilised single faecal samples. The current study utilised sequential calprotectin and M2-PK matched samples and attempted to correlate the means.

A Pearson correlation was performed to determine whether there was a significant relationship between the total mean concentrations of calprotectin and lactoferrin. The analysis indicated that there was a significant moderate positive relationship between the two variables ($r = 0.66$, $n = 24$, $p < 0.001$), (see Table 3-21 and Figure 3-6) These results should, however, be viewed with caution as the lactoferrin sample size was small following the accidental discard of some samples which was outside the control of the author (see appendix 10).

No known study to date has used sequential faecal biochemical markers to assess GI inflammation. Previous studies have used a single sample to attempt a correlation between calprotectin and M2-PK, suggesting a strong linear relationship between the two markers (Chung-Faye et al., 2007). Another study supports the idea that lactoferrin is a useful marker in predicting IBDs and it correlates well with capsule endoscopic assessment (Sidhu et al., 2010a). Previous studies also suggested that calprotectin and lactoferrin correlate well with endoscopic examination. (Yamamoto et al., 2013).

After matching the appropriate samples, the data was analysed using a Pearson's correlation test. The results of the current study suggest that there is a weak linear correlation between calprotectin and M2-PK mean values suggesting that these markers may potentially be used together as markers of inflammation or disease activity in autistic children suffering from GI problems. However, due to the weak linear correlation the results should be viewed with caution.

There was moderate correlation between calprotectin and lactoferrin concentrations. This indicates that these two markers may also potentially be used together as markers of inflammation in autistic children.

Even though the results from the correlations between markes were positive, these results should be treated with caution. The sample sizes were small, a limitation of the study, particularly regarding the correlations between calprotectin and lactoferrin.

No known study to date has published data on the analysis of sequential calprotectin, M2-PK and lactoferrin faecal samples in children displaying the autistic spectrum disorder (ASD) who also show GI symptoms. Despite the limitations of the study the results are encouraging and may stimulate more research in this field.

4.3.6 Discussion Study II - Twenty-four parameter S.O.S questionnaires evaluation at Period I, Period II and Period III

In the past fifteen years, research into ASD has suggested that there is a possible gut-brain axis, where the worsening of behavioural symptoms may possibly be due to inflammatory gut reactions meditated by immunological signals (Jyonouchi et al., 2005a, Reichelt and Knivsberg, 2009, Forsythe et al., 2010).

This suggests that the positive comparative results between the periods I, II and III, suggested and presented in the results section of this thesis, may be of high importance. Nock and Kazdin (2001), suggest that these results may reflect a much greater significance of real positive changes.

The significant overall results, demonstrated by the repeated measure ANOVA analysis of the mean values, between the periods and the post hoc test (see results section 3.5.1), revealed that VOT may be effective in decreasing overall signs and symptoms of autistic children as perceived by their parents. This is reflected in a significant decrease in signs and symptoms when comparing the control/baseline period, the treatment period and the post treatment period.

Significant changes were also perceived in the individual questionnaire subscales reported on section 3.5.3 of this thesis. The subscales, 'social behaviour and communication' and 'digestive signs', produced a significant main effect on the repeated measures ANOVA and also demonstrated significant changes between the pre-treatment and the treatment post hoc test. This suggests that there was an overall positive significant improvement ($p < 0.05$) between before and after VOT treatment in autistic children suffering from GI symptoms.

Individual analysis of three of the twenty four parameters tested also showed significant changes (see Figure 3-9, Figure 3-10 and Figure 3-11). Seventy five percent of the study population showed an improvement in the parameter 'vomiting' ($p = 0.00029$) by 2 points on a 10 point scale, fifty percent showed improvement in 'poor Appetite' by 2 points on a 10 point scale ($p = 0.039$) and fifty percent showed improvement in the parameter 'lack of eye contact' by 1 point on a 10 point scale ($p = 0.035$). The data analysis of the 'vomiting', 'poor appetite' and 'lack of eye contact' parameters demonstrated statistically significant improvements, suggesting that the use of VOTs may be of benefit to children with autism.

VOTs are a low-invasive form of treatment that uses manipulation of the abdominal tissues and organs, possibly improving blood and lymphatic circulation within the abdomen. However, the purpose of this study was to determine whether there were any changes resulting from a VOT treatment intervention; not to investigate any mechanism behind such GI and/or behavioural symptomatic changes.

It is possible to speculate that the changes which occurred after application of VOT on autistic children may be the result of improvements in the lymphatic system. Data from an animal model (dogs) support the idea that rhythmic compressions of the abdominal area may facilitate the flow of inflammatory mediators into the lymphatic circulation (Huff et al., 2008). Huff et al. (2008) also found that the rhythmic pumping of the abdominal area increases the number of T and B cells within the lymphatic circulation and an abdominal pumping action helps the transport of leukocytes from the mesenteric lymph nodes to the thoracic duct. Similarly, Hodge et al. (2010) found that a pumping action of the lymphatic system of dogs might also result in the mobilisation of inflammatory mediators into the lymphatic circulation. The authors postulated that this could support a potential therapeutic rationale for the enhancement of lymphatic system efficiency in the treatment of infections (Hodge et al., 2010). It has also been proposed that visceral homeostasis may be supported by the relief of congestion, which may possibly be achieved via mobilisation of GI structures (Huff et al., 2008). Therefore, it may be possible that the significant changes reported on the GI and behavioural patterns of autistic children (result section 3.5), may be the response of positive changes in the lymphatic system, although this is currently speculative.

As discussed in the introduction of this thesis (section 1.10), the brain-gut axis may play a role in the severity of the GI and behavioural signs and symptoms of autistic children. The significant improvement after application of VOT in some of the parameters tested using the S.O.S questionnaire may have been influenced by changes in this bi-directional system. Since D'Eufemia et al. (1996) published a paper suggesting the occurrence of GI abnormalities in autistic children, researchers have been investigating the influence of GI imbalances on the behaviour of autistic children (D'Eufemia et al., 1996, Horvath et al., 1999, Ibrahim et al., 2009, Nikolov et al., 2009). It may be possible that the enteric nervous system, via the IPANS with its stretch receptors (Furness, 2000), may be influenced by the application of VOT. Consequently, this may influence the neuro-regulatory pathway in the autistic children resulting in positive GI and behaviour changes after application of VOT, although again this is speculative.

Gershon (1999) and Goehler et al. (2007) reported that changes in the mood and cognition of autistic children may be the result of ENS imbalances. Therefore, it is possible that treating the GI problems in these children may also potentially improve behavioural changes. In this current study, lack of eye contact, one of the most noticeable behavioural patterns in autism, demonstrated a significant improvement. Even though the mechanism of this change cannot be explained it might be hypothesised that positive changes in the ENS could be effected by manual treatment.

One of the limitations of the present study is that autistic children in the low spectrum usually lack the ability to describe their symptoms. Therefore, the present research relied on the parents' perceptions of their child's symptomatic changes. Due to ethical constraints relating to the awareness of autistic children, the study was designed as a before and after intervention (internally controlled study). All designs have intrinsic limitations, but the most appropriate design is that which affords the research an ability to achieve adequate results and conclusions, but with minimal, if any, negative impact on the participants. Using the same subjects as their own control provides a secure and well established method of achieving a baseline which may then be used as the control.

The novel approach of using VOTs for this project to treat children with autism, has indicated that this low-invasive form of treatment could have a significant and important impact on the quality of life and wellbeing of children with autism who also suffer GI symptoms. This research reports some promising positive results, specifically behavioural and GI symptoms of autistic children following VOT treatment. The results may be considered to add weight to the gut-brain axis hypothesis postulated in recent papers (Mayer et al., 2006, Reichelt and Knivsberg, 2009, Forsythe et al., 2010, Mayer and Tillisch, 2011).

4.3.7 Discussion Study III Calprotectin, M2-PK and lactoferrin concentrations measured at Period I, II and III

This study assessed levels of nine sequential samples of faecal biochemical inflammatory markers, calprotectin, M2-PK and lactoferrin, before VOT

(Period I – control Period), during VOT (Period II – treatment period) and after VOT (Period III – post-treatment period) in autistic children.

Studies have suggested that the cognitive deficit in autistic children may be linked to GI symptoms (Horvath, 2000, Horvath and Perman, 2002, Koves et al., 2004, Valicenti-McDermott et al., 2008). Autistic individuals at the lower end of the spectrum and with impaired language skills, may potentially express GI pain and discomfort through body mannerism and self-harm (Carr and Owen-Deschryver, 2007). According to Breau et al. (2003), the greater the behavioural or mental impairment the higher the pain and discomfort. The action of pushing the abdominal area against objects, running around in circles or jumping several times, may possibly be a way to alleviate GI discomfort.

The bowel symptoms of autistic children resemble elements of IBS; however, a specific link has not been found. Carr and Owen-Deschryver (2007), postulate that the intensity and frequency of pain in autistic individuals could lead to problem behaviour. A consensus report analysed anecdotal reports from parents in an attempt to establish the importance of assessing the GI function of autistic children. This report suggested that autistic children should not only be investigated and treated for their behavioural problems, but should also be investigated and possibly treated for GI problems as these may be atypical and therefore may go undiagnosed (Buie et al., 2010). Kearney and Brown-Chang (2008) support the idea of complementary and alternative methods for the treatment of IBS patients due to the psychological benefits of combined ‘body and mind’ methods.

The faecal markers used for this analysis have already been presented in the discussion of the baseline results of Study I – Parts A, B and C (see sections 4.3.1; 4.3.2 and 4.3.3). All three markers displayed high levels of sensitivity and specificity for diagnosing IBDs and differentiating IBS. The three markers’ baseline presented in Study I – Parts A, B and C have created a satisfactory overall reliability for the mean values data for all three markers. This baseline has been used to compare the results of calprotectin, M2-PK and lactoferrin during period II and III of this thesis.

The analysis of the repeated measure ANOVAs suggests that there are no significant changes in the inflammatory biochemical markers calprotectin, M2-PK and lactoferrin between Period I - control, Period II - treatment and Period III - post-treatment. However, there is a progressive increase in calprotectin mean concentrations between Periods I, II and III of the study (see Table 3-27 and Figure 3-12). Although, this increase is not significant, it may suggest that the use of VOTs between Period I - control period and Period II - treatment period has possibly influenced the changes in the inflammatory marker levels. There is also a progressive increase in calprotectin mean levels between Period II - treatment period and Period III - post-treatment period, which was measured six weeks after the last VOT session.

Analysis of the M2-PK and lactoferrin levels also demonstrates changes in the mean scores between Period I - control, Period II - treatment and Period III - post-treatment (see Table 3-29 and Table 3-31) (see Figure 3-13 and Figure 3-14). These changes occurred during the application of VOTs, between Period I - control period and Period II - treatment period, where a drop in the mean levels of both biochemical markers was observed. During Period III – post-treatment period, six weeks after the last VOT session, the mean concentrations of M2-PK and lactoferrin increased.

Although there were no significant changes in the mean concentrations of the faecal biochemical markers, further analysis demonstrated notable changes when comparing the three distinct study periods, which although not statistically significant should not be ignored.

This is the first study to employ the use of biochemical markers to measure symptomatic changes before, during and after VOTs and to date, no comparative study has been published. Further studies on the effect of VOTs on autistic children suffering from GI may provide more robust answers.

4.3.8 The relationship between calprotectin and twenty-four parameter S.O.S questionnaires

Another aim of this study was to determine whether a correlation exists between the very stable faecal GI inflammatory marker calprotectin, the main

marker for this thesis, and the perception of the parents of autistic children to their child's GI symptoms. A twenty-four parameter questionnaire was used, the S.O.S questionnaire, to assess parents' perceptions and a standard ELISA test used to assess calprotectin levels present in the stools (see methodology chapter for information on the marker and the S.O.S questionnaire 2.19 and 2.22).

There are a number of methods for determining bowel inflammation, including biopsy, endoscopy, colonoscopy and stool analysis for biochemical markers (Sherwood, 2012, Berthold et al., 2013). The least invasive, particularly important in children, utilises biochemical markers, including the S100 group of proteins (Aomatsu et al., 2011). Calprotectin is a member of the S100 protein family, also known as MRP8/14, and is a heterodimer of two calcium binding-proteins (Striz and Trebichavsky, 2004, Stroncek et al., 2005) and accounts for 30% to 60% of total cytosolic protein found within neutrophils (Olafsdottir et al., 2002, Yui et al., 2003, Stroncek et al., 2005).

Currently, the main application for the calprotectin ELISA test is to differentiate between IBD and IBS (Tibble et al., 2002). The concentration of calprotectin decreases during successful IBD therapy and higher levels may be indicative of an IBD relapse (D'Inca et al., 2008). Assessing faecal calprotectin has been found to be a reliable diagnostic tool for patients who present with IBD symptoms (Xiang et al., 2008). Furthermore, a meta-analysis by (Van Rheenen et al., 2010) determined that calprotectin as a predictor of inflammation in adults had a sensitivity of 93% and a specificity of 96%, whilst 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 can be used before more invasive procedures (Bunn et al., 2001, Van Rheenen et al., 2010, Zippi et al., 2010).

This study sought to investigate a twenty-four parameter questionnaire and determine whether an association existed with the biochemical marker calprotectin. The study found a significant correlation between the parameters 'need for a fixed routine' and 'constipation', and the inflammatory biochemical

faecal marker calprotectin. These results suggest that these two parameters could be used together as an initial screen in a standardised questionnaire, which would be a simple to use, non-invasive and inexpensive predictor of inflammatory bowel processes in autistic children between the ages of 3 ½ and 8 years old.

The results from the univariate study indicate a further four parameters as having a possible association with levels of calprotectin (see Table 3-32 and Table 3-33). Although they are not independent predictors of GI inflammation, they could be appropriately used in combination with the two multivariable parameters ‘need for a fixed routine’ and ‘constipation’.

This final set of six parameters could be used as a standard questionnaire within clinical settings in order to simplify the diagnostic process, thereby enabling greater cost efficiency and more effectively directing subjects to further diagnostic examination (see Table 3-33). Combining these six parameters into one single questionnaire could enable a reduction in the number of parameters to be tested from twenty-four to six, without any loss of power or sensitivity. Such a standardised six parameter questionnaire would not replace any other form of diagnostic testing for GI inflammation, but could be a cost effective, non-invasive initial screen for clinical diagnosis.

The correlation between the behavioural 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 exhibited by autistic children.

From the study results it may be possible to create a useful, short questionnaire that is simple to use, reliable, non-invasive and inexpensive to be used as a screening tool which is capable of evaluating GI inflammation in autistic children. Such a questionnaire could be easily completed by parents or guardians and could potentially facilitate initial screening of GI inflammation in autistic children.

Chapter 5 - Conclusions and Summary of Future Work

The conclusion of the outcome measures used before, during and after visceral osteopathic sessions in autistic children is presented in this chapter. The chapter is subdivided into conclusion of study I, II, III and IV, as well as a summary of future work, limitations and contribution to knowledge.

No known study to date has used sequential faecal biochemical markers and questionnaires to assess autistic children also suffering from GI symptoms before and during the application of VOTs. The results showed large biochemical marker concentration variations within faecal samples over time which is strongly suggestive of wide intra-individual biological variation. Since this study used multiple sampling, also described by Gilbert et al. (1996) and by Husebye et al. (2001), rather than the single sampling techniques of other studies (Fagerberg et al., 2003, Lundberg et al., 2005, Sherwood et al., 2005, Langhorst et al., 2008, Aomatsu et al., 2011, Sherwood, 2012) this finding of biological variation of the markers over time is a potentially important finding for future diagnostic protocols.

The novel approach of this project in using VOTs to treat GI symptoms of ASD children has indicated that this low-invasive form of treatment could have a significant and important impact on their quality of life and wellbeing. This research may be considered, also, to add weight to the gut-brain axis hypothesis postulated in recent papers (Mayer et al., 2006, Reichelt and Knivsberg, 2009, Forsythe et al., 2010, Mayer and Tillisch, 2011).

An important contribution of this research is that it shows sufficiently positive outcomes to support and stimulate further investigation into the use of VOTs in these children.

This innovative study has involved treatment of behaviourally challenging autistic subjects (McClintock et al., 2003, Murphy et al., 2005, Chiang and Lin, 2008) over an extended period of time (18 weeks) and taken serial measurements using questionnaires and biochemical markers. Moreover, involving the same subjects throughout the study has avoided the introduction

of variation that could occur between subject groups displaying a complex behavioural disorder, for example when using a different control population that could potentially create unmatched data for analysis (Mallinckrodt et al., 2003, Nakai, 2009, Rosner, 2011).

5.1 Conclusion Study I – Period I – Parts A, B and C

An important feature of this study is the inclusion of multiple sampling of individual subjects. Multiple sampling creates an individual trend of inflammatory levels that reflects a more accurate appraisal of disease activity. The result is a more robust reflection of the real inflammatory state than given by an analysis using single sample testing. Other studies (Bunn et al., 2001, Aadland and Fagerhol, 2002, Summerton et al., 2002, Szarszewski et al., 2003, Yui et al., 2003, Fagerberg et al., 2005, Chung-Faye et al., 2007, Sidhu et al., 2010a, Sidhu et al., 2010b, Day et al., 2012, Manz et al., 2012) have used single samples and taken the mean of the population. The design study of this work has enabled an innovative approach to the assessment of faecal biochemical marker levels. This study determined a mean concentration level for each biochemical marker from four sequential faecal samples, thus providing a more reliable baseline for Periods II and III of the study.

5.1.1 Intra-Individual Biological Variability

The biomarkers are not unstable as has been clearly stated (see sections 2.19; 2.20 and 2.21), but there has been shown to be considerable intra-individual biological variability in some subjects when multiple samples are assessed over time (see section 4.3.1). The method of taking multiple samples is considerably more robust than taking a single sample and then basing a diagnosis on that one result. Currently, the recommendation for diagnosticians to help distinguish between IBD from IBS is the use of a single faecal calprotectin analysis (NICE, 2013). NICE recommends further investigation such as blood tests, i.e. C-reactive protein and full blood count if the result of the faecal calprotectin analysis is raised (NICE, 2013). However, in this current project up to 4 samples were taken to obtain a background, or control level, during the pre-treatment period of each biochemical marker.

From this approach it can be seen that for some patients there is large biological variability even when there has been no overt intervention or treatment given. This background has shown some subjects with considerable outlier results, whereas other subjects have relatively constant levels. The implication of this intra-individual biological variability has been discussed further in results sections 3.3 and discussion sections 4.2; 4.3.1; 4.3.2; 4.3.3 and 4.3.7.

Table 1-3 in section 1.11.1 clearly shows the levels of sensitivity and specificity of calprotectin, M2-PK and lactoferrin (Bunn et al., 2001, Fagerberg et al., 2003, Kane et al., 2003, Fagerberg et al., 2005, Chung-Faye et al., 2007, Langhorst et al., 2008). Ton et al. (2000) have shown that calprotectin, the main faecal biochemical marker of the current study, has high levels of stability and reproducibility. Gilbert et al. (1996), Husebye et al. (2001) and Ricós et al. (2009) suggest that faecal markers may be influenced by the day to day physiological intra-individual biological variability of each particular individual. More recently, Calafat et al. (2015) have suggested significant intra-individual variation occurring within a day. However, the suggested intra-individual biological variability does not impact on the high reproducibility, sensitivity and specificity of the markers.

Calprotectin, M2-PK and lactoferrin were assessed weekly for individual scores (Table 3-5, 3-10 and 3-12). The data from Period I suggest that using one random stool sample to assess calprotectin, M2-PK and lactoferrin levels, is not a reliable measure of the presence or absence of inflammation and that it is probably necessary to perform multiple sample tests; either to corroborate the clinical symptoms or to establish a biochemical diagnosis. Utilising only one faecal sample could create an artificially higher chance, in either direction, of the calprotectin, M2-PK or lactoferrin results, not reflecting the true clinical picture/pathological changes. Autistic children who display clear clinical symptoms of IBS or an IBD should be screened via the analysis of more than one faecal calprotectin and/or M2-PK and/or lactoferrin sample, in order to ascertain that the assessment of these markers accurately reflects the real situation within their GI system.

There is to date, no known study of the analysis of sequential faecal samples in autistic children who also display GI symptoms. It is suggested that a larger sample population should be investigated using multiple sample testing to determine the validity of these markers as a tool in the identification of IBD or IBS in autistic who suffer from GI signs and symptoms. Also, future studies may consider attempting to correlate a standard examination tool, such as an endoscopic GI assessment, with sequential faecal markers, to further examine this non-invasive investigative procedure in autistic children.

The statistical analysis of Study-I Parts A, B and C, concluded that changes in calprotectin, M2-PK and lactoferrin weekly scores may potentially be due to the results of individual physiological intra-individual biological variability , suggested by Husebye et al. (2001) and Ricós et al. (2009). Even though there is a possibility that individual intra-individual biological variability is a feature of the markers, analysis suggested that the mean baseline data for the pre-treatment sequential samples for calprotectin, M2-PK and lactoferrin, were reliable.

5.1.2 Conclusion Period I – Study I (Part-D): Sequential S.0.S Questionnaire

The S.O.S questionnaire was used to measure 24 parameters subdivided into four sub scales. The data from four sequential questionnaires were used as baseline for later comparison with Periods II and III of the research. Hence, no conclusion was drawn from this study as it was used as comparative data only.

5.1.3 Conclusion Period I – Study I (Part-E): Correlations between calprotectin, M2-PK and lactoferrin

Analysis was performed to assess whether a correlation existed for the markers calprotectin and M2-PK during control/baseline – Period I. After matching the corresponding sequential sample results, the data were analysed using a Pearson's linear correlation test. The results obtained suggest that there is a significant moderate linear correlation between the total calprotectin and total M2-PK mean values ($r = 0.40$, $n = 38$, $p = 0.01$), thereby suggesting that these markers may be interpreted together as

markers of inflammation or disease activity in autistic children suffering from GI disturbances.

A strong correlation was found between the calprotectin and lactoferrin sample results ($r = 0.65$, $n = 24$, $p < 0.001$), indicating that these two markers may potentially be interpreted together as linked markers of inflammation in autistic children. However, caution should be applied owing to small sample size (lactoferrin $n = 24$), as a result of the loss of a number of lactoferrin samples outside the author's control (Appendix 10). Consequently, this correlation between mean scores for calprotectin and lactoferrin should be considered as being of potential interest until further research to confirm or refutes this.

This is the first study to assess a possible correlation between biochemical markers and GI problems and the positive correlation presented here should enhance the published literature.

5.1.4 Conclusion – Study II: Twenty-four parameter S.O.S questionnaires evaluation at Period I, Period II and Period III

The analysis of the nine questionnaires completed by the parents during the different periods of the study, suggests that the use of VOTs in autistic children suffering from GI symptoms may be beneficial. The employment of a parental questionnaire was necessary in order to measure a child's symptomatic status, since young children in the low autistic spectrum are not usually able to provide this information independently. Results of the repeated measures ANOVA for questionnaires 5-8, collected during Period II – treatment, and questionnaire 9 from Period III - post-treatment, demonstrated a significant difference ($p<0.05$) compared with the control questionnaires (1 – 4), collected during Period I. This indicates that there was an observational appreciation of symptomatic improvement after application of VOTs within this study population. There was also significant positive change ($p <0.05$) between the control period scores and the post-treatment period scores indicating that the behavioural and gastrointestinal symptoms remained at an improved level compared with the control period, six weeks after treatment. This indicates, according to parental perception, a positive and lasting

statistically significant effect of VOT intervention on the symptoms experienced by autistic children at six weeks post treatment (Period III - post-treatment).

Analysis of individual parameters showed significant changes in three of the twenty four parameters tested: ‘vomiting’ ‘poor appetite’ and ‘lack of eye contact’.

Statistical improvement is mirrored in the clinical findings reported by the parents and recorded in the case history. Although, this material is anecdotal and was not used as an outcome measure for this thesis, it follows the statistical trend, supporting the beneficial effect of VOT in the study group (result section 3.8, case history anecdotal observation). More often than not, the child would make eye contact with the practitioner and/or the parent during the treatment session, a remarkable event within the ASD experience, even for immediate family (see section 3.8 case history anecdotal observation). Interestingly, eye contact demonstrated statistically significant differences comparing the pre-treatment with the treatment outcome measures in the S.O.S questionnaire.

The mechanism underlying the changes perceived and reported by the parents cannot be known. However, the gut-brain connection may play a role in giving possible clues to answers. Study II of this thesis, suggested positive changes, not only in the gastrointestinal symptoms but in behaviour patterns of autistic children after VOT application. The use of VOT potentially might have activated this complex bidirectional connection between the brain and the gut. However, this is speculation and specific research in this field is required.

Several aspects of the data indicated that VOT is of benefit to autistic children suffering from GI symptoms. However, a limitation was apparent since the low spectrum autistic child is unable to interact with direct, reasoned discussion. Direct clinical benefit from the subjects’ perspective was therefore not possible. Further studies are advised in this field to determine if the same results are extended to a larger ASD cohort.

5.1.5 Conclusion – Study III: Calprotectin, M2-PK and lactoferrin concentrations measured at Period I, II and III

The use of VOTs and their effect on faecal biochemical markers was analysed by comparing the concentrations of calprotectin, M2-PK and lactoferrin in samples collected during Period I – control, Period II – treatment, and Period III - post-treatment.

A repeated measure ANOVA suggests that there were no significant changes for the inflammatory biochemical markers during any period of the study. However, a progressive increase in mean levels of calprotectin was observed between the three distinct periods of the study. Although this increase was not significant, it may suggest that the use of VOTs, between Periods I and II, influenced the change in calprotectin levels. A further increase in mean calprotectin levels was observed between Periods II and III, six weeks after the last VOT session.

The increase in mean levels of calprotectin between the Periods II and III may have been the result of VOT treatment. Possibly, the increase of calprotectin may be supported by Schander et al. (2008). This author suggests that inflammatory mediators are pumped into the lymphatic circulation after manual mobilisation of the abdominal area. The progressive rise of calprotectin may possibly be explained by the increase in number of T and B cells within the lymphatic circulation, resulting in an inflammatory response, also supported byHodge et al. (2010). This study is not attempting to postulate a mechanism for biochemical changes between the treatment and post-treatment periods but it might be the effect of tissue repair within the gut lining.

Analysis of the M2-PK and lactoferrin levels also demonstrated a change in these mean values between the three study periods, and following the application of VOTs there was a decrease in the mean scores for both biochemical markers between Periods I and II. The mean values for M2-PK and lactoferrin during Period III, indicated an increase in these faecal markers compared to Period II. The rationale for these results may possibly be that the

VOTs positively influenced inflammatory responses, resulting in a reduction in the levels of these inflammatory markers whilst treatment was applied.

In Study III, the effect of VOT on the levels of objective markers was attempted. M2-PK and lactoferrin demonstrated a clear decrease in the mean scores during Period II – treatment and a clear increase in the mean score levels during Period III - post-treatment, whilst calprotectin values showed increasing levels from Period I - control through to Period III - post-treatment. The increasing levels of calprotectin during the treatment and post treatment period is, therefore, in direct contrast to the lactoferrin and M2-PK results, where the levels decreased during treatment but rose again post treatment. The reasons for these conflicting results are unknown, and larger studies will be required to investigate this phenomenon further. Whether the markers are reflecting more subtle changes in the inflammatory process, or whether they are measuring different stages of the process remains unclear. However, the aim of this research was not to analyse the mechanism of any changes.

5.1.6 Conclusion Study IV – The relationship between calprotectin and twenty-four parameter S.O.S questionnaires

In study IV, an attempt was made to test parental perception of symptomatic changes objectively and to correlate the faecal biochemical marker calprotectin results with the questionnaire results (parental perception to treatment).

The potential correlation between the 24 parameters of the questionnaire and the biochemical markers was investigated. A significant correlation was found between the parameters, ‘need for a fixed routine’ and ‘constipation’ and the inflammatory biochemical faecal marker calprotectin. These results suggest that these two parameters could be used together as an initial screen in a standardised questionnaires a simple to use, non-invasive and inexpensive predictor of inflammatory bowel processes in autistic children between the ages of 3 ½ and 8 years.

The results from the univariate analysis indicated four further parameters could have an association with levels of calprotectin. Although they are not

independent predictors of GI inflammation, they could be appropriately used in combination with the two multivariable parameters previously identified. Together, this set of six parameters could be used as a standard questionnaire within clinical settings in order to simplify the diagnostic process, thereby enabling greater cost efficiency and more effectively directing subjects to further appropriate diagnostic examination (Table 3-33). Combining these six parameters into one single questionnaire could enable a reduction in the number of questions from twenty-four to six, without any loss of power or sensitivity. Such a standardised six parameter questionnaire would not replace any other form of diagnostic testing for GI inflammation, but could be a cost effective, non-invasive initial screen for clinical diagnosis. Such a questionnaire could be completed by parents or guardians easily and could potentially facilitate initial screening of GI inflammation in autistic children.

The study demonstrated a positive correlation between the marker and specific components of the questionnaires. This information may potentially be used as a standard questionnaire within clinical settings in order to aid the GI diagnostic process.

5.2 Limitations of the Study

One of the limitations of the study is that low spectrum autistic children usually lack the ability to describe or grade their symptoms. Therefore, the results from the questionnaires relied on the parents' perception of their child's symptomatic changes and it was not possible to determine how the subject personally felt in response to the VOT intervention. This limitation may possibly result in parents over or under estimating the rate value in the Likert scale, potentially creating biasing the results. An independent assessor rating the parameters blind could possibly prevent the introduction of 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

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 2.6, was that the subjects were required to present a formal diagnosis of autism and be assigned at 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-IV 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. 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. According to Chandler et al. (2013) and Gorrindo et al. (2012) 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.

Another limitation to this trial is that it was not designed as a randomised control trial owing to constraints imposed by the Ethics Committee. The research was designed as a before and after intervention and does not

compare two intervention or placebo group measures that could potentially minimise bias. However, all designs have intrinsic limitations, and the most appropriate design is that which affords the research an ability to achieve adequate results and conclusions, with minimal, negative impacts upon the participants. Using the same subjects as their own control provides a secure and well established method of achieving a baseline that may then be used as the control.

It was not the aim of this research to analyse the interaction between researcher and the subjects during application of VOT. However, the interaction between practitioner and child cannot be disregarded due to the nature of the VOT. Several studies have shown that ASD children may present with hypo or hyper sensitivity to touch, leading to challenging behaviour such as tactile defensiveness, withdrawal or avoidance of touch (Grandin, 1992, Cascio et al., 2013, Puts et al., 2014). Cullen et al. (2005), explored the effects of positive touch between parents and autistic children. The study suggests that the use of positive touch resulted in improvement of sleep patterns, relaxation and an overall positive effect on child-parent interaction. The general symptomatic changes of the current study support the findings of Cullen et al (2005). However, it was not possible to know if the patient/practitioner interaction influenced this positive result. This is because the application of the techniques was done by a sole osteopathic practitioner. Future studies may include a group of practitioners applying the VOT techniques so that a concordance among raters could be investigated to analyse the effectiveness of VOT on autistic children.

This study followed clear guidelines for clinical safety and practice standards. According to the Osteopathic Practice Standards (General Osteopathic Council, 2012) it is the professional duty of an osteopath to take an appropriate case history for each patient who may potentially be treated by an osteopath (General Osteopathic Council, 2012). The data generated from the case histories were not part of the original hypothesis of the study but were recorded for professional clinical best practice and patient safety. Similarly, issues about utilising data from these case histories were not raised during

the MPhil/PhD registration document and the PhD transfer report. The main focus of this research was to help to fill the knowledge gap in understanding the impact of VOT on the behaviour and GI signs and symptoms of the study ASD subjects. Any collected information was designed to build on the pilot study (Bramati-Castellarin and Janossa, 2002), by using both questionnaires and GI markers which were included to generate objective quantitative data. The case histories have generated interesting qualitative/anecdotal information which has been described in section 3.8. The analysis of the case histories, however, was not part of the study design and was neither submitted to nor, therefore, approved by the Ethics Committee. However, the interesting data collected may potentially be used in subsequent qualitative research if Ethics is granted.

The same applies to the information generated from the screening questionnaire (Appendix 5). This information was used to assess the inclusion and/or exclusion criteria of the study and the data it generated was not designed to be included as quantitative data and it was not, therefore, approved by the Ethics committee to be used as part of this research.

The number of subjects in the study could be considered as a further limitation and this was exacerbated by the loss of the lactoferrin samples by the analysing laboratory thus reducing the power of the statistics through a substantial decrease in sample numbers for comparison. This resulted in a loss of power for this specific analysis and therefore further study is necessary to determine the validity of the results found.

The implication of being a practitioner and researcher is a recognised limitation of this study. In mitigation measures were put in place when designing the methodology. The observed behavioural changes were assessed by parents and not the practitioner, and the biochemical markers were assessed independently to exclude researcher bias during measurement.

It could be argued that the inclusion of more than one practitioner within the study would decrease any potential bias. Also any ‘bed side manner’ effect

potentially generated by a single practitioner applying the treatment protocol could decrease the nonspecific effects of treatment (Caspi et al., 2000, Licciardone and Russo, 2006). However, inclusion of a second practitioner or researcher would have incurred financial constrains that would have been prohibitive for this study and could have introduced other errors such as standardisation of treatment and heterogeneity of osteopathic manipulation (Licciardone and Russo (2006). Also, it could not be certain that the inclusion of more practitioners for the study would have been ethically acceptable as autistic children do not respond well to change (Wing, 1998).

It cannot be argued that the role of practitioner/researcher does carry a potential for bias and forms a limitation of this study. However, the nature of the osteopathic profession cannot be separated from the interaction between practitioner and patient when establishing the effectiveness of a treatment application, particularly when the researcher meets the subject at each treatment session. According to Licciardone and Russo (2006), in this situation the non-specific effects of the treatment may be stronger. However, it is the professional duty of an osteopath or manual therapist to ensure the wellbeing of a patient and therefore the inclusion of a bias may possibly be in the nature of the profession (Herbert et al., 2001, Rogers, 2005). It is possible that only in a double blind control trial, where the researcher is often not present during the active research, that the subjectivity of the research is mostly removed (Caspi et al., 2000, Licciardone and Russo, 2006).

In the current study, questionnaires were used as one of the outcome measures and assessed the perception of the parents regarding the signs and symptoms of the child. The questionnaire completion could have been influenced by the belief of parents that either a positive or negative outcome would occur (Rattray and Jones, 2007), and thus represent perception credibility of the practitioner/researcher or of the treatment itself (Caspi et al., 2000, Stewart, 2010). Further studies may address this limitation and shed light on this complex area.

5.3 Contribution to Knowledge and Final Conclusions

No known study to date has used sequential faecal biochemical markers and questionnaires to assess GI inflammation in autistic children suffering from GI symptoms. This thesis assessed levels of biochemical markers in sequential samples taken before and during the application of VOTs, and discovered unexpected score variations within faecal samples only previously described by Gilbert et al. (1996) and Husebye et al. (2001) as possibly due to biological variation of the markers over time. This is a potentially important finding suggesting the need for more studies in this area.

The novel approach of this project in using VOTs to treat autistic children suffering from GI symptoms has indicated that this low-invasive form of treatment could have a significant and important impact on their quality of life and wellbeing. This research reports some promising positive results for specific behavioural and GI symptoms of autistic children following VOT treatment and can be considered to add weight to the gut-brain axis hypothesis postulated in recent papers (Mayer et al., 2006, Reichelt and Knivsberg, 2009, Forsythe et al., 2010, Mayer and Tillisch, 2011).

An important contribution of this research is that it shows sufficiently positive outcomes to support and stimulate further investigation into the use of VOTs in autistic children suffering from GI symptoms. Correlation of the three markers with questionnaires on nine separate occasions resulted in the generation of a large volume of data and robust statistical analysis.

This study is the first to use VOT on autistic children displaying GI signs and symptoms whilst assessing therapeutic outcome. Even though challenging, due to the nature of the ASD, the study is unique as it observes autistic subjects over an extended period of time (18 weeks). Moreover, the use of the same subjects displaying a range of complex behavioural disorder avoids the introduction of variations within subjects that could potentially create data inconsistency.

In the current study, clinical changes were perceived and reported by the parents. Initially, parents were apprehensive about the prospect of the child displaying touch avoidance during the treatment sessions, and were surprised to see the child accepting the treatment and relaxing. On more than one occasion, during the second or third sessions onwards, the child would run into the treatment room and lie down on the couch, taking the practitioner's hand and placing it on his/her abdomen. This action seems to indicate that the child was accepting and perhaps desiring the intervention rather than being frightened of treatment.

Additionally, parents often reported a general improvement in symptoms after treatment. The children appeared to be calmer and more socially interactive, while also showing signs of general GI symptom improvement and eye contact after treatment sessions. Children were also reported to have improved appetite, with two parents reporting that, during the weeks of treatment, their children started to eat a wider variety of food, including fish and meat, an otherwise very rare occurrence. Information regarding parents' comments was recorded on the case history during each consultation, as part of the standard of care required of osteopathic treatment.

Techniques used in this study may have had a positive influence on the immune system through the mobilisation of the greater momentum owing to its immunological function (Carlow et al., 2009). In addition, mobilisation of the internal organs following VOT treatment and the resulting increase in circulation and peristalsis, may have a positive effect on the pumping action of the lymphatic system, thereby enhancing its function (Huff et al., 2008, Schander et al., 2008, Hodge et al., 2010).

5.3.1 Future Work

The positive findings generated from the use of VOT in autistic children might possibly be implemented in clinical osteopathic settings. Moreover, visceral osteopathic treatment could become part of the treatment procedures within special schools for autistic children alongside other therapies such as speech therapy, behavioural therapy and occupational therapy. Future studies are

necessary to determine whether the outcome measures used for this thesis may be replicated.

An interest in low-invasive gastrointestinal treatment for autistic children who also experience GI symptoms led to this thesis. The application of standardized VOT to the abdominal area of autistic children suffering from GI symptoms was a novel idea that may lead to a new application of a standardized low-invasive form of therapy previously unexplored in autistic patients. The results of this thesis may potentially be applied both in clinical settings and by parents as a form of home therapy to help to create a closer parent/child relationship. However, more studies are needed in this field to explore other potential effects of VOT.

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APPENDICES

Appendix 1 - Ethical Approval

UNIVERSITY OF WESTMINSTER

Ms Iona Bramati-Castellarin
1 Lloyd Villas
Lewisham Way
London
SE4 1US

11 December 2006

Dear Ms Bramati-Castellarin

App. No. 06/07/19
Iona Bramati Castellarin, School of Biosciences
Effect of Visceral Osteopathy on Gastrointestinal Abnormalities in children with autistic disorders.

I am writing to confirm that your application for ethics approval has been considered by the University Ethics Committee and approval has been granted.

Please inform me if you plan to make any further changes to your research.

Yours sincerely

Carl Hornsey
Assistant Registrar (Student Information)
Secretary, University Ethics Committee

THE QUEEN'S AWARDS FOR ENTERPRISE 2000

ACADEMIC REGISTRAR'S DEPARTMENT
Educating for professional life 115 New Cavendish Street London W1W 6UW
www.wmin.ac.uk Tel 020 7911 5000 Fax 020 7911 5118

Telex 25964 Telegrams University of Westminster W1 A charity and a company limited by guarantee Registration number 977818 England Registered office 309 Regent Street London W1

BRITISH COLLEGE OF
BCOM
OSTEOPATHIC MEDICINE

Lief House
120-122 Finchley Road
London NW3 5HR

Telephone:
Admin (020) 7435 6464
Clinic (020) 7435 7830

Facsimile:
(020) 7431 3630

Email: info@bcom.ac.uk

Website: www.bcom.ac.uk

This is to confirm that ethical approval was granted to IONA BRAMATI CASTELLARIN at the meeting of Research Ethics on 13th October 2004. The study is entitled: EFFECT OF VISCERAL OSTEOPATHY ON GASTROINTESTINAL ABNORMALITIES IN CHILDREN WITH AUTISTIC DISCORDERS.

Professor J. Dickerson Chair of Research Ethics Committee

Date: 21/11/05

The National Autistic Society

2nd Floor
Church House
Church Road
Filton
Bristol BS34 7BD

T: 0117 974 8400
F: 0117 987 2576
www.autism.org.uk

Dr Drysdale
Bristish College of Osteopathic Medicine
Lief House
120-122 Finchley Rd
London
NW3 5HR
UK

22 April 2010

Dear Dr Drysdale

I am pleased to confirm that the NAS endorses the research project that has been submitted by Iona Bramati.

I understand that there has been some misunderstanding of the status of this so for clarification and to avoid possible future problems I need to reiterate that endorsement of any research project does not imply endorsement of any product, intervention or approach that may be associated with such research. .

Hoping this is helpful.

With kind regards

pp

Richard Mills
Director of Research
The National Autistic Society

Appendix 2 – Study information and recruitment letters

Research Proposal

Information FOR parents

Background of investigation

Although autism has been closely investigated over the last decade it still remains a disorder with no clear cause and no current effective treatment. There have been many hypotheses regarding the causes of autism, but recent theories focus on the possibility of autism being due to imbalances in gut function. A recent study suggests an association between the onset of gut symptoms, such as abdominal pain, diarrhoea, and bloating and in some cases food intolerance, with developmental regression.

Our research group has decided to use visceral osteopathic techniques[1] (abdominal massage) to help the gut function of children who have been diagnosed as autistic. It is believed that manual stimulation of the gut may help to decrease inflammatory processes as well as aid in a decrease in constipation, diarrhoea and bloating suffered by autistic children. It may also help with amelioration of abnormal behavioural symptoms characteristic of autism.

Aims of investigation:

The aim of this investigation is to apply a soft tissue technique (massage) to the abdominal area to attempt to decrease bowel irritation.

Methods/design of investigation:

The proposal is to analyse bowel function and behaviour of the autistic children before and after the application of soft tissue techniques on the abdominal area

[1] The abdominal visceral soft tissue technique may change bowel movements, but apart from this there are not known risks.

(tummy area). The subjects' ages will range from 3 to 8 years old. The evaluation of the study will be performed via four questionnaires given to the parents; who will be able to observe the child's response to the massage techniques, e.g. differences in frequency of bowel movements. These questionnaires will be given to the parents before treatment, twice during the treatment phase and after the last treatment. Also, the children will be assessed by analysing stool samples. Parents will be asked to collect stool samples before the initiation of the treatment, during treatment and after the last treatment session. Parents will be provided with a 'Stool Collection Kit' in which samples may be sent to King's College Hospital in a stamped addressed Safebox™. More details on how to collect the material will be provided in the 'Stool Collection Kit'. The purpose of stool analysis is to measure bowel inflammation.

There will be six treatment sessions, each lasting for 30 minutes, depending on the child's co-operation. In the event of a child being distressed or oncooperative, the parent/guardian together with the researcher will decide on the merits of continuing treatment.

The children will be assessed at their own school, although a parent or a guardian must be present during application of the technique.

Duration of investigation:

Study Period	Weeks
Period I. The control period	1-6
Period II. The treatment period	7-12
Phase III. The post-treatment period	18

Letter to Parents

IPD/IB-C

March 2007

Dear Parents

I am a Registered Osteopath practising in London with a special interest in children suffering from autism. I have been developing a research project as part of my MPhil/PhD studies at BCOM/University of Westminster. The aim of the study is to attempt to positively influence behaviour and bowel function in the children suffering from autism.

The study involves the treatment of the child's abdomen using osteopathic visceral treatment – tummy massage. Behaviour patterns and biochemical analysis of stool samples before and after treatment would be scrutinised to determine the effects on the child's gut function and behaviour. For the study to be effective volunteers are necessary and I would like to invite your child to participate. The treatment is planned to take place at your child's school premises to minimise disruption and to cause as little inconvenience to you and the children.

I am enclosing a brief information pack which will help you to understand a little more about how the treatment would work. Also, it may indicate how you and your child can help the study determine whether osteopathic treatment is an effective treatment to improve the gut function of children suffering from autism.

If you would like to be involved then perhaps you could please inform Ms/Mrs.....at the school.

The treatments are due to start next year (2007) and you would be informed in plenty of time of the start date, probably in March. If you need any further information please do not hesitate to contact me at my e-mail address – contact@ibccare.co.uk.

Thank you very much for your time and consideration,

Iona Bramati Castellarin BSc(Hons) Ost Med DO,ND

Osteopath – MPhil/PhD Researcher

Dr Ian Drysdale BSc, ND, DO, PhD

Director of Studies

THIS PROJECT IS UNDERTAKEN WITH THE SUPPORT OF KING'S COLLEGE HOSPITAL AND THE NATIONAL AUTISTIC SOCIETY.

Letter to Headteachers

Dear Headteacher

I am a Registered Osteopath practising in London and possessing a special interest in children suffering from Autism. I graduated from the British College of Osteopathic Medicine (BCOM) in 2001 and subsequently, the Principal, Dr Ian Drysdale, offered me the opportunity to continue with my undergraduate research.

In November 2000 as part of my undergraduate degree I developed a clinical trial where I treated autistic children using Osteopathic techniques. The treatment consisted of 6 treatment sessions using Osteopathic techniques to the children's abdominal area – essentially a tummy massage. I am now enrolled in my second year with BCOM/University of Westminster as an MPhil/ PhD student. King's College Hospital has agreed to support the project and specifically to undertake responsibility for the laboratory analysis of the collected samples.

The purpose of this letter is to enquire whether it would be possible to include the children of..... School for the next phase of this study.

The original study included children aged 3 to 8 years old, who had been diagnosed as autistic. To satisfy the inclusion criteria the children also suffered from gastrointestinal problems symptoms, impaired social relationship and poor communication skills. Each child was given six 30-minute treatments, once a week for six weeks, focusing on the abdominal area.

Parents and teachers were advised how the treatment would work via a brief presentation during a school meeting. The trial study was very successful and showed some very positive results in terms of the positively changed behaviour of the participating children. Parents and teachers were very supportive of the research project at the time.

The second phase of the project is planned to start next year when the plan is to start treating autistic children with the same techniques used in the pilot study using osteopathic abdominal massage, and to include objective measurements

and assessments. The gastrointestinal parameters are to be measured in the children before and after osteopathic treatment and include analysis of collected faecal/stool material and quantitative assessment of a biochemical marker. Neither the treatment nor the stool collection is invasive. The parents will be asked to send the children's stool samples for laboratory analysis to enable assessment of the levels of the tissue inflammation biochemical marker.

I would be delighted to include The.....School in this study if you and the parents consider this research project appropriate, and I would be happy to discuss this further with you.

Please do not hesitate to contact me by post at:

London.....

or on my mobile 079.....

I look forward to hearing from you.

With kind regards,

Iona Bramati Castellarin BSc (Hons) Ost Med, DO,ND

Osteopath - MPhil/PhD researcher

Dr Ian Drysdale BSc, ND, DO, PhD

Director of Studies

Stool Collection Kit information

1. Write the date and time of the test on the label of the blue top plastic tube. Collect one faecal (stool) motion at any time of day from a potty or nappy and place several scoops of faeces in the tube using the spoon provided in the tube. Seal the tube tightly, place it in the box provided.
2. Each Safebox™ is numbered. Please use the Box number 1 for the first stool collection test and fill the form that is inside with the time and date of stool collection. Place the form back into the safe box. Please repeat the same procedure for each consecutive stool collection. Each stool collection needs to be at least a week apart. Your child needs to be tested for 4 calprotectin assays before initiation of the Osteopathic treatment.
3. Post the sample to the Dept of Clinical Biochemistry, King's College Hospital, Denmark Hill, London SE5 9RS.
4. Please note that I am including a questionnaire that needs to be completed up each time you collect a stool sample from your child. Date the questionnaire and send it back in the s.a.e provided. Each questionnaire is also numbered from 1 to 4. Please use the appropriate questionnaire to match the same Safebox™ number.
4.
5. Please find attached instructions on how to use the Safebox™.

Thank you very much for being part on this research study.

Iona Bramati Castellarin

CONSENT FORM

For voluntary participation in:

Effects of Visceral Osteopathy on the Gastrointestinal and Behavioural Changes in Children with Autistic Disorder.

We are inviting you and your child to participate in an investigation that we believe to be of potential importance. In order to help you to understand what the investigation is about, we are providing you with the Research Proposal – Information for Parents. If you agree to participate, please be sure you are comfortable with the contents of the Information sheet. If you have any questions or concerns regarding the procedures used in this study please do not hesitate to contact us for further information.

Contact may be via:

Mrs Ioná Bramati Castellarin BSc DO, ND,

Dr Ian Drysdale BSc,PhD,ND,DO or

Dr Margit Janossa MD,DO at

BCOM clinic:

Frazer House 6 Netherhall Gardens

London NW3 5RR

Tel:020 7435 7830

Or

Mrs Ioná Bramati Castellarin BSc DO, ND, at

The Notting Hill Osteocare

26 C…. C…. C….

London W2 ……

Tel: 0207 7……..

Or by e-mail at:

research@.....

Thank you for agreeing to take part in this project.

Please note that:

- Your child's participation in this research project is entirely voluntary;
- You are free to refuse to answer any questions;
- You are free to withdraw your child from the research at any time;
- The treatment is a type of massage and there are no known risks associated with this;
- The technique cannot be applied through clothing, therefore your child's abdominal area needs to be exposed;
- Assessment will be performed via:

1. A questionnaire completed by the child's parents and teacher before and after treatment session;

2. Stool Samples analysis.

There will be complete confidentiality of records

- No names will be disclosed at any stage of the research;
- The research will take place at BCOM clinic - no fee will be required.

If you agree to your child taking part in the study please sign and date below to indicate that you have read and understood the content of this form and return it a.s.a.p. together with the Confidential Health Questionnaire in the accompanying s.a.e.

Child's name:

_____

Parent's name:

_____

Parent's signature:

_____

Date: _____

PERMISSION FORM FOR SCHOOLS

TITLE OF INVESTIGATION: An assessment of gastrointestinal inflammation on autistic children using faecal biochemical markers.

Name of School Participant:.....

I have read the attached information about the research the School has been asked to participate in. I have been given a copy of the Protocol to keep for the school record and I have had the opportunity to discuss the details and ask questions about this information.

The Investigator has explained the nature and purpose of the research and I believe that I understand what is being proposed.

I have been informed what the proposed study involves. I understand that all data from this trial will remain strictly confidential. Only researchers involved in the investigation will have access to the data.

I hereby fully and freely consent, on behalf of the School Governors, to participation in the study which has been fully explained to me.

PRINCIPAL'S NAME: (BLOCK CAPITALS):

Name of School:……

SIGNATURE of Principal…..……

(ON BEHALF OF PARTICIPATING SCHOOL)

DATE: ……../……/……

As the Investigator responsible for this investigation, I confirm that I have explained to the participant named above the nature and purpose of the research to be undertaken.

INVESTIGATOR'S NAME: Ioná Bramati Castellarin

INVESTIGATOR'S SIGNATURE:

DATE: ……./……/…….

Appendix 3 – Example Diagnostic Statement together with the statement of special educational needs provided by the ELB

NHS Islington

INITIAL MULTIDISCIPLINARY ASSESSMENT REPORT

The Islington Child Development Team (CDT)
The Northern Health Centre
580 Holloway Road
London N7 6LB
Tel: 020 7445 8205

NAME:	[redacted]
DOB:	15/09/2006
ADDRESS:	[redacted]
DATE OF ASSESSMENT:	July- September 2009
AGE AT ASSESSMENT:	2 years 10-11 months.
CDT CASE COORDINATOR:	[redacted]
NURSERY:	Bennett Court playgroup and Four Corners Preschool (Hertfordshire), moving to Archway Children's Centre in September 2009.

SUMMARY

[redacted] is a nearly 3 year old [redacted] who is currently making great progress in several areas of development.

He has many areas of strength including:

- His interest in adults and ability to initiate interaction to request things, e.g., books, continuation of songs.
- His ability to learn, especially visually e.g., letters, number, actions.
- His response to routines e.g., sitting for group-time at nursery.
- His emerging range of gestures.
- His recent improvements in receptive and expressive language.

Both formal and informal assessment and observation of [redacted] across a variety of settings indicates that he presents with an **Autism Spectrum Disorder**, consistent with the diagnostic criteria for autism from the ICD 10 (International Classification of Diseases, 10th revision). This means he shows difficulties in the following three areas:

1. Social interaction e.g. difficulty in developing peer relationships (relative isolation in unstructured time at playgroup etc), limited or unusual use of eye gaze, over familiarity with strangers, difficulty picking up on social cues around him.

2. Communication (verbal & non-verbal) e.g., stereotyped use of language, difficulty with directing all his communication.

3. Restrictive and repetitive patterns of behaviour e.g. repetitive patterns of play, interest in books to an unusual degree, and sometimes the details within them, stereotyped motor mannerisms e.g., flapping, hand movements.

REFERRAL & PREVIOUS CONTACT WITH SERVICES

[redacted] was originally referred by his GP to Dr [redacted], Consultant Paediatrician at the Whittington at the age of 18 months. This was due to parental concerns about unusual body gestures such as rocking in a chair, flapping his arms when excited and slow speech and language development. Dr [redacted] assessed [redacted] and referred on to the community paediatric services for further developmental assessment. [redacted] was then seen by the Consultant Paediatrician in the community, Dr [redacted], at the Northern Health Centre on 22 July 2008 who felt he had mild delay in speech and social communication skills, and also displayed some immature gestures with some sensory-seeking behaviour. As it appeared [redacted] at this stage had made great progress in the few months up to this date she decided to review his progress in October 2008. It was at the appointment on 12 November 2008 when he was reviewed by [redacted] that he needed a referral him to this team, This was because although he had progressed, he was showing features of autistic spectrum disorder, which his mother was particularly aware of as there is a family history of autism and possible learning difficulties. Until July [redacted] was attending the Bennett Court playgroup (previously Aubert Court) on a part-time basis, Monday, Wednesday and Thursday 9.15-1pm and they had been trying to provide some additional help for him.

CURRENT FAMILY SITUATION & DEVELOPMENTAL HISTORY

[redacted] is the second of three children to [redacted] and [redacted]; they have [redacted] born April 2005 who attends Yerbury school nursery and [redacted] who was born 20.11.08. [redacted] works as a builder and [redacted] is a full-time mother who has done some part-time administrative work. They have lots of family around for support and their accommodation is currently adequate for their needs.

[redacted] was born at the Whittington hospital following a normal pregnancy, but delivery was helped with forceps as it was found that [redacted] had inhaled meconium. He then required admission to the neonatal unit where he stayed for one week, being ventilated as he had developed a pneumothorax which requiring draining. He also required 5 days nasogastric feeding following which he established breast-feeding well. At four months he developed loose stools which have not stopped, although have lessened somewhat with the introduction of soya milk to replace cow's milk.

[redacted] is reported to have smiled by six weeks of age but his mother does not feel he looked at her properly; she thinks this has improved over time and as a baby he babbled in a similar fashion as his sister, using many single words describing objects actions & greetings by age 22 months.

ASSESSMENTS USED:

This report is based on assessment at home, playgroup and the NHC, using the following tools/methods:

- Autism Diagnostic Observation Schedule (ADOS, Lord et al, module 1) The ADOS is a standardised assessment of communication, social interaction, and play or imaginative use of materials for individuals who have been referred because of a possible autistic spectrum disorder.
- Informal observations at home, in clinic and at nursery.
- Parent and teacher interviews.
- Play based assessment at home, in clinic and at nursery.

Lewisham

Educational Psychology & Learning Support Services
Children and Young People Directorate

Kaleidoscope
Lewisham Centre for Children & Young People
32 Rushey Green
Catford
SE6 4JF
tel: 020 7138 1417
fax: 020 7138 1411
email: [redacted].gov.uk

PSYCHOLOGICAL REPORT (APPENDIX D)

EDUCATIONAL PSYCHOLOGIST	[redacted]
PUPIL	[redacted]
D.O.B.	10.8.03
SCHOOL/NURSERY	Turnham Primary Foundation School
PARENTS	Mr and Mrs [redacted]
HOME ADDRESS	[redacted]
First language of young person	English

EVIDENCE ON WHICH THIS ADVICE HAS BEEN BASED

Observation	2.4.08
Interview with young person	2.4.08
Parent interview	2.4.08
Staff discussion	2.4.08
Other agencies involved	Speech and Language Therapy Service

This advice constitutes the psychologist's contribution to the statutory assessment of [redacted] special educational needs

BACKGROUND

[redacted] is a four year old [redacted] who has attended Turnham Foundation Primary School since September 2006. She attends part-time and is currently in the Nursery.

[redacted] underwent an initial developmental assessment with Dr [redacted], Community Paediatrician, at Kaleidoscope in February 2007. Dr [redacted] concluded [redacted] had 'severe global developmental delay' and reported her behaviours suggested social

communication disorder. She referred [redacted] to the Communication Clinic at Kaleidoscope. [redacted] was assessed at the Communication Clinic in November 2007 by Dr [redacted], Associate Specialist in Community Paediatrics, and Dr T[redacted]. They concluded [redacted] had impairments in the areas of speech and language, communication, imagination and constructive play, and social interaction. This was alongside 'repetitive and stereotypical behaviour and unusual motor mannerisms and sensory responses.' They found these impairments and behaviours fulfilled the criteria for a diagnosis of Autism.

Background information on [redacted] early development is provided within Mr and Mrs [redacted] supporting letter to the application for statutory assessment, and the reports arising from the Developmental Assessment (seen 7.2.07) and the Communication Clinic (seen 21.11.07).

When I spoke with [redacted] teacher, Ms [redacted], she stated her main concern was [redacted] language, both expressive and receptive (understanding). She feels [redacted]'s language difficulties are preventing her from accessing the Foundation Curriculum and has led to the attainment gap between [redacted] and her peers widening. She is concerned that the Reception curriculum will be beyond [redacted]'s abilities and the attainment gap will widen further as [redacted] moves through the year groups. She believes [redacted] would benefit from 1:1 support. She feels support is required to support [redacted] in remaining engaged in activities, during group activities, when working independently, and to make resources for inclusion. She believes [redacted] would require support at lunchtime to support her eating and toileting when in Reception.

Currently, [redacted] receives additional support from the Reception teacher, Ms [redacted], for one session a week. The Early Intervention Team has provided [redacted] with a block of 6 sessions, with accompanying strategy suggestions for nursery staff. [redacted] and her parents have received direct support from the Speech and Language Therapy Service, with advice on strategies to implement in the home.

The SENCo, Mrs [redacted] applied for statutory assessment of [redacted] needs in January 2008. She reported the school requires funding that will reflect [redacted] 'complex and severe needs'. [redacted] entry into Reception has been delayed since January 2008. I understand she is due to transfer to Reception on a part-time basis at the start of the Summer Term 2008. [redacted]' intention is to gradually extend [redacted] hours, with a view to including her for a full day with support.

[redacted] lives at home with her parents and two sisters. [redacted] nearly two years old (22 months) and [redacted] who is 6 years old. I understand [redacted] had speech and language therapy for a while due to mild speech and language delay. There are no concerns about her speech now. She attends Turnham Primary School and is doing well.

VIEWS OF PARENT

I spoke with [redacted]'s mother, Mrs [redacted] at Turnham Primary School on 2.4.08. She feels that [redacted]'s main difficulties are with speech and communication, and her ability to interact with other children. She stated she would like [redacted] to receive support

Appendix 4 – Recruitment Advertisements

'Tummy massage' for AUTISTIC CHILDREN at Notting Hill Osteocare

We are inviting children with a diagnosis of Autism between the ages of 3-8 years of age to take part in osteopathic treatment sessions– tummy massage at Notting Hill Osteocare.

Iona Bramati is a Registered Osteopath practising in London and for the past 10 years has possessed a special interest in children suffering from Autism. Iona graduated from the British College of Osteopathic Medicine (BCOM) in 2001 and is currently obtaining an MPhil/ PhD degree. Initial studies that Iona carried out looked at the behavioural response of autistic children following tummy massage. Due to the positive results, this has led onto a more detailed research project studying autism with the University of Westminster/ BCOM in collaboration with King's College Hospital, London. Recently the project has been endorsed by the National Autistic Society (NAS).

Iona is using tummy massage to help improve gut function of children who have been diagnosed autistic. Previous studies have shown that manual stimulation of the gut may help to decrease inflammatory processes as well as aid a decrease in constipation, diarrhoea and bloating suffered by autistic children. It may also help to reduce some of the behavioural symptoms characteristic of autism.

If your child suffers from gut problems such as: diarrhoea, constipation, bloating he or she might benefit from this treatment.

Please contact research@..... or call 07---------- for specific details

or log in at www.……..

* There is no cost involved in participating in this study. Any participant may withdraw from the study at any time.

UNIVERSITY OF WESTMINSTER

BRITISH COLLEGE OF BCOM OSTEOPATHIC MEDICINE

'Tummy Massage'

for Autistic Children

We are inviting children with a diagnosis of Autism between the ages of 3-8 years old to take part in osteopathic treatment sessions– tummy massage at Notting Hill Osteocare.

Iona Bramati is a Registered Osteopath practicing in London and for the past 10 years has possessed a special interest in children suffering from Autism. Iona graduated from the British College of Osteopathic Medicine (BCOM) in 2001 and is currently obtaining a MPhil/ PhD degree.

Initial studies that Iona carried out looked at the behavioural response of autistic children following tummy massage. Due to the positive results, this has led onto a more detailed research project studying autism with the University of Westminster/ BCOM in collaboration with King's College Hospital, London. Recently the project has been endorsed by the National Autistic Society (NAS).

Iona is using tummy massage to help improve gut function of children who have been diagnosed autistic. Previous studies have shown that manual stimulation of the gut may help to decrease inflammatory processes as well as aid a decrease in constipation, diarrhoea and bloating suffered by autistic children. It may also help to reduce some of the behavioural symptoms characteristic of autism.

If your child suffers from gut problems such as: diarrhoea, constipation, bloating he or she might benefit from this treatment.

Please contact research@nottinghillosteocare.co.uk

or call 07950119715 for specific details

or log in www.nottinghillosteocare.co.uk

*

There is no cost involved in participating in this study. Any participant may withdraw from the study at any time.

angels & urchins
AUTUMN 08
London's indispensable parents' magazine

LOCAL NOTICEBOARD

RICHMOND, KEW & KINGSTON NCT NEARLY NEW SALE
Saturday 27 September, Richmond Adult Community College, Open 1–3pm. Entrance £2. Quality second hand items, clothes, toys and nursery kit. To reserve a selling place (£25) call Sabine on 8940 0934 or e-mail: events@nctrichmond.co.uk. Priority entry for NCT members in first 15mins. Parking, café.

CLAPHAM NCT AUTUMN SALE
Saturday 4th October, Broomwood Methodist Church Hall, Kyrle Road, SW11. Open 11am–1pm. Entrance £1.50 (free after 12pm). Priority entry to card-carrying NCT members. Nearly-new baby equipment, clothes, toys and books for babies and young children. For general Nearly New Sale enquiries or to sell items e-mail: nearlynewsales@claphamnct.com.

TIME & TALENTS CHARITY CHRISTMAS GIFT FAIR
Wednesday 15th October 10am–5pm, The Hurlingham Club, Ranelagh Gardens, London SW6. Entrance £5. Free Crèche, Raffle, Tombola and Café. Parking £5. "One-stop Christmas Shop". Stocking Fillers; toys; baby gifts; books; chocolate; handbags; jewellery; men's gifts and more. For further details and a personal invitation, ring 7231 7845 or e-mail: info@timeandtalents.org.uk.

WANTED
After-school help approx 2 nights a week. 5-8pm. Help with homework, tidying house and putting children to bed. For more details, e-mail areid@primex.co.uk.

OFFERED
Autistic children between aged 3–8 are invited to participate in a study investigating the effects of visceral osteopathy on autistic children. Treatment involves tummy massage and analysis of behavioral and gastrointestinal function after six osteopathic treatment sessions. Study being conducted by Notting Hill Osteocare/Westminster University and British College of Osteopathic Medicine, in collaboration with King's College Hospital. The National Autistic Society (NAS) is also endorsing the research. Preliminary results are extremely positive. If interested, please contact Iona Bramati on 07950 119715, e-mail: research@nottinghillosteocare.co.uk or visit: www.nottinghillosteocare.co.uk for more specific details. There is no cost involved in participating in this study. Any participant may withdraw from the study any time.

Appendix 5 – Questionnaires employed in the study

Admin Number: | | | | | | | | | |

Osteopathic treatment of GI symptoms in Autistic Children
Confidential Health Questionnaire – Screening Questionnaire

Child's Name:_____

Date of Birth: _____
Age:_____Sex:_____

Parent's name_____ Tel: (home):_____

(mob):_____

e-mail address:_____

1. How old were you during your pregnancy? _____

2. Was your child full term? (please circle) Yes
No

If **no,** give brief details.

_____
_____
_____
_____
_____

3. Did you have any problems during pregnancy?

If **yes,** please give brief details

_____
_____
_____

4. Did you have any problem in delivering you baby?

If **yes**, please give brief details

_____
_____
_____

_____
_____
_____

5. Were the following used : (tick the relevant box)

a) Forceps •
b) Ventouse •
c) Amniotomy (artificial rupture of the membrane) •
d) Pethadine (a potent analgesic drug) •
e) Epidural •

6. Was you labour induced? (please circle) Yes
No
If **yes**, please give brief details.
_____
_____
_____
_____
_____

7. Was your child delivered via caesarian? (please circle) Yes
No
If **yes**, please give brief details.
_____
_____
_____

8. Was your child breast fed? (please circle) Yes
No
If **yes**, for how long?_____

9. Was your child bottle fed? (please circle) Yes
No
Was he/she fed on: (please tick the relevant box)
a) Formula (cow's milk) •
•
b) Unmodified cow's milk • •

c) other (please give details)_____ •
•

10. At what age were solids first introduced? _____

11. At what age were gluten containing foods introduced?_____

12. Are there any known food allergies? (please circle) Yes
No

If **yes,** what are the associated symptoms? (tick the relevant box)

a) Skin rash •
b) Eczema •
c) Diarrohoea •
d) Itching •

13. Has your child been immunised? (please circle) Yes
No

(see below for list)

14. Do you remember any side effects post vaccination? (please circle) Yes
No

15. If **yes**, tick on the vaccines that in your view may have caused adverse side effects.

a) DTP (Triplice) •
b) HIP (Haemophilus influenza) •
c) OPV (Oral Polio Vaccine) •
d) MMR (measles, mumps, rubella) •

16. Has your child ever suffered from: (please circle)

a) Asthma	Yes	No
b) Diabetes	Yes	No
c) Bronchitis	Yes	No
d) Epilepsy	Yes	No
e) Kidney problems	Yes	No
f) Bowel problems	Yes	No

17. If **yes**, for any of the questions above please give brief details.

_____
_____
_____
_____

18. Bowel function - stool characteristics (please give one answer for each of the following)

1. **Colour**	dark	very dark	pale	very pale
2. **Consistency**	loose	very loose	hard	very hard

3. **Smell** no smell light smell strong very strong

4. **Frequency** once twice three more (daily)

19. Has your child had any surgery in the past? (please circle) Yes No

If **yes**, give brief details

_____
_____
_____

20. Is your child on any medication? (please circle) Yes No

If **yes**, please list it below.

_____
_____
_____

21. Has your child had any accidents? (please circle) Yes No

If **yes**, give brief details.

_____
_____
_____

22. Was he/she hospitalised?(please circle) Yes No

If **yes**, brief state why and for how long

_____
_____
_____

23. Does your child suffer or has he/she ever suffered from ear, nose or throat infections? (Please circle)

Yes No

24. Is he/she been treated with antibiotics? (Please circle) Yes No

If **yes**, give brief details why.

_____
_____
_____

25. Does your child sleep well at night time? Yes
No

26. When was your child diagnosed with Autism? _____

27. Who diagnosed your child? (tick the relevant box)

a) GP •
b) Paediatrician •
c) Health visitor •
d) Other •

please specify_____

Please sign below and send this questionnaire with the consent form in the stamped addressed envelope.

Parent/guardian name:
_____(Mother/Father) delete as appropriate

Parent/guardian signature: _____

Date: _____

This questionnaire is based on ROME II Integrative Questionnaire: Research Diagnostic Questions for Functional Gastrointestinal Disorders

Before Treatment/ During Treatment/Post Treatment Questionnaire

Osteopathic Treatment of GI symptoms in Autistic Children

Child's Name:
Child's Age:
Date:
Please rate the child's behaviour and digestive symptoms using the scale below which ranges from:

0= never shows this particular symptom or behaviour
1= slight/unobtrusive
2
3= mild
4
5=moderate
6
7=severe
8
9=extreme/incapacitating

Tick the relevant box to the following:

Social behaviour and communication.

	0	1	2	3	4	5	6	7	8	9
Lack of awareness and interaction with parent										
Abnormal greeting behaviour										
Abnormal comfort seeking										
Can't make friends										
Lack of awareness of social rules										
Lack of spontaneous speech										
Abnormal word utilisation										
Poor compression of verbal instructions										
Lack of eye contact										

Ritual and Repetitive Activities

	0	1	2	3	4	5	6	7	8	9
Abnormal repetitive gestures										
Need to maintain sameness										
Need of fixed routine										

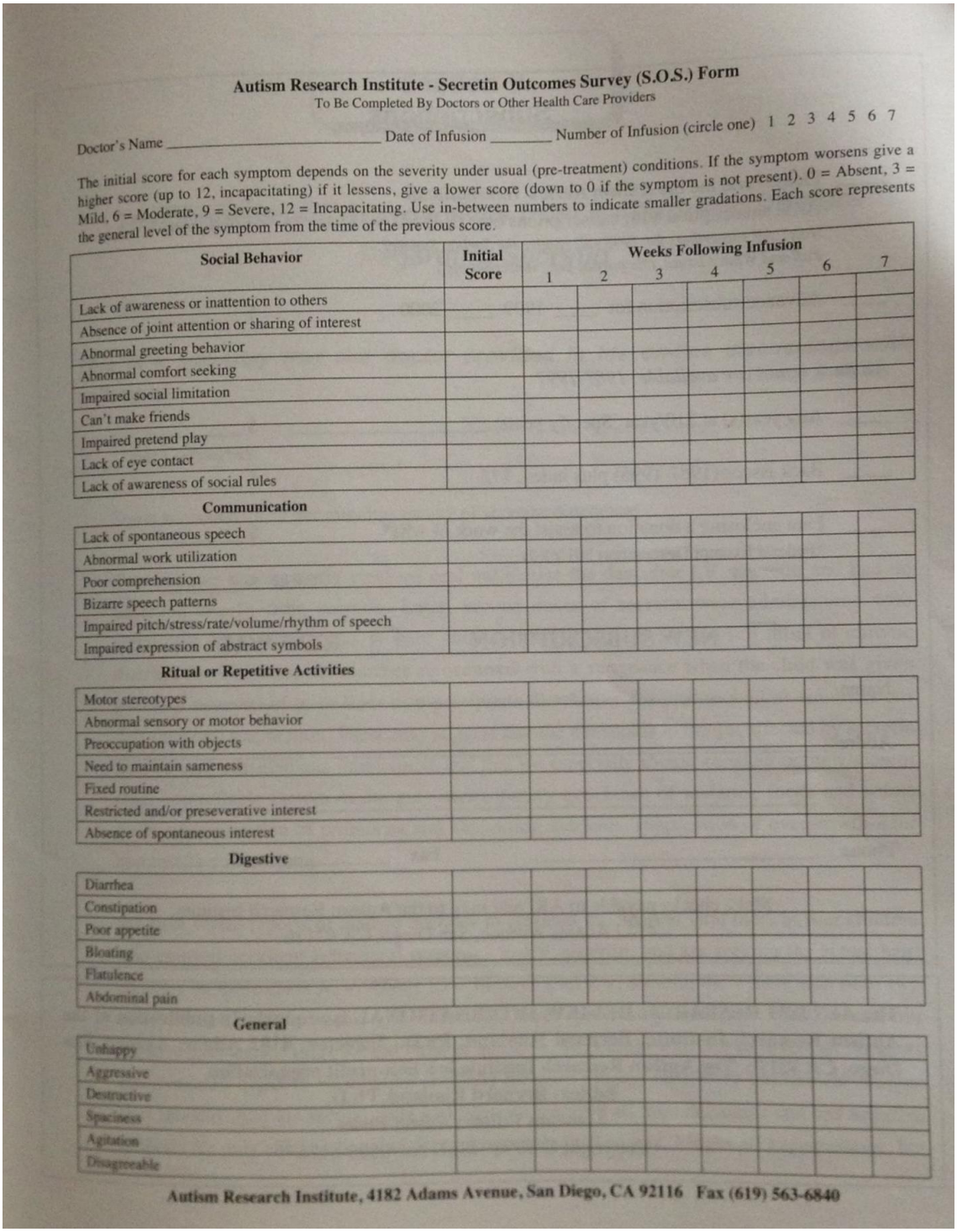
Digestive Symptoms

	0	1	2	3	4	5	6	7	8	9
Diarrhoea										
Constipation										
Poor Appetite										
Bloating										
Flatulence										
Vomiting										

General Symptoms

	0	1	2	3	4	5	6	7	8	9
Unhappy										
Aggressive										
Destructive										
Spaced out/Non Interactive										
Agitated										
Disagreeable										

Secretin Outcomes Survey (S.O.S.) Form



Autism Research Institute - Secretin Outcomes Survey (S.O.S.) Form

To Be Completed By Doctors or Other Health Care Providers

Doctor's Name _____ Date of Infusion _____ Number of Infusion (circle one) 1 2 3 4 5 6 7

The initial score for each symptom depends on the severity under usual (pre-treatment) conditions. If the symptom worsens give a higher score (up to 12, incapacitating) if it lessens, give a lower score (down to 0 if the symptom is not present). 0 = Absent, 3 = Mild, 6 = Moderate, 9 = Severe, 12 = Incapacitating. Use in-between numbers to indicate smaller gradations. Each score represents the general level of the symptom from the time of the previous score.

Social Behavior	Initial Score	Weeks Following Infusion 1	2	3	4	5	6	7
Lack of awareness or inattention to others								
Absence of joint attention or sharing of interest								
Abnormal greeting behavior								
Abnormal comfort seeking								
Impaired social limitation								
Can't make friends								
Impaired pretend play								
Lack of eye contact								
Lack of awareness of social rules								
Communication								
Lack of spontaneous speech								
Abnormal work utilization								
Poor comprehension								
Bizarre speech patterns								
Impaired pitch/stress/rate/volume/rhythm of speech								
Impaired expression of abstract symbols								
Ritual or Repetitive Activities								
Motor stereotypes								
Abnormal sensory or motor behavior								
Preoccupation with objects								
Need to maintain sameness								
Fixed routine								
Restricted and/or preseverative interest								
Absence of spontaneous interest								
Digestive								
Diarrhea								
Constipation								
Poor appetite								
Bloating								
Flatulence								
Abdominal pain								
General								
Unhappy								
Aggressive								
Destructive								
Spaciness								
Agitation								
Disagreeable								

Autism Research Institute, 4182 Adams Avenue, San Diego, CA 92116 Fax (619) 563-6840

Appendix 6 – Permission to Reproduce Printed Material

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United Kingdom

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Facsimile: +44 (0)23 8064 3701
Email: alpha@alphalabs.co.uk

Iona Bramat Castellarin
IBC Care
9 Upper Wimpole St
London
W1G 6LJ

24th March, 2014

Dear Ms Castellarin,

I confirm that Alpha Laboratories and Buhlmann grants permission for the text from the Instructions For Use for the Buhlmann EK-CAL Calprotectin ELISA Test to be quoted as part of your thesis on the condition that full acknowledgement is given to the company as the legal holder of the copyright.

Your Sincerely

Amanda Williams
Diagnostics Product Manager

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Telefax: +44 (0)1256 327889
http://www.schebo.co.uk

Iona Bromati Castellarin
IBC Care
9 Upper Wimpole Street
London
W1G 6LJ

12 March 2014

Dear Ms Castellarin,

I confirm that ScheBo® • Biotech AG grants permission for the text from the Instruction Manual for the ScheBo® • Tumor M2 PK™ ELISA Stool Test to be quoted as part of your thesis, on the condition that full acknowledgement is given to the company as the legal holder of the copyright.

Yours sincerely,

Ivor Smith, B.Sc.,
Managing Director

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James Boone

To: Iona Bramati
Subject: RE: Template letter - complete and return

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17/03/2014
9 Upper Wimpole Street
London W1G 6LJ

Dear Sir or Madam:

In a thesis I am preparing to be entitled 'A study of gastrointestinal and behavioural symptoms of autistic children during Visceral Osteopathic treatment using questionnaires and biochemical markers ' scheduled for publication in 2014 by the University of Westminster Repository (http://westminsterresearch.wmin.ac.uk/cgi/latest) , I would like to include the following material, which originally appeared in **IBD-SCAN® by TechLab®**, published in 2008:

Page 2 – Principle of the Test
Page 3 – Precautions and preliminary preparations
Page 4 – Collection of the specimen and preparation for dilutions
Page 5 – Test procedure and Quality control
Page 7 – example calculation, Interpretation of Values, shelf-life and storage, performance characteristics
Page 8 – IBD-SCAN® Test results, Limitation of the procedure

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Sincerely yours,

Iona Bramati Castellarin

AGREED TO AND ACCEPTED:

BY _____ DATE 3/18/14

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Appendix 7 – Raw Data Biochemical Markers (Study I-IV)

Compliancy table of faecal samples analysed

	Faecal Samples received for analysis of Calprotectin and M2-PK	PERCENTAGE OF COMPLIANCE OVER THE PHASES	PERCENTAGE OF NON COMPLIANCE OVER THE PHASES
SAMPLE 1	49	100%	0%
SAMPLE 2	48	95.91%	4.09%
SAMPLE 3	48	95.91%	4.09%
SAMPLE 4	46	85.71%	14.29%
SAMPLE 5	45	89.79%	10.21%
SAMPLE 6	43	87.75%	10.20%
SAMPLE 7	44	89.79%	20.21%
SAMPLE 8	39	79.59%	20.41%
SAMPLE 9	33	59.18%	40.82%

Legend to the table: This table represents the number of faecal samples received from the total subject number (n=49).

Calprotectin (1-4) - Raw Data

patient	initials	Calpro 1	Calpro 2	Calpro 3	Calpro 4
1	EAOD	21	16	108	70
2	EB	19	10	21	10
3	YB	42	40	43	16
4	SC	20	38	40	10
5	GM	37	13	15	31
6	MA	46	43	112	10
7	FM	10	11	56	16
8	IO	22	37	48	15
9	RR	13	39	10	39
10	PW	10	10	10	11
11	JEW	10	21	10	14
12	OF	10	13	12	54
13	BD	164	23	32	56
14	HH	10	10	10	10
15	DB	10	10	10	34
16	SMcA	18	10	10	33
17	RA	12	31	48	17
18	JS	10	10	10	10
19	AD	10	10	10	10
20	HH2	36	25	43	
21	VS	10	10	10	14
22	JAW	10	10	10	10
23	AK	21	Excluded from study 1		
24	CA	10	121	14	11
25	ZRS	10	10	10	12
26	NL	10	10	10	10
27	GN	10	10	70	10
28	JA	10	10	10	10
29	ZR	29	51	18	10
30	NR	30	14	22	13

31	LB	11	10	15	25
32	EC	10	11	10	27
33	NB	24	27	23	14
34	JB	27	18	14	15
35	SR	121	30	41	47
36	AP	10	69	12	34
37	RO	30	40	14	17
38	BG	41	36	97	55
39	FAM	511	89	224	66
40	OB	14	551	640	40
41	TO'D	10	12	15	35
42	N-JF	409	1592	151	391
43	JB	15	10	22	10
44	AD	10	34	88	59
45	MMR	83	234	153	108
46	RSM	38	14	21	
47	KMK	30	30	59	43
48	AM	50	18	16	13
49	SAH	138	195	121	21
		49	48	48	46

M2-PK (1-4) Raw Data

patient	initials	M2PK-1	M2PK-2	M2PK-3	M2PK-4
1	EAOD	1.65	1.13	2.07	1.16
2	EB	1.00	1.00	1.00	1.00
3	YB	1.98	1.00	8.28	1.49
4	SC	2.16	1.00	3.96	1.00
5	GM	1.00	1.22	1.00	3.14
6	MA	20.00	20.00	20.00	17.65
7	FM	1.66	1.00	12.72	1.00
8	IO	4.66	2.16	5.44	1.00
9	RR	2.78	1.00	1.00	1.00
10	PW	1.00	1.00	1.00	1.00
11	JEW	1.00	2.10	1.00	1.00
12	OF	1.00	1.00	1.00	1.00
13	BD	1.00	1.00	1.00	1.00
14	HH	1.00	1.00	1.00	1.00
15	DB	1.00	1.00	1.00	1.86
16	SMcA	1.00	1.00	1.00	1.00
17	RA	1.46	12.54	8.40	1.43
18	JS	1.00	1.00	1.00	1.00
19	AD	1.00	1.00	1.00	1.00
20	HH2	1.00	1.00	1.00	
21	VS	1.00	1.00	1.00	3.50
22	JAW	1.10	2.16	1.31	1.00
23	AK	1.00	Excluded from study 1		
24	CA	1.04	1.00	1.93	6.24
25	ZRS	1.00	1.00	1.03	1.97
26	NL	1.00	1.00	1.00	1.00
27	GN	1.00	1.00	3.42	1.00
28	JA	1.00	1.00	1.00	1.00
29	ZR	3.34	2.19	3.16	1.00
30	NR	1.00	1.00	1.00	1.00

31	LB	1.00	1.14	1.20	2.00
32	EC	1.00	1.00	1.58	1.00
33	NB	1.00	1.00	1.00	1.00
34	JB	1.00	1.00	1.00	1.00
35	SR	15.37	1.39	3.81	1.00
36	AP	1.00	1.00	2.02	2.79
37	RO	1.54	1.00	1.00	1.00
38	BG	1.00	1.00	4.40	1.21
39	FAM	20.00	2.60	13.91	1.00
40	OB	2.89	20.00	20.00	1.00
41	TO'D	1.00	1.00	1.00	2.69
42	N-JF	4.05	7.95	3.10	1.00
43	JB	1.00	1.00	2.86	1.00
44	AD	1.00	5.32	2.25	3.21
45	MMR	6.24	2.37	20.00	3.53
46	RSM	1.00	1.00		
47	KMK	1.00	1.00	1.00	1.00
48	AM	1.61	1.00	1.00	8.54
49	SAH	5.66	1.09	1.00	1.00
		49	48	47	46

Lactoferrin 1-4 (Raw Data)

patient	initials	Lactoferrin ng/mL-1	Lactoferrin ng/mL-2	Lactoferrin ng/mL-3	Lactoferrin ng/mL-4
1	EAOD	62.5	Thrown away	Thrown away	2,569.8
2	EB	Thrown away	62.5	62.5	1,424.1
3	YB	2,356.0	4,392.2	Thrown away	Thrown away
4	SC	356.9	1,466.6	Thrown away	62.5
5	GM	6,045.9	504.0	Thrown away	1,868.1
6	MA	10,000.0	Thrown away	Thrown away	1,222.2
7	FM	Thrown away	625.8	Thrown away	803.1
8	IO	196.3	229.2	Thrown away	127.8
9	RR	1,048.2	1,126.3	62.5	260.3
10	PW	62.5	<62.5	Thrown away	172.8
11	JEW	62.5	235.6	62.5	Thrown away
12	OF	Thrown away	289.5	305.6	Thrown away
13	BD	Thrown away	962.6	825.4	Thrown away
14	HH	Thrown away	240.0	Thrown away	1,952.8
15	DB	Thrown away	62.5	Thrown away	3,454.7

16	SMcA	219.5	806.4	Thrown away	361.2
17	RA	Thrown away	7,529.3	Thrown away	7,093.4
18	JS	309.3	452.7	Thrown away	410.4
19	AD	Thrown away	1,063.8	Thrown away	874.6
20	HH2	95.7	634.4	1,232.2	
21	VS	241.2	186.4	527.4	547.1
22	JAW	Thrown away	720.4	Thrown away	304.6
23	AK	3,683.0			
24	CA	Thrown away	4,383.5	Thrown away	4,396.7
25	ZRS	Thrown away	62.5	Thrown away	5,050.7
26	NL	985.0	1,250.0	1,499.0	1,217.2
27	GN	304.8	111.5	19,805.3	1,711.7
28	JA	62.5	1,845.4	201.3	4,234.5
29	ZR	2,497.2	1,637.3	1,342.9	434.4
30	NR	830.6	587.8	713.6	732.3
31	LB	1,048.9	173.8	5,542.9	4,999.2
32	EC	62.5	216.8	434.4	403.8
33	NB	3,600.0	918.2	4,066.9	1,067.5
34	JB	598.6	258.1	72.7	98.3
35	SR	7,030.0	837.4	0.0	886.1
36	AP	188.9	2,331.7	1,700.0	2,442.6
37	RO	236.4	623.5	236.4	529.1
38	BG	558.7	426.9	1,604.8	549.6
39	FAM	10,000.0	3,624.0	10,000.0	2,973.6
40	OB	195.0	10,000.0	10,000.0	772.4
41	TO'D	699.4	650.7	62.5	1,029.8

42	N-JF	10,000.0	1,017.8	8,221.4	2,632.2
43	JB	712.0	62.5	0.0	62.5
44	AD	62.5	1,022.2	9,260.0	1,266.1
45	MMR	3,566.2	3,340.8	2,400.0	3,373.6
46	RSM	1,646.5	<62.5		
47	KMK	0.0	0.0	0.0	911.2
48	AM	92.6	62.5	0.0	92.6
49	SAH	7,900.1	2,688.4	559.8	775.6
		24	24	24	24

Calprotectin (5-9) Raw Data

patient	initials	Calpro 5	Calpro 6	Calpro 7	Calpro 8	Calpro 9
1	EAOD	12	64	37	14	15
2	EB	17	11	23	19	16
3	YB	10	24	90	21	135
4	SC	31	35	96		18
5	GM	70	73	160	64	284
6	MA	32	112	40	45	91
7	FM	10	10	73	32	12
8	IO	17	11	14	39	10
9	RR					10
10	PW	10	47	17	10	10
11	JEW	10	11	10	50	10
12	OF	30				20
13	BD	39	20	31	22	110
14	HH	10	12	10	10	10
15	DB	10	10	10	10	10
16	SMcA	10	10	10		12
17	RA	43	34	246	114	
18	JS	13	10	10	10	26
19	AD	10	10	10	10	12
20	HH2					
21	VS					
22	JAW	10	10	10	10	10
23	AK	31	104	41		
24	CA	24	10	16	10	13
25	ZRS	10	10	14	10	10
26	NL	10	10	10	10	10
27	GN	10	15	10	10	12
28	JA	33	10	10	10	
29	ZR	10	14	10	12	

30	NR	10	34	127		
31	LB	11	26	26		15
32	EC	14	12	11	10	12
33	NB	12	10	17	11	41
34	JB	12	13	36	20	62
35	SR	63	19	66	17	36
36	AP	10	10	21	15	82
37	RO	22	10	18	104	10
38	BG	18		57	28	108
39	FAM	123	11	12	40	
40	OB	10	10	59	56	25
41	TO'D	18	30	21	483	16
42	N-JF	455		50	323	
43	JB	10	10	10	14	10
44	AD	35	15	54		
45	MMR	482	44	34	471	
46	RSM		10		103	109
47	KMK	71	89	99	11	88
48	AM	21	54	27	30	
49	SAH	129	137	43	124	
total		45	43	44	39	36

M2-PK (5-9) Raw Data

patient	initials	M2PK-5	M2PK-6	M2PK-7	M2PK-8	M2PK-9
1	EAOD	1.00	1.89	1.00	1.00	1.00
2	EB	1.00	1.00	1.00	1.00	1.00
3	YB	1.00	1.49	1.00	3.43	1.00
4	SC	1.00	1.00	11.81		1.4
5	GM	1.00	1.74	3.71	2.59	1.00
6	MA	20.00	20.00	4.44	20.00	20.00
7	FM	1.00	2.05	2.12	1.95	1.00
8	IO	1.00	1.00	1.00	1.76	1.00
9	RR					1
10	PW	1.43	1.22	1.00	1.00	1.00
11	JEW	3.77	1.00	1.00	1.00	1.00
12	OF	1.00				1.25
13	BD	1.00	1.00	1.00	1.00	1.00
14	HH	1.00	1.00	1.00	1.20	1.30
15	DB	1.00	1.00	1.33	1.00	3.32
16	SMcA	1.00	1.00	1.00		1.00
17	RA	4.08	4.19	3.36	20.00	
18	JS	1.00	1.00	1.00	3.29	1.00
19	AD	1.00	1.04	1.00	1.23	1.00
20	HH2					
21	VS					
22	JAW	1.00	1.00	1.00	1.00	1.24
23	AK	1.00	1.00	1.00		
24	CA	3.99	1.00	2.45	1.00	2.59
25	ZRS	2.23	2.09	1.00	1.00	1.00
26	NL	1.00	1.00	1.00	1.00	1.00
27	GN	1.00	1.00	1.00	1.00	1.01
28	JA	12.08	2.48	1.88	1.44	
29	ZR	1.00	1.00	2.62	1.67	
30	NR	1.00	1.00	1.86		

31	LB	1.00	1.00	1.00		1.31
32	EC	1.29	5.42	1.68	1.00	1.00
33	NB	1.00	1.00	1.33	1.00	1.00
34	JB	1.00	1.00	1.00	1.00	1.79
35	SR	1.00	1.00	1.00	1.00	10.19
36	AP	1.00	1.00	2.09	1.00	8.75
37	RO	2.38	1.49	1.00	1.00	1.99
38	BG			4.74	2.85	5.68
39	FAM	10.48	2.75	1.00	4.56	
40	OB	1.00	1.68	2.59	1.00	8.24
41	TO'D	1.00	1.19	2.04	19.86	1.00
42	N-JF	6.89		1.00	7.89	
43	JB	1.83	1.00	1.00	1.00	1.52
44	AD	2.56	3.89	5.43		
45	MMR	20.00	1.68	1.00	20.00	
46	RSM		1.00		4.02	5.94
47	KMK	8.94	5.64	15.90	1.00	2.67
48	AM	1.00	2.59	1.00	1.00	
49	SAH	2.69	4.78	1.00	1.00	
		44	43	44	39	36

Lactoferrin (5-9) Raw Data

patient	initials	Lactoferrin ng/mL-5	Lactoferrin ng/mL-6	Lactoferrin ng/mL-7	Lactoferrin ng/mL-8	Lactoferrin ng/mL-9
1	EAOD	62.5	1,269.8	Thrown away	62.5	Thrown away
2	EB	62.5	Thrown away	Thrown away	Thrown away	1,269.7
3	YB	62.5	1,674.6	Thrown away	Thrown away	Thrown away
4	SC	879.5	322.0	Thrown away		Thrown away
5	GM	2,984.7	3,209.9	Thrown away	Thrown away	5,269.8
6	MA	2,925.4	10,000.0	Thrown away	1,835.5	Thrown away
7	FM	710.3	1,375.7	Thrown away	Thrown away	62.5
8	IO	224.4	62.5	Thrown away	79.0	Thrown away
9	RR					
10	PW	62.5	1,241.3	Thrown away	130.3	<62.5
11	JEW	Thrown away	484.3	Thrown away	1,406.3	Thrown away
12	OF	2,160.0				Thrown away
13	BD	1,645.5	1,930.2	Thrown away	921.3	Thrown away
14	HH	9,773.1	147.8	62.5	62.5	455.2
15	DB	629.7	744.3	Thrown away	62.5	Thrown away
16	SMcA	749.0	Thrown	62.5		4,812.9

			away			
17	RA	6,527.1	5,756.5	Thrown away	7,987.7	
18	JS	1,093.8	787.6	1,424.4	Thrown away	331.2
19	AD	369.3	202.4	Thrown away	221.9	<62.5
20	HH2					
21	VS					
22	JAW	383.3	62.5	Thrown away	106.5	912.3
23	AK	1,246.0	10,000.0	1,382.4		
24	CA	4,985.5	3,156.8	Thrown away	8,349.5	Thrown away
25	ZRS	62.5	62.5	Thrown away	62.5	Thrown away
26	NL	2,946.8	715.0	6,421.8	7,344.1	4,096.5
27	GN	1,030.8	172.8	174.2	1,833.7	1,363.3
28	JA	7,923.4	5,188.4	3,001.8	1,469.7	
29	ZR	1,881.3	1,731.8	500.7	1,820.4	
30	NR	62.5	732.3	937.8		
31	LB	1,214.6	2,709.7	1,042.8		452.3
32	EC	836.4	1,749.3	92.8	62.5	790.4
33	NB	62.5	871.4	2,355.9	62.5	1,044.4
34	JB	105.9	205.7	1,777.2	675.0	6,752.9
35	SR	986.0	225.7	0.0	216.3	0.0
36	AP	1,345.4	62.5	2,435.4	902.3	
37	RO	753.6	62.5	816.9	759.2	62.5
38	BG			855.0	2,697.4	
39	FAM	10,000.0	3,345.8	1,569.1	3,578.4	
40	OB	62.5	62.5	1,017.8	2,290.6	1,020.3
41	TO'D	62.5	62.5	62.5	10,000.0	62.5
42	N-JF	7,429.7		#VALUE!	6,259.2	

43	JB	1,024.8	921.3	62.5	105.9	959.4
44	AD	2,596.2	1,368.7	3,974.9		
45	MMR	10,000.0	546.4	95.3	10,000.0	
46	RSM		62.5		1,599.8	2,568.4
47	KMK	4,563.1	3,591.2	2,569.8	62.5	2,739.3
48	AM	62.5	1,025.3	556.2	109.8	462.3
49	SAH	889.5	1,023.6	79.0	566.9	

Appendix 8 – Raw Data Questionnaires 1-9 (Study I – IV)

Compliancy table of Questionnaires received

	S.0.S Questionnaire (N)	PERCENTAGE OF COMPLIANCE OVER THE PHASES	PERCENTAGE OF NON COMPLIANCE OVER THE PHASES
Quest 1	48	98%	2%
Quest 2	47	96%	4%
Quest 3	44	90%	10%
Quest 4	44	90%	10%
Quest 5	46	94%	6%
Quest 6	43	88%	12%
Quest 7	44	90%	10%
Quest 8	42	86%	14%
Quest 9	29	59%	41%

Legend to the table: This table represents the number of questionnaire received from the total subject number (n=49).

Lack of awareness and interaction with parent Q1	Abnormal greeting behaviour Q1	Abnormal comfort seeking Q1	Can't make friends Q1	Lack of awareness of social rules Q1	Lack of spontaneous speech Q1	Abnormal word utilisation Q1	Poor comprehension of verbal instructions Q1	Lack of eye contact Q1	Abnormal repetitive gestures Q1	Need to maintain sameness Q1	Need of fixed routine Q1	Diarrhoea Q1	Constipation Q1	Poor Appetite Q1	Blotting Q1	Flatulence Q1	Vomiting Q1	Unhappy Q1	Aggressive Q1	Destructive Q1	Spaced out/ Non Interactive Q1	Agitated Q1	Disagreeable Q1
5	1	3	7	9	1	1	4	3	6	5	5	1	0	0	0	2	1	0	1	0	6	2	3
1	1	7	0	2	7	7	2	2	3	3	7	0	5	[redacted]					8	8	4	4	4
4	6	3	7	7	9	9	8	3	4	1	2	6	7	5	9	[redacted]	0	4	1	[redacted]	5	[redacted]	3
3	3	2	3	5	5	5	4	0	1	1	3	[redacted]	5	5	7	5	5	5	2	1	0	6	6
0	1	5	0	0	0	3	0	5	0	0	1	0	0	7	7	3	0	1	5	7	7	7	7
[redacted]	9	9	9	6	9	9	8	8	9	7	9	0	0	0	0	1	0	0	3	7	8	7	6
0	2	2	6	3	7	9	5	5	5	5	5	0	6	6	0	4	0	1	5	1	3	6	3
0	1	1	0	5	0	5	0	3	5	5	7	0	7	0	0	0	0	0	3	3	[redacted]	5	3
0	1	0	0	0	1	1	0	0	[redacted]		0	0	0	0	1	0	0	0	0	0	0	0	1
3	5	4	6	6	5	5	6	4	7	8	8	0	6	5	5	2	3	3	3	3	2	5	4
5	7	7	9	9	8	9	7	6	9	5	1	5	2	1	0	3	0	0	1	5	1	5	5
0	0	1	2	2	1	1	1	3	5	3	3	6	1	5	1	1	0	1	0	0	2	2	2
0	2	0	0	0	1	1	0	4	0	1	1	1	0	2	1	2	0	0	1	0	0	1	1

3				9	9		9	5	5	3	3	1	2	5		1	0	0	0	0		4	
1	2	3	6	6	3	2	5	3	3	4	3	2	1	4	3	3	1	2	3	3	3	3	5
7	6	8	9	9	9		7	7	8	8	8	0	4	3	1	1	0	2	4	0	0	6	4
3	5	1	9	7	3	7	6	4	1	6	6	3	5	1	1	0	0	1	0	0	6	3	3
5	3	5	3	3	3	0	3	3	5	3	3	0	7	0	1	0	0	3	3	0	5	5	5
3	4	3	NA	3	3	3	7	7	6	9	9	6	9	9	1	9	2	NA	2	2	NA	9	7
0	0	1	3	2	0		1	0	0	1	0	2	0	0	0	0	0	1	3	0	0	2	2
3	1	5	1	2	5	1	0	0	0	0	0	0	5	5	5	1	0	5	0	0	2	4	4
0	0	0	0	0	0	0	0	0	0	0	0		0				9	0	0	0	0	0	0
7	7	8	8	9	8	9	7	5	7	7	7	5	7	3	8	3	0	7	9	9	3	7	5
1	3	5	5	6	7	5	2	1	5	3	3	5	8	5	7	7	0	3	0	1	1	1	1
2	1	1	8	7	3	4	5	3	2	2	1	1	3	5	6	2	1	1	3	4	4	2	1
5	7	6	9	9	9	9	7	7	2	7	0	9	9	0	7	3	0	0	0	3	5	6	0
1	1	3	6	7	7	7	7	3	5	5	5	5	7	5	7	7	1	1	3	3	2	3	3
1	3	3	6	9	5	6	4	6	5	1	1	8	8	6	5	7	0	7	1	1	8	7	1
3	8	9	9	9	9	0	3	5	9	8	6	0	7	9	7	0	0	2	3	7	2	8	8
1	5	1	7	6	3	6	3	2	0	1	1	0	8	0	8	0	0	3	3	1	0	0	1
3	4	5	5	7	3	4	5	6	7	7	7	3	0	7	5	0	5	5	8	6	2	2	6
3	5	3	3	5	3	5	5	5	1	3	6	2	6	0	3	3	0	3	6	6	6	6	7
3	3	4	6	8	9	9	3	5	9	5	4	9	1	5	3	3	1	2	1	3	7	7	7
1	4	4	9	8	4	3	4	4	6	4	3	3	7	1	7	5	0	2	0	0	3	4	2
0	0	2	2	3	0	1	0	0	7	7	7	7	6	3	2	4	1	3	1	2	0	5	6
5	3	3	7	7	9	7	9	5	7	5	5		7	8	1	0	0	1	3	3	3	3	6
7	7	7	7	7	9	9	8	6	6	7	7	6	0	0	2	2	1	3	7	8	3	6	6
6	0	7	9	9	9	6	7	6	9	9	9	8	8	6	6	1	1	6	7	6	6	7	6
2	0	3	7	9	7	7	2	3	3	3	7	7	9		3	3	2	5	7	8	6	8	9

5	5	5	7	5	5	0	7	1	0	8	8	0	9	7	0	0	0	5	0	0	5	9	9
3	6	5	5	7	5	7	6	5	6	7	7	4	0	0	4	4	0	0	0	3	3	0	1
0	4	4	8	7	9	7	4	1	7	5	5	7	3	3	6	7	0	2	1	0	3	0	1
7	7	7	9	9	9	9	8	5	8	9	8	0	9	0	5	7	0	5	5	7	7	6	8
4	2	2	1	1	1	1	1	1	7	4	3	0	0	0	0	0	4	6		5	4	5	5
2	2	0		0	0	2	2	5	3	3	3	4		0	0	3	0	0	1	0	1	3	3
3	9		9	9	9		9	5	8	8	6	3	8	3	5		4	4	7	9			2
3	4	5	3	3	5	4	4	4	3	3	3	5	5	3	6	4	6	3	4	5	4	5	5

Lack of awareness and interaction with parent Q2	Abnormal greeting behaviour Q2	Abnormal comfort seeking Q2	Can't make friends Q2	Lack of awareness of social rules Q2	Lack of spontaneous speech Q2	Abnormal word utilisation Q2	Poor comprehension of verbal instructions Q2	Lack of eye contact Q2	Abnormal repetitive gestures Q2	Need to maintain sameness Q2	Need of fixed routine Q2	Diarrhoea Q2	Constipation Q2	Poor Appetite Q2	Blotting Q2	Flatulence Q2	Vomiting Q2	Unhappy Q2	Aggressive Q2	Destructive Q2	Spaced out/ Non Interactive Q2	Agitated Q2	Disagreeable Q2
3	5	7	7	9	6	6	4	4	6	5	5	0	0	0	0	0	0	1	0	0	1	3	4
1	1	1	1	3	2	7	3	1	7	4	4	7	0	0	0	0	0	1	2	2	0	6	6
4	7	3	8	7	9	9	9	3	3	2	3	2	4	3	8	[redacted]	0	4	1	[redacted]	6	[redacted]	4
2	3	3	2	3	5	5	5	1	3	2	4	0	5	1	7	5	5	[redacted]					
1	1	5	0	0	0	5	0	3	0	0	0	0	0	3	0	0	0	0	7	3	0	0	7
9	9	9	[redacted]	3	7	7	7	6	9	8	9	0	0	0	1	1	0	0	3	5	9	5	5
3	5	3	7	3	5	7	3	3	5	1	2	0	3	0	0	[redacted]	0	0	3	0	0	3	0
0	3	3	0	3	0	3	0	3	5	5	5	0	7	0	0	[redacted]	0	0	3	3	[redacted]	3	3
0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	0
4	4	3	6	6	6	6	6	5	6	7	7	3	4	3	4	3	2	3	2	2	4	3	4
5	6	7	7	9	5	7	5	5	6	4	4	4	4	3	2	4	0	1	1	4	2	3	4
1	0	1	1	2	0	0	1	1	4	6	5	4	1	3	2	0	0	1	1	1	2	1	1
0	1	0	0	0	3	1	0	1	0	0	0	0	2	1	1	0	0	0	0	0	0	1	1

3	3	3	8	9	9	9	9	3	3	3	3	[illegible]	6	6	7	0	0	1	0	0	2	3	0
1	3	4	6	4	1	4	5	3	3	3	3	4	3	3	4	2	1	2	3	3	3	4	4
5	5	6	9	9	9	9	6	6	5	5	4	3	1	5	1	1	0	2	3	0	5	4	2
3	6	3	9	8	7	7	7	5	2	5	5	1	8	3	5	1	0	2	0	0	4	4	2
3	0	3	3	5	3	0	3	0	3	3	3	0	6	0	3	0	0	3	0	0	3	7	3

0	1	0	1	1	0	1	1	0	0	1	0	1	0	1	1	1	0	0	0	0	1	1	1
0	0	0	2	2	3	1	1	1	0	0	0	0	4	1	2	2	0	0	0	0	1	0	0
3	7	3	9	9	9	9	7	3	7	7	7	9	9	1	1	0	0	2	2	2	2	2	2
7	6	4	8	7	5	8	6	4	6	7	7	0	9	2	8	8	0	7	9	9	7	7	7
0	0	5	2	5	5	6	5	0	1	0		6	7	5	7	5	0	1	0	1	0	2	2
2	1	4	8	7	3	4	4	3	5	2	1	1	4	4	6	3	0	1	4	4	1	1	2
4	7	8	9	9	9	9	9	7	4	9	3	6	8	0	6	6	0	1	0	2	9	9	6
1	2	2	4	4	4	5	4	2	3	3	3	0	0	2	3	3	[illegible]	1	1	1	1	1	1
1	3	3	6	9	4	6	4	7	6	1	1	8	8	8	6	8	0	6	1	1	9	6	1
6	9	4	9	9	8	[illegible]	5	6	9	6	6	6	0	1	1	6	0	5	7	8	7	8	8
4	7	7	9	7	0	0	5	5	6	7	6	1	6	8	7	4	0	4	4	5	3	6	6
1	1	6	6	5	3	6	3	2	0	1	1	0	7	0	8	[illegible]	0	2	4	0	0	1	2
5	7	7	7	8	5	5	6	6	5	5	6	0	9	9	5	0	6	5	7	7	1	5	9
3	3	1	3	5	3	1	5	4	1	4	4	1	5	0	3	3	0	3	6	4	4	6	6
2	3	4	4	2	3	3	2	3	5	3	3	3	1	1	2	1	0	2	2	2	3	4	3
1	1	4	5	5	2	3	3	4	[illegible]	[illegible]	1	1	5	1	4	4	0	8	3	2	[illegible]	[illegible]	8
0	0	2	2	3	0	1	0	0	7	7	7	7	6	3	2	4	1	3	1	2	0	5	6
6	7	5	5	7	7	7	7	5	5	6	7	0	7	6	3	2	0	5	3	3	5	5	5
6	6	7	6	7	8	8	6	7	7	7	7	7	0	0	1	1	0	5	6	7	7	7	6
5	7	6	9	8	9	9	8	7	9	8	6	5	5	1	3	2	0	4	6	6	8	8	6
7	8	9	9	9	5	5	4	7	5	8	8	6	1	9	[illegible]	[illegible]	6	6	8	8	4	7	8

5	3	3	3	5	5	1	5	6	3	7	7	0	9	8	0	0	0	5	0	0	5	5	5
3	7	5	3	5	3	5	5	2	5	6	6	2	0	0	2	2	0	0	1	1	2	0	0
0	3	6	8	6	9	9	7	7	5	5	5	5	5	2	6	6	1	1	1	0	3	0	1
9	8	8	9	9	9	9	9	7	8	9	9	0	3	0	6	6	0	7	8	8	8	7	9
3	1	3	1	1	1	1	1	1	7	7	4	1	0	0	0	0	0	2	5	6	6	3	7
1	2	1			9	9	2	2	2	3	3	4	3	0	1	3	0	1	1	1	1	1	1
3	9		9	9	9	9	4						8	3			4	4	7	9			2
5	6	4	3	3	2	3	3	6	3	2	4	7	7	7	7	7	5	5	4	3	4	4	4

Lack of awareness and interaction with parent Q3	Abnormal greeting behaviour Q3	Abnormal comfort seeking Q3	Can't make friends Q3	Lack of awareness of social rules Q3	Lack of spontaneous speech Q3	Abnormal word utilisation Q3	Poor comprehension of verbal instructions Q3	Lack of eye contact Q3	Abnormal repetitive gestures Q3	Need to maintain sameness Q3	Need of fixed routine Q3	Diarrhoea Q3	Constipation Q3	Poor Appetite Q3	Bloating Q3	Flatulence Q3	Vomiting Q3	Unhappy Q3	Aggressive Q3	Destructive Q3	Spaced out/ Non Interactive Q3	Agitated Q3	Disagreeable Q3
1	3	5	7	7	6	1		3	7	6	5	1	0	0	2	3	1	0	1	0	2	3	5
5	1	1	1	5	1	5	1	4	1	1	1	[illegible]	3	[illegible]	3	[illegible]	[illegible]	1	1	1	3	3	3
4	5	5	8	7	9	9	7	5	2	2	2	0	1	6	7	[illegible]	0	5	1	1	7	3	4
1	1	2	1	2	2	2	2	0	2	0	1	0	3	0	5	4	2	2	1	1	1	2	3
3	0	3	5	0	0	0	3	7	0	1	0	0	0	0	0	0	0	5	7	7	5	3	7
9	9	[illegible]	4	9	9	7	9	6	8	7	9	0	1	0	0	0	0	0	7	7	9	6	5
[illegible]																							
0	3	3	3	3	1	5	3	5	5	5	5	0	7	0	0	[illegible]	0	0	1	3	[illegible]	3	3
0	0	[illegible]	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0
[illegible]																							
5	7	7	7	9	6	7	5	5	7	6	4	1	4	4	0	6	0	1	1	4	0	5	6
1	1	0	2	3	1	0	0	1	3	4	3	0	6	1	0	0	0	1	2	0	1	2	1
0	1	0	0	1	2	2	4	0	0	0	0	0	1	2	1	2	0	0	0	0	0	0	0

3	5	5	9	9	9	0	7	3	3	5	5	0	2	2	2	0	0	0	0	0	[redacted]	2	0
1	3	5	6	7	1	3	4	1	3	3	1	4	3	3	3	3	1	2	2	2	1	3	3
4	4	3	8	8	9	[redacted]	5	4	4	6	6	4	1	5	4	[redacted]	0	0	0	0	4	2	[redacted]
4	7	3	9	8	7	7	7	6	3	4	4	3	8	4	4	2	0	2	0	1	5	4	3
7	7	0	5	7	7	0	3	3	7	3	3	0	7	0	0	0	0	3	3	0	7	7	7
1	0	0	1	1	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	1	1	1
0	0	0	0	0	2	2	2	1	0	0	1	0	5	0	4	3	0	0	0	0	1	0	0
[redacted]																							
7	7	8	9	8	9	9	6	7	6	8	7	2	8	0	8	8	0	7	4	5	4	7	7
0	1	3	2	3	7	7	5	1	4	2	2	7	7	6	7	6	0	4	4	4	1	4	4
1	0	1	8	6	2	4	3	2	3	2	1	1	2	1	2	1	0	1	2	3	1	1	2
5	5	5	9	9	9	9	7	6	6	6	2	2	8	0	6	5	0	1	0	3	8	8	2
0	1	1	3	4	7	7	6	0	5	5	5	0	3	0	2	1	0	1	1	1	1	1	1
1	3	3	8	9	5	5	4	7	6	1	1	8	8	6	5	6	0	3	1	1	5	4	1
5	8	6	9	8	9	9	4	4	9	6	5	2	6	0	2	7	0	4	7	7	6	5	6
5	9	9	9	7	9	0	5	4	8	8	7	0	8	9	8	7	0	4	6	8	4	8	8
1	1	1	6	5	3	6	3	2	0	1	1	0	8	0	7	0	0	1	2	0	0	1	1
5	5	5	7	7	5	5	4	7	6	6	6	0	8	8	8	0	5	1	5	3	3	2	4
2	2	3	6	7	0	1	2	4	0	2	4	0	6	0	1	3	0	2	7	6	2	2	6
1	2	2	2	7	2	2	1	2	5	3	2	3	0	0	2	2	0	1	1	2	3	3	2
4	3	7	7	5	4	2	2	2	3	2	2	1	7	2	7	3	0	3	2	1	3	5	5
0	1	1	3	2	0	2	1	0	7	5	5	4	3	1	2	3	0	0	4	3	0	2	5
5	5	3	6	6	7	7	7	5	5	6	6	0	6	7	2	3	0	5	4	4	6	4	6
7	7	7	7	7	9	8	8	7	7	7	7	6	0	0	3	3	0	6	6	6	6	5	6
[redacted]																							
8	3	6	8	9	4	7	7	1	5	6	7	4	1	5	0	[redacted]	3	8	7	7	7	8	9

5	5	8	5	6	5	0	5	6	7	8	9	0	9	8	7	[illegible]	5	5	1	1	5	[illegible]	7
3	6	1	4	4	1	3	4	3	4	5	5	4	0	0	0	2	0	1	0	2	1	0	0
1	3	3	7	7	9	9	5	5	7	7	5	7	0	7	7	5	0	3	1	0	1	0	5
9	9	8	9	9	9	9	9	7	9	9	9	0	8	0	6	6	0	8	9	8	7	7	9
4	4	4	4		1	1	1	3	1	5	3	0	0	0	0	0	0	1	4	5	1	1	3
[illegible]																							
3	9		9	9	9		9	4	[illegible]				8	3			4	4		7			2
4	4	4	4	5	4	4	5	5	4	4	4	0	0	0	0	0	0	3	5	5	5	5	5

Lack of awareness and interaction with parent Q4	Abnormal greeting behaviour Q4	Abnormal comfort seeking Q4	Can't make friends Q4	Lack of awareness of social rules Q4	Lack of spontaneous speech Q4	Abnormal word utilisation Q4	Poor comprehension of verbal instructions Q4	Lack of eye contact Q4	Abnormal repetitive gestures Q4	Need to maintain sameness Q4	Need of fixed routine Q4	Diarrhoea Q4	Constipation Q4	Poor Appetite Q4	Bloating Q4	Flatulence Q4	Vomiting Q4	Unhappy Q4	Aggressive Q4	Destructive Q4	Spaced out/ Non Interactive Q4	Agitated Q4	Disagreeable Q4
2	6	3	7	8	6	5	5	4	2	6	2	1	0	0	0	1	1	1	0	3	4	4	5
7	3	7	1	7	7	7	3	7	3	3	3	3	3	3	[illegible]				4	4	7	4	4
2	6	3	9	7	9	9	9	3	3	2	1	0	1	6	5	4	0	0	1	6	5	4	0
0	1	0	0	2	1	1	2	0	1	1	0	0	5	0	6	5	4	0	1	2	1	2	2
3	0	3	5	0	0	0	3	7	0	1	0	0	0	0	0	0	0	5	7	7	5	3	7
9	7	7	6	9	6	9	[illegible]	7	9	9	9	0	0	0	1	1	1	0	3	3	7	5	5
2	1	1	3	4	7	7	5	3	1	2	3	0	3	0	0	0	0	1	0	0	3	3	1
0	3	3	3	3	1	5	3	5	5	5	5	0	7	0	0	[illegible]	0	0	1	0	[illegible]	3	3
0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
[illegible]																							
5	6	7	7	9	6	6	6	5	5	5	3	2	3	5	0	5	0	1	0	1	5	3	3
1	1	1	2	2	0	0	0	1	4	4	4	3	3	1	1	1	0	1	1	2	0	1	1
0	1	0	0	0	2	2	5	1	0	0	0	0	2	3	0	1	0	0	0	0	0	0	0

3	2	2	9	9	9	9	7	2	3	5	5	9	9	3	4	9	9	0	0	0	5	5	5	
1	3	4	5	5	1	3	3	1	3	2	1	3	3	3	4	3	1	1	2	3	2	3	3	
7	6	9	8	8	9	9	6	[illegible]	7	8	5	2	2	7	2	[illegible]	0	1	3	0	0	2	0	
3	3	1	7	5	2	2	5	5	3	3	3	3	5	5	0	0	0	1	1	0	3	1	1	
3	7	7	3	7	3	0	3	5	7	7	3	0	7	0	0	0	0	3	5	0	7	7	5	
0	0	0	1	1	0	0	1	0	1	0	0	2	0	0	0	1	0	0	0	0	0	1	1	
0	0	0	0	0	0	2	2	1	1	1	1	0	3	0	3	2	0	0	0	0	0	0	0	
[illegible]																								
7	7	7	7	8	7	6	9	7	7	8	8	[illegible]	8	8	6	7	0	6	6	8	7	8	7	
0	3	3	3	4	7	7	5	0	4	3	1	5	8	5	7	7	0	4	1	1	1	4	4	
2	1	2	6	6	2	3	3	2	2	1	1	3	3	4	2	2	1	1	1	1	1	2	1	
5	6	7	9	9	9	9	7	7	1	6	1	0	5	0	5	5	0	1	0	8	8	8	0	
[illegible]																								
1	3	3	8	9	5	5	2	4	4	1	1	6	8	7	7	7	0	3	1	0	5	3	1	
[illegible]																								
3	6	8	8	8	0	0	7	6	7	7	7	0	8	9	8	7	0	2	3	4	2	7	8	
1	1	1	6	4	3	5	2	1	0	1	0	0	7	0	6	0	0	1	1	0	0	1	1	
5	6	6	9	8	5	5	5	8	5	5	6	0	9	9	9	0	6	0	6	6	4	5	8	
3	4	1	3	5	1	1	2	4	1	3	3	1	5	0	1	1	0	3	6	5	4	5	5	
3	3	3	3	8	4	2	3	3	7	3	3	1	8	0	3	3	0	6	1	6	1	7	7	
2	3	3	7	7	5	5	3	2	4	1	1	8	1	8	8	4	0	3	2	0	3	7	8	
0	1	2	2	3	0	1	0	0	7	6	5	5	1	0	2	3	0	2	2	3	0	4	7	
4	4	4	7	7	7	6	7	4	6	6	6	0	7	7	4	4	0	3	3	3	6	5	5	
7	7	7	7	7	8	7	7	7	7	7	7	7	0	0	2	2	0	7	7	7	7	7	7	
8	5	8	9	9	9	7	8	7	7	9	9	6	0	9	1	7	9	8	8	1	9	1	[illegible]	
2	1	5	6	8	4	5	4	1	6	4	7	6	6	7	[illegible]		2	3	2	6	2	5	8	

5	5	5	2	5	5	5	5	6	1	5	7	0	9	7	6	0	0	5	0	0	5	5	5
6	[redacted]	8	5	8	7	8	7	2	4	7	8	6	0	0	0	0	0	0	0	0	5	5	0
1	1	3	7	7	9	7	5	3	7	5	7	5	2	8	5	5	0	3	1	0	1	0	1
8	8	8	9	9	9	1	9	6	8	9	9	0	8	6	6	6	0	6	7	8	9	7	9
3	3	2	1	1	1	1	1	1	6	6	6	2	1	1	1	1	1	0	6	7	7	7	7
[redacted]																							
3	9		9	9	9		9	4	[redacted]				8	3	[redacted]		4	4	[redacted]	7	[redacted]		2
4	5	4	4	4	4	4	4	4	4	3	2	0	0	0	0	0	0	4	5	4	3	3	5

Lack of awareness and interaction with parent Q5	Abnormal greeting behaviour Q5	Abnormal comfort seeking Q5	Can't make friends Q5	Lack of awareness of social rules Q5	Lack of spontaneous speech Q5	Abnormal word utilisation Q5	Poor comprehension of verbal instructions Q5	Lack of eye contact Q5	Abnormal repetitive gestures Q5	Need to maintain sameness Q5	Need of fixed routine Q5	Diarrhoea Q5	Constipation Q5	Poor Appetite Q5	Bloating Q5	Flatulence Q5	Vomiting Q5	Unhappy Q5	Aggressive Q5	Destructive Q5	Spaced out/ Non Interactive Q5	Agitated Q5	Disagreeable Q5
1	5	5	9	9	7	3	5	3	7	7	6	3	0	0	0	5	0	3	1	0	2	5	3
1	1	1	1	1	3	6	2	0	1	3	3	5	[redacted]					1	1	1	1	4	4
3	6	5	9	8	9	9	7	2	3	2	1	0	1	6	7	[redacted]	0	4	2	3	5	3	1
0	1	2	2	2	2	4	2	0	1	0	0	0	2	0	1	2	0	0	0	0	0	1	3
5	4	3	7	5	5	4	7	3	7	7	7	0	0	1	0	0	0	1	7	3	3	3	7
6	7	7	6	9	7	9	9	7	9	9	6	0	0	0	0	2	0	1	4	4	3	6	7
0	1	2	5	5	4	4	2	1	4	3	4	0	1	0	0	0	0	4	0	0	1	1	0
0	3	3	3	3	0	4	0	3	3	5	5	0	7	0	0	0	0	0	3	3	1	5	3
[redacted]																							
	4	5	3	5	5	4	4	4	4	7	7	7	1	4	3	4	1	1	1	1	1	5	5
3	5	6	8	9	[redacted]	5	6	3	5	4	2	1	4	1	0	2	0	0	0	5	0	3	4
2	1	1	1	2	0	1	3	3	2	3	2	2	1	1	1	1	1	1	1	1	3	2	1
1	4	0	7	7		4	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	0	0

3	3	3	9	9	9	9	9	3	3	1	2	0	3	3	4	0	0	0	0	0	3	3	0
0	1	0	5	5	3	5	4	3	5	3	1	6	3	4	7	5	1	2	3	2	2	2	3
3	2	1	8	9	9	9	3	4	5	6	2	0	[illegible]	1	1	0	0	0	0	0	3	4	0
2	7	2	9	8	7	7	6	5	5	4	3	4	7	2	5	2	0	1	0	0	5	3	3
0	5	3	0	3	3	0	0	1	3	3	5	0	5	0	5	0	0	1	1	0	3	1	3

[illegible]																							
0	1	0	0	1	2	2	2	1	1	0	0	1	1	0	2	1	1	0	0	0	1	0	0
1	3	4	9	6	9	9	5	3	5	5	5	4	9	7	7	0	0	2	3	3	3	3	3
5	6	7	8	7	6	9	7	5	6	7	7	2	6	0	4	3	0	6	6	7	7	6	6
1	1	0	1	4	7	[illegible]	8	1	2	3	2	7	8	8	8	8	0	3	5	5	0	4	5
2	2	2	5	4	[illegible]	4	2	1	1	3	2	3	3	4	6	2	0	1	1	2	1	2	2
5	6	6	9	9	9	9	7	6	6	6	0	2	6	0	4	4	0	[illegible]	1	5	5	6	7
5	7	7	7	7	7	7	6	3	7	5	5	2	7	3	6	4	1	6	6	6	6	6	6
1	3	3	8	8	5	5	4	7	4	1	1	7	7	3	4	4	0	4	0	0	8	5	2
1	4	3	8	9	9	9	2	1	8	6	6	0	2	0	1	1	0	3	7	9	5	7	7
3	4	7	8	8			5	5	7	7	7	0	8	9	9	9	0	8	4	8	7	7	8
1	1	1	7	4		3	2	1	0	1	1	0	5	0	5		0	4	1	0	0	0	3
4	4	3	5	4	3	2	3	5	4	4	4	1	6	7	6	1	3	5	6	6	1	3	6
1	1	1	3	3	0	0	2	3	0	2	1	0	2	0	5	3	0	1	5	4	1	1	3
1	1	5	5	6	1	6	2	2	7	2	1	4	1	0	3	1	0	3	3	5	3	6	6
1	1	6	3	1	1	1	1	1	0	0	0	0	9	3	4	1	8	1	0	0	0	1	1
0	1	3	2	1	0	3	0	0	8	6	5	5	1	0	2	4	0	2	1	5	0	6	6
3	3	1	4	7	7	5	8	3	5	7	7	0	7	6	4	4	0	3	5	3	5	5	5
7	8	8	8	8	9	9	8	8	9	1	1	1	1	3	4	8	8	8	8	8	7	7	7
5	7	7	7	7	9	9	9	7	7	7	7	3	4	3	7	[illegible]	0	5	6	7	7	9	7
3	2	2	6	7	3	2	2	3	3	2	6	3	0	5	0	0	0	4	1	2	0	3	6

5	5	5	5	[redacted]			2	4	6	6	6	[redacted]	1	5	[redacted]	0	0	2	0	0	[redacted]			
3	6	2	4	4	4	5	3	3	4	7	6	5	0	0	4	4	0	0	0	2	2	1	1	
0	3	3	7	7	9	7	6	1	7	5	5	5	3	1	5	5	0	1	3	0	0	3	3	
8	8	8	9	9	8	9	9	6	9	9	9	0	9	3	3	2	0	7	6	7	8	7	9	
2	1	1	1	1	1	1	1	1	5	5	1	0	2	1	1	1	1	3	7	8	7		6	
3	3	2	5	5				3	1	3	3	3	3	2	3	3	0	2	2	1	2		2	
3	9		9	9	9		9	4	[redacted]				8	3	[redacted]		4	4	7		9		2	
5	5	5	5	5	4	5	5	5	4	4	5	1	1	1	1	1	1	4	3	4	4	4	4	

Lack of awareness and interaction with parent Q6	Abnormal greeting behaviour Q6	Abnormal comfort seeking Q6	Can't make friends Q6	Lack of awareness of social rules Q6	Lack of spontaneous speech Q6	Abnormal word utilisation Q6	Poor comprehension of verbal instructions Q6	Lack of eye contact Q6	Abnormal repetitive gestures Q6	Need to maintain sameness Q6	Need of fixed routine Q6	Diarrhoea Q6	Constipation Q6	Poor Appetite Q6	Blotting Q6	Flatulence Q6	Vomiting Q6	Unhappy Q6	Aggressive Q6	Destructive Q6	Spaced out/ Non Interactive Q6	Agitated Q6	Disagreeable Q6
3	6	6	9	8	6	4	4	3	4	3	3	6	0	2	0	4	0	1	0	1	4	3	2
3	3	5	1	3	3	5	5	1	6	3	3	5	[redacted]							4	[redacted]	4	[redacted]
3	4	3	9	9	8	9	7	3	2	1	3	0	1	2	4	5	0	2	4	6	6	4	7
0	0	0	3	[redacted]	2	3	3	1	0	0	0	0	0	0	2	1	0	0	0	0	0	1	1
3	3	2	[redacted]	3	3	3	4	2	3	3	3	0	0	1	0	0	0	1	1	1	1	1	2
9	7	7	3	9	[redacted]	6	6	4	7	6	8	1	3	1	1	1	1	2	3	5	7	3	3
0	1	0	5	3	5	0	0	1	2	2	1	0	2	0	0	0	0	0	0	2	0	3	0
0	3	3	3	3	0	0	3	5	5	7	7	0	7	0	0	0	0	0	3	3	5	5	5
[redacted]																							
5	4	4	4	3	4	4	4	4	4	4	6	6	0	5	3	2	1	2	2	2	3	3	5
5	5	7	9	9	7	7	5	2	6	5	1	5	1	3	0	2	0	0	1	5	0	2	2
2	3	3	2	3	1	3	2	2	6	5	4	7	0	1	1	1	0	1	1	1	1	1	1
0	1	0	7	5	2	2	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0

3	6	6	9	9	9	0	0	3	3	3	3	0	3	1	3	0	0	0	0	0	3	3	0
1	2	2	5	5	2	3	4	3	5	2	2	3	3	3	5	2	0	2	1	2	2	2	3
2	2	0	1	8	[illegible]	1	[illegible]		3	3	0	0	1	0	0	0	0	1	1	1	2	0	0
2	3	3	9	8	6	7	7	6	4	5	5	1	5	1	3	1	0	1	0	0	6	2	2
5	5	7	5	7	1	0	3	5	7	7	5	0	5	0	7	0	0	1	5	0	7	7	7

0	1	0	0	1	1	1	1	0	0	0	0	0	3	0	3	3	0	0	0	0	0	0	0
2	2	2	2	1	9	9	9	1	9	9	9	[illegible]	5	4	[illegible]	9	9	1	5	0	0	9	9
6	7	7	8	8	7	9	6	5	5	6	6	2	4	5	2	3	0	5	4	6	7	6	6
1	2	3	5	4	8	8	5	0	3	5	2	5	3	3	5	5	6	1	2	2	0	1	1
2	2	2	5	3	5	4	2	1	3	2	2	2	2	3	5	1	0	1	1	1	1	1	1
3	5	7	9	9	9	9	6	6	5	5	1	3	5	0	4	5	0	1	0	5	6	7	6
1	3	5	5	5	7	7	4	1	7	6	4	1	1	3	7	7	0	2	2	2	2	2	2
2	3	3	8	8	7	[illegible]	4	7	4	0	0	7	7	1	3	1	0	4	0	0	9	8	3
4	9	4	9	9	9	9	3	2	9	7	7	4	1	1	2	2	0	3	6	6	9	7	7
5	6	7	9		6		5	6	6	6	6	0	6	8	6	5	0	6	3	5	3	7	6
1	1	1	7	3	2	2	2	1	0	1	1	0	1	0	2		0	5	2	0	0	4	4
4	5	5	5	5	4	4	4	5	5	5	5	0	4	5	2	2	4	4	6	6	2	3	6
4	4	1	4	5	3	1	3	4	1	3	3	1	3	1	5	1	0	3	5	4	3	4	4
4	6	5	5	7	7	6	4	4	7	5	4	3	3	0	2	1	0	5	5	5	4	6	6
1	1	4	4	4	2	2	2	2	1	1	1	2	5	2	7	5	0	3	0	0	2	4	3
0	1	1	2	1	0	0	0	0	6	3	3	5	0	0	4	5	0	1	2	3	0	5	6

7	7	7	7	7	9	9	6	6	6	7	7	6	0	1	0	0	1	4	7	7	5	6	5
7	7	7	7	7	7	7	7	7	7	7	7	1	5	5	7	1	3	5	5	7	7	7	5
1	0	4	5	6	7	3	2	2	2	3	6	0	0	3	0	0	0	3	1	1	0	3	4

1	2	2	2	1	1	1	1	1	3	3	2	0	0	0	1	0	0	0	0	0	1	0	0
1	3	3	9	7	7	7	6	1	5	5	5	5	1	1	5	5	0	0	0	0	0	0	0
8	8	8	9	9	9	9	9	6	9	9	9	0	8	0	4	4	0	7	7	9	8	8	9
3	2	2		6			3	2	3	3	4	4	2	2	2	2	0	1	1	2	3	3	2
3	9		9	9	9		9	4					8	3			4	4	6	9			2
3	3		3	4	4	4	4	4	5	5	5	2	1	2	2	2	2	3	3	3	4	4	4

Lack of awareness and interaction with parent Q7	Abnormal greeting behaviour Q7	Abnormal comfort seeking Q7	Can't make friends Q7	Lack of awareness of social rules Q7	Lack of spontaneous speech Q7	Abnormal word utilisation Q7	Poor comprehension of verbal instructions Q7	Lack of eye contact Q7	Abnormal repetitive gestures Q7	Need to maintain sameness Q7	Need of fixed routine Q7	Diarrhoea Q7	Constipation Q7	Poor Appetite Q7	Blotting Q7	Flatulence Q7	Vomiting Q7	Unhappy Q7	Aggressive Q7	Destructive Q7	Spaced out/ Non Interactive Q7	Agitated Q7	Disagreeable Q7
5	5	7	8	8	5	5	6	3	7	6	6	1	1	0	1	1	0	1	0	0	5	5	4
3	1	4	4	4	4	4	2	1	1	2	2	3	[illegible]						1	2	[illegible]		
3	4	6	9	8	8	9	8	4	4	2	3	1	1	6	2	1	0	2	3	4	5	4	6
[illegible]																							
3	4	3	3	3	4	3	4	2	5	5	5	0	0	0	0	0	0	3	3	3	3	3	3
9	9	9	3	9	9	9	4	3	7	7	9	0	0	0	1	1	0	1	4	4	5	6	6
0	0	1	4	4	5	1	0	1	1	1	1	0	1	0	0	1	0	0	0	0	1	1	0
0	3	3	3	3	0	3	0	3	5	5	5	0	7	0	0	0	0	0	3	3	0	3	3
[illegible]																							
5																							
5	3	5	8	9	7	7	7	2	4	2	1	1	1	1	0	5	0	0	0	5	0	2	3
2	2	1	2	3	1	2	2	2	6	2	2	0	0	0	0	0	0	2	2	1	3	3	2
0	1	0	2	4	1	1	0	0	0	0	0	0	2	0	0	1	0	0	0	0	0	1	1

4	3	4	9	9	9	9	9	3	3	3	3	0	2	3	2	0	0	0	0	0	3	3	0
2	2	2	5	5	2	3	5	3	3	1	1	3	2	3	3	2	0	1	1	2	1	2	2
5	4	8	8	8	9	3	4	5	5	6	5	[illegible]	8	3	6	[illegible]	[illegible]	4	3	0	0	3	3
5	7	2	9	8	7	7	5	5	3	3	3	3	6	1	5	0	0	2	1	0	7	5	2
2	0	3	7	9	3	0	7	7	7	7	7	0	3	0	7	5	0	3	7	7	7	7	7

1	0	0	0	1	3	2	1	1	1	0	0	0	2	0	3	3	0	0	0	0	2	0	0
												[illegible]			[illegible]								
5	6	6	7	8	6	9	5	5	5	5	5	0	0	5	5	3	0	6	6	6	6	6	6
1	0	3	3	4	6	7	5	0	3	2	1	5	5	1	5	5	0	4	3	1	0	4	1
3	4	4	4	4	4	4	4	2	3	3	2	1	4	4	4	6	1	4	1	2	3	4	2
4	5	4	7	9	8	9	7	6	5	3	3	6	6	0	2	6	0	0	0	5	5	5	0
2	2	2	5	5	7	7	7	1	4	4	4	1	0	1	2	2	0	1	2	2	1	1	1
0	1	3	7	8	4	8	4	8	4	1	1	6	6	4	6	6	0	5	0	0	8	5	3

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6	6	6	9	7	9	9	9	7	8	8	8	3	6	3	6	6	0	6	2	6	7	7	7
3	1	5	7	8	3	1	2	3	3	2	5	2	0	7	0	1	0	1	5	5	2	2	5

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1	1	1	7	7	7	7	5	1	5	5	7	1	2	0	3	3	0	0	0	0	0	0	0
7	7	7	9	9	8	9	5	8	9	9	0	8	0	4	4	0	0	5	6	8	8	7	8
3	2	3											2	5				2	4	6		4	4
3	9		9	9	9		9	4					8	3			4	4		7			2

Lack of awareness and interaction with parent Q8	Abnormal greeting behaviour Q8	Abnormal comfort seeking Q8	Can't make friends Q8	Lack of awareness of social rules Q8	Lack of spontaneous speech Q8	Abnormal word utilisation Q8	Poor comprehension of verbal instructions Q8	Lack of eye contact Q8	Abnormal repetitive gestures Q8	Need to maintain sameness Q8	Need of fixed routine Q8	Diarrhoea Q8	Constipation Q8	Poor Appetite Q8	Bloating Q8	Flatulence Q8	Vomiting Q8	Unhappy Q8	Aggressive Q8	Destructive Q8	Spaced out/ Non Interactive Q8	Agitated Q8	Disagreeable Q8
4	6	6	7	7	6	4	5	3	6	6	4	1	1	0	1	4	0	1	0	1	4	1	1
2	1	4	4	2	2	4	1	1	1	2	2	[redacted]											
3	6	3	9	9	9	9	7	3	1	0	2	0	9	8	9	[redacted]	0	3	2	[redacted]	8	4	0
[redacted]	2	2	2	2	0	2	3	0	1	3	3	0	1	0	1	1	0	1	3	3	2	2	2
3	3	3	4	4	3	3	3	1	3	3	3	0	0	0	0	0	0	2	3	2	3	3	3
9	9	9	3	8	9	[redacted]	7	6	7	7	7	0	0	0	0	0	0	0	3	3	3	2	4
1	0	0	4	2	5	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
0	3	3	3	3	0	3	0	3	5	5	5	0	7	0	0	0	0	0	3	3	3	5	5
[redacted]																							
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3	4	5	8	9	6	6	7	2	4	4	3	1	2	1	0	5	0	0	0	4	0	2	2
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3	2	2	9	9	9	9	9	3	3	3	3	0	3	5	2	2	0	0	0	5	4	5	0
4	4	3	5	5	2	3	3	5	3	1	1	1	1	3	1	3	1	1	1	1	1	1	2
2	2	5	8	7	9	[illegible]	3	3	5	3	4	0	2	2	3	[illegible]	0	3	4	0	0	4	3
3	4	4	9	8	5	7	5	6	5	5	5	2	5	0	5	1	0	2	0	1	5	5	5
5	1	1	3	7	3	[illegible]	3	3	5	5	3	0	5	0	7	0	0	7	7	7	7	7	7
[illegible]																							
0	1	1	1	2	2	2	1	0	1	0	0	2	2	0	2	1	0	0	0	0	2	0	0
5	6	5	8	8	7	9	5	4	5	6	6	0	1	0	5	4	0	4	5	5	5	5	5
0	0	1	1	2	5	5	1	0	4	1	0	0	3	0	3	1	0	0	0	0	1	1	1
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4	5	7	9	9	9	9	7	7	3	9	3	0	7	0	3	3	0	6	6	6	9	7	5
[illegible]																							
2	3	3	7	8	5	7	3	5	3	0	0	7	7	1	7	1	0	4	0	0	7	4	0
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4	5	7	8	7	9	0	5	5	7	8	6	0	7	7	7	7	0	5	5	7	5	6	6
0	1	0	7	3	2	2	1	0	0	0	0	0	1	0	1		0	2	2	0	0	2	1
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1	2	3	2	6	4	4	1	2	5	3	2	3	1	0	0	0	0	2	2	3	2	4	4
1	1	7	4	2	2	2	1	2	1	1	1	0	1	0	4	7	0	1	0	0	0	0	0
0	0	1	1	1	0	0	2	0	6	3	4	5	3	7	5	4	5	3	0	0	0	5	5
3	4	4	7	7	7	5	7	3	5	7	7	0	7	7	3	3	0	3	5	4	4	6	7
[illegible]																							
5	5	5	9	6	8	8	8	8	6	6	6	0	0	7	8	8	0	7	7	7	6	7	7
2	0	4	5	[illegible]	4	2	2	2	1	1	4	1	0	4	0	0	0	3	2	3	2	3	4

2	1	1	0	0	1	0	0	0	0	0	1	2	0	0	0	0	7	0	0	0	0	0	0
1	3	2	3	2	2	1	1	1	2	4	4	0	0	0	0	0	0	0	0	0	2	0	0
1	1	5	8	8	8	8	5	1	5	5	5	1	0	0	3	5	0	0	1	0	0	0	0
8	9	8	9	9	7	8	7	5	8	9	9	0	70	3	4	0	4	6	8	8	7	9	8
3								4					2	1					3	3		5	5
3	9		9	9	9		9	4					8	3			4	4	7	9			2
4	4	4	4	4	4	4	5	5	4	4	4	4	4	5	5	5	5	3	3	2	2	2	2

Appendix 9 – Univariate Analysis Study IV – Step by Step analysis

Variable	Value	Std.Error	p-value
(Intercept)	22.269808	3.1779737	P<0.001
parent	2.110164	0.9021045	0.02005

Variable	Value	Std.Error	p-value
(Intercept)	25.2866675	3.5179010	P<0.001
greeting	0.7561233	0.8154401	0.35461

Variable	Value	Std.Error	p-value
(Intercept)	24.3918211	3.505568	P<0.001
comfort	0.8077814	0.814360	0.32213

Variable	Value	Std.Error	p-value
(Intercept)	28.2817628	4.1137820	P<0.001
friends	-0.1644126	0.6670985	0.80551

Variable	Value	Std.Error	p-value
(Intercept)	26.5936344	4.4251636	P<0.001
social_rules	0.1853427	0.7029702	0.79224

Variable	Value	Std.Error	p-value
(Intercept)	26.4239013	3.7849563	P<0.001
word_utilisation	0.2595251	0.6702091	0.69890

Variable	Value	Std.Error	p-value
(Intercept)	24.4134058	3.8184330	P<0.001
verbal_instructions	0.7664847	0.7780968	0.32547

Variable	Value	Std.Error	p-value
(Intercept)	26.044005	3.6641116	P<0.001
eye	0.518118	0.9218233	0.57454

Variable	Value	Std.Error	p-value
(Intercept)	17.63134	3.9223333	P<0.001
repetitive	2.58051	0.7736294	0.97009

Variable	Value	Std.Error	p-value
(Intercept)	20.156811	3.7824729	P<0.001
sameness	2.056389	0.7850955	0.00931

Variable	Value	Std.Error	p-value
(Intercept)	18.202121	3.4942286	P<0.001
routine	2.905437	0.7995067	0.00033

Variable	Value	Std.Error	p-value
(Intercept)	27.0713582	2.6718799	P<0.001
Diarrhoea	-0.1301251	0.7815099	0.86789

Variable	Value	Std.Error	p-value
(Intercept)	31.735587	3.4218727	P<0.001
Constipation	-1.119046	0.6954069	0.10875

Variable	Value	Std.Error	p-value
(Intercept)	27.0656649	2.9488893	P<0.001
Appetite	0.3348045	0.7486577	0.65509

Variable	Value	Std.Error	p-value
(Intercept)	31.940579	3.4610308	P<0.001
Bloating	-1.307219	0.7600441	0.08663

Variable	Value	Std.Error	p-value
(Intercept)	28.326840	2.8242489	P<0.001
Flatulence	-0.691903	0.8504919	0.41670

Variable	Value	Std.Error	p-value
(Intercept)	26.757117	2.350903	P<0.001
Vomiting	1.074328	1.262311	0.39550

Variable	Value	Std.Error	p-value
(Intercept)	25.6661145	3.015553	P<0.001
Unhappy	0.9952029	1.070505	0.35339

Variable	Value	Std.Error	p-value
(Intercept)	24.939714	2.9679088	P<0.001
Aggressive	1.131391	0.8934463	0.20649

Variable	Value	Std.Error	p-value
(Intercept)	25.4818082	2.9900487	P<0.001
Destructive	0.7490692	0.8208432	0.36230

Variable	Value	Std.Error	p-value
(Intercept)	24.009535	3.2516380	P<0.001
Non_Interactive	1.153383	0.7934077	0.14723

Variable	Value	Std.Error	p-value
(Intercept)	23.307871	3.9876881	P<0.001
Agitated	1.102448	0.9121076	0.22786

Variable	Value	Std.Error	p-valuee
(Intercept)	20.30515	3.6454420	P<0.001
Disagreeable	2.13007	0.8213322	0.01003

Multivartiate Analysis Study IV – Step by Step analysis

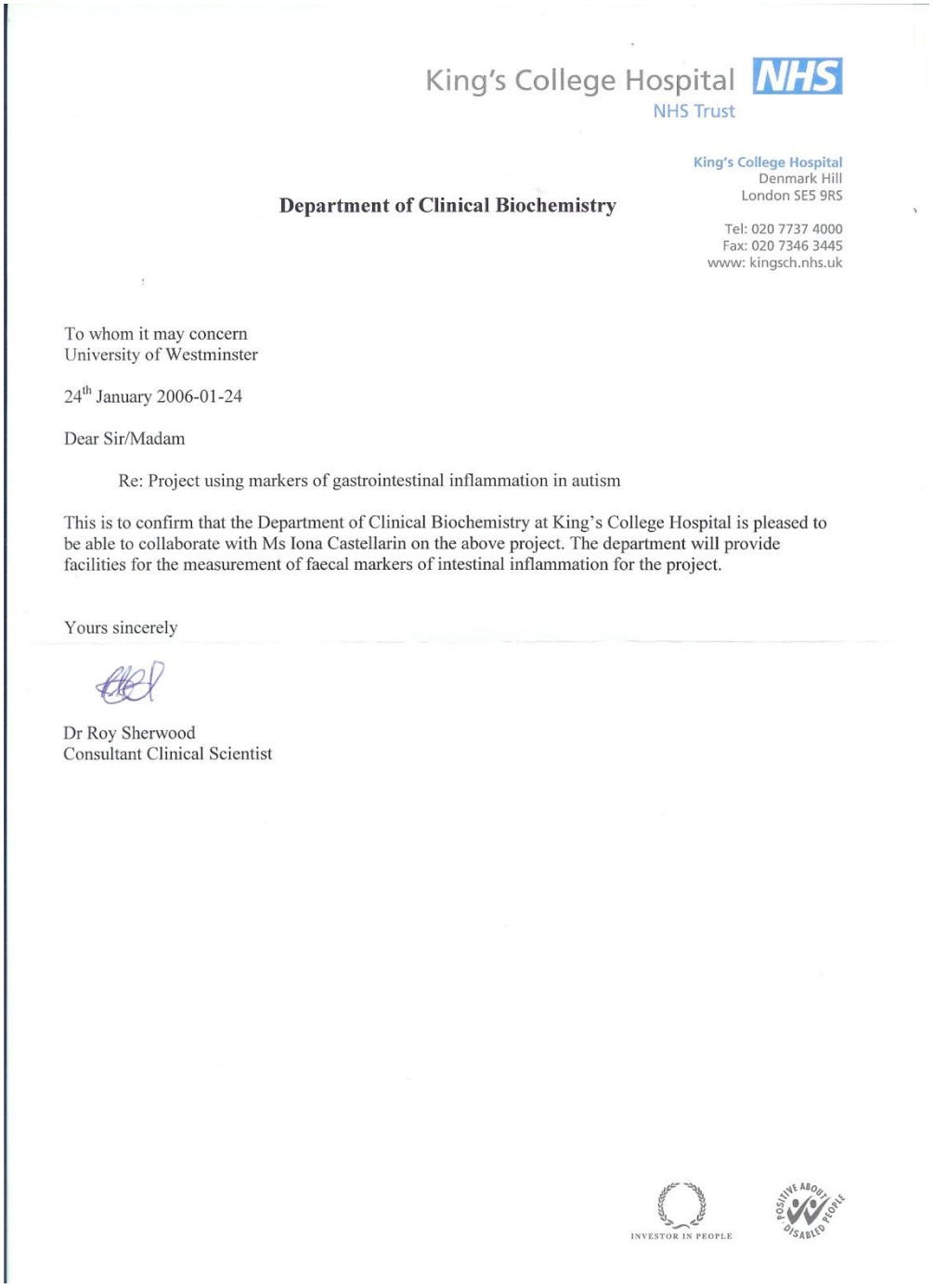
Variable	Value	Std.Error	p-value
(Intercept)	22.9931883	4.8535384	P<0.001
parent	1.2828019	1.1471293	0.26452
repetitive	1.2848881	1.2489797	0.30459
sameness	-2.4204905	1.6967762	0.15496
routine	3.6855561	1.5960845	0.02175
Constipation	-1.2326576	0.8615343	0.15374
Bloating	-0.6784147	0.9253896	0.46418

Final Table

Variable	Value	Std.Error	p-valuee
(Intercept)	22.894636	4.023740	P<0.001
Routine	3.226851	0.813211	0.00009
Constipation	-1.584028	0.69109	0.02269

Appendix 10 – Loss of Samples for Lactoferrin Analysis

All biochemical analysis of samples was performed by the Department of Clinical Biochemistry at King' College Hospital.



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Department of Clinical Biochemistry

To whom it may concern
University of Westminster

24th January 2006-01-24

Dear Sir/Madam

Re: Project using markers of gastrointestinal inflammation in autism

This is to confirm that the Department of Clinical Biochemistry at King's College Hospital is pleased to be able to collaborate with Ms Iona Castellarin on the above project. The department will provide facilities for the measurement of faecal markers of intestinal inflammation for the project.

Yours sincerely

Dr Roy Sherwood
Consultant Clinical Scientist

INVESTOR IN PEOPLE

POSITIVE ABOUT DISABLED PEOPLE

A large number of lactoferrin samples went missing due to a clerical error at the Department of Clinical Biochemistry, King's College Hospital, London, the institution responsible for storing and analysing the samples in this thesis. For this reason, the number of subjects included in the lactoferrin analysis was smaller than for calprotectin or M2-PK.

KingsPath
Clinical Diagnostic Pathology Services
King's College Hospital NHS
NHS Foundation Trust

GSTS Pathology

Clinical Biochemistry Dept
King's College Hospital
Denmark Hill
London SE5 9RS

06 March 2013

Dr Ian Drysdale
British College of Osteopathic Medicine
Fraser House
6 Netherhall Gardens
London
NW3 5RR

Re: Iona Bramati-Castellarin, PhD candidate.

The Department of Clinical Biochemistry at King's College Hospital carried out testing for faecal calprotectin as a marker of GI tract inflammation in a study on treatment of autism as part of the above student's PhD project. A total of 440 samples from 50 patients were analysed between 2008 and May 2011.

Samples were stored in the freezer within the Gastrointestinal function section of the laboratory in case further analysis was required. It was decided by the student and her supervisors in June 2011 to test some samples for M2-PK as an additional marker. Three sets of samples from within the 50 subjects involved in the research were selected to represent the most significant time-points in the sub-set of patients. M2PK - ELISA kits were ordered by BCOM and provided to our lab by University of Westminster in August 2011. The 150 samples used for the M2PK analysis were completed by December 2011 and the 150 samples were placed in distinctive blue bags in the GI section freezer clearly marked with the study number and the assay date. In June 2012 the student and her supervisors decided to test for lactoferrin in these samples. It was not possible to locate 80 samples, corresponding to 25 subjects, from the total of 150 samples tested for M2PK, when it came to testing and it would appear that they had been inappropriately disposed of by personnel working in the GI section at King's although they were clearly told not to throw any of these samples away. It is impossible to ascertain who the individual responsible for disposing of the samples was and therefore it is difficult to take any further action other than to institute a system for careful monitoring and registering of study samples kept in the GI section freezer to prevent any similar occurrence in the future.

As a gesture of goodwill it was agreed that all remaining samples be analysed for both M2PK and lactoferrin at no charge. This entailed 280 samples for M2PK and 352 samples for lactoferrin at a total cost of £7000. It is hoped this will go some way to mitigating the potential adverse effect and the time delay incurred. We apologise that this has happened and that this represents a failure to meet the normal standards in this laboratory.

Yours sincerely

Dr Roy Sherwood, Scientific Director, KingsPath.