An Investigation of Potential Biomarkers in Children with Autism Spectrum Disorder

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Glossary of Abbreviations

Glossary of uncommon terms and general abbreviations used throughout the text are listed below.

3MT 3- methoxytyramine
5-HIAA 5-hydroxyindole acetic acid
5-HT serotonin
8-OHdG 8-hydroxy-2-deoxyguanosine
A adrenaline
AD autistic disorder
ADHD attention deficit hyperactivity disorder
AGAT arginine: glycine amidinotransferase
APGC autism genome project consortium
AS Asperger syndrome
ASD autism spectrum disorder
ASSQ autism spectrum screening questionnaire
BSA bovine serum albumin
CAD collision
CAMs complementary and alternative medicines
CDS creatine deficiency syndrome
CD cluster of differentiation
CE collision energy potentials
CNS central nervous system
CNVs copy number variants
CON community controls
COPRO coproporphyrin
CRTR creatine transporter
CR creatine
CUR curtain
CV coefficient of variation
CXP collision exit potentials
DA dopamine
DMPS 2,3-dimercapto-1-propanesulfonic acid
DOPAC 3,4-dihydroxyphenylacetic acid
DPI dietary protein intolerance
EIRP early intervention research program
ESI electrospray ionisation
FGID functional gastrointestinal disorder
FP focusing potential
FXS fragile X syndrome
GAA guanidinoacetate
g *g*ravity (e.g. 12000 x *g*)
GABA gamma-aminobutryic acid
GAMT guanidinoacetate methyltransferase
GC gas chromatography
GOR gastro-oesophageal reflux
GORD gastro-oesophageal reflux disease

GI	gastrointestinal	**NA**	noradrenaline
GSH	glutathione	**NEB**	nebuliser
HPLC	high-performance liquid chromatography	**PCR**	polymerase chain reaction
		PDD	pervasive developmental disorder
HVA	homovanillic acid	**PMA**	phorbol myristate acetate
HPHPA	3-(3-hydroxyphenyl)-3-hydroxypropionic acid	**PSG**	polysomnography
IAG	indolyl-3-acryloylglycine	**QC**	quality control
IFN	interferon	**RNase**	ribonuclease
IL	interleukin	**ROS**	reactive oxygen specie
IS	internal standard	**S.E.M**	standard error of the mean
LC-MS/MS	liquid chromatography with tandem mass spectrometry	**SCFA**	short chain fatty acids
LNH	lymphoid nodular hyperplasia	**SDS**	sodium dodecyl sulphate
LPS	lipopolysaccharide	**SIB**	typically developmental siblings
MHPG	methoxyhydroxy phenylglycol	**TNF**	tumour necrosis factor
MRI	magnetic resonance imaging	**URO**	uroporphyrin
MMR	measles-mumps-rubella	**VMA**	vanillylmandelic acid

Publication to Support This Thesis

Published Research Papers

Wang L, Angley MT, Gerber JP, Sorich MJ. 2011. *A review of candidate urinary biomarkers for autism spectrum disorder.* Biomarkers (in press)

Wang L, Christophersen CT, Sorich MJ, Gerber, JP, Angley MT & Conlon MA. 2011. *Low Relative Abundances of the Mucolytic Bacterium Akkermansia muciniphila and Bifidobacterium spp. in Feces of Children with Autism.* Applied and Environmental Microbiology 77: 6718-21.

Wang L, Angley MT, Sorich MJ, Young RL, McKinnon RA & Gerber JP. 2010. *Is there a role for routinely screening children with autism spectrum disorder for creatine deficiency syndrome?* Autism Research 3: 268-72.

Wang L, Angley MT, Gerber JP, Young RL, Abarno DV, McKinnon RA, Sorich MJ. 2009. *Is urinary indolyl-3-acryloylglycine a biomarker for autism with gastrointestinal symptoms?* Biomarkers 14: 596-603.

Papers for Submission

Wang L, Christophersen CT, Sorich MJ, Gerber, JP, Angley MT & Conlon MA. *Faecal short chain fatty acid and ammonia are elevated in children with autism spectrum disorder.*

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Published Abstracts of Conference Presentations

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Wang L, Angley MT, Gerber JP, Young RL , Abarno DV, McKinnon RA & Sorich MJ (2007) 'Investigation of the urinary indolyl-3-acryloyglycine (IAG):creatinine ratio in children with autism versus typical siblings: A putative diagnostic marker for autism spectrum disorder?' Australasian Pharmaceutical Sciences Association (APSA) Meeting, Sydney.

Wang L, Angley MT, Gerber JP, Young RL , Abarno DV, McKinnon RA & Sorich MJ (2007) 'Investigation of the urinary indolyl-3-acryloyglycine (IAG):creatinine ratio in children with autism versus typical siblings.' Australasian Scientific and Clinical Experimental Pharmacologists and Toxicologists Meeting, Adelaide.

Summary

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with poorly defined aetiology. Recent well-designed studies using whole-genome scanning methods, cytogenetics and genetic linkage/association analyses indicate genetic factors play a key role in the aetiology of ASD. ASDs carry a greater risk of co-morbidities. Between 6% and 10% of children have a medical condition that might have led to the autistic disorder (e.g. fragile X syndrome, tuberous sclerosis and neurofibromatosis) leaving 90% of the cases to be idiopathic (Fombonne *et al*. 2005). Thus, environmental and epigenetic factors have also been suggested impacting on susceptibility to ASD. Evidence is building that ASD represents a cluster of syndromes that have distinct aetiologies involving inflammation, increased oxidative stress, impaired gastrointestinal (GI) health, mitochondrial dysfunction, autoimmune processes, and impaired ability to neutralise toxins. Currently, ASD diagnosis is based on a set of clinical behaviours. Laboratory-based diagnosis remains elusive.

For the purpose of this study, urine and faecal samples were collected from children with ASD, their siblings and community controls in order to investigate the aetiology and develop a non-invasive laboratory test for the early diagnosis of ASD. Urinary and faecal metabolites were measured, and GI microbiota profiles investigated.

Investigation of urinary biomarkers showed significantly higher levels of urinary indolyl-3-acryloylglycine (IAG) were found in children with ASD experiencing GI disturbance. In contrast, the differences were not observed in combined controls (siblings plus community controls) with and without GI disturbance. Moreover, ASD children with GI disturbance had significantly higher levels of IAG: creatinine than controls with GI disturbance. These findings suggest that the raised IAG may be a specific biomarker to identify children with ASD and GI disturbance.

Creatine deficiency syndrome (CDS) is an inborn error of metabolism which shares some clinical features with ASD. It has been speculated that ASD and CDS have overlapping aetiologies. Urinary levels of creatine and guanidinoacetate were analysed to determine whether CDS biomarkers were raised in the ASD cohort. Although it was found that levels of creatine and guanidinoacetate were similar in ASD and controls, screening of urinary

creatine and guanidinoacetate should still be recommended during routine diagnostic tests for ASD. Early detection of CDS cases can guide implementation of dietary interventions which can dramatically improve behavioural outcomes for affected individuals.

In terms of GI health, 48 h faecal samples were collected from children with and without ASD. Altered faecal fermentation products were found in children with ASD compared with their siblings and community controls. In particular, levels of total short chain fatty acids, propionate, acetate and ammonia were elevated in children with ASD compared with controls. Furthermore, a distinctive GI microbiota profile was revealed in children with ASD. Significantly decreased abundances of *Bifidobacterium spp.* and *Akkermansia muciniphila* were found in ASD children compared with controls. The numbers of the *Bacteroides fragilis* group were significantly greater in ASD children with GI disturbance compared with those without. These results contribute to the knowledge of changes in the GI tract that occur in children with ASD and potentially guide dietary and medical interventions that optimise gut health in this population.

Findings in this thesis indicate that GI factors play an important role in the aetiology of ASD. The potential ASD biomarkers identified in this body of work may enable identification of specific GI subtypes in ASD, guild the early diagnosis, enable development of specific interventions as well as monitor physiological responses to treatments.

Declaration

I declare that:

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university;

To the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged.

_____ Date: _____

Lv Wang

I dedicate this thesis to my parents,
Lilin Wang & Shiqiu Zang,
whose love, support and generosity is endless.

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1 General Introduction

1.1 Overview of Autism Spectrum Disorder

Autism is a neurodevelopmental disorder with an estimated prevalence of approximately 1-2% in the general population. Phenotypic variability between individuals with differing severity suggests that autism is a complex multi-aetiological disorder. Twins and family studies provide evidence to support a genetic basis in the pathophysiology of autism (Trottier *et al.* 1999; Nicolson and Szatmari 2003). However, the lack of complete concordance in identical twins indicates environmental factors also contribute to the aetiology of autism (Horvath *et al.* 1999; Kern and Jones 2006; Slotkin *et al.* 2006; Careaga *et al.* 2010; Main *et al.* 2010; Hallmayer *et al.* 2011). A range of theories have been proposed to account for the deficits in affected individuals from changes in brain structures to metabolic impairment (Kidd 2002).

1.1.1 Definition and prevalence

Autism was first described by Kanner (1943), and presents as a triad of deficits in communication, socialisation and behaviour (Bacchelli and Maestrini 2006). Initially, it was assumed that autism was a rare, seriously handicapping disorder, usually associated with intellectual disability (Rutter 2005). Recently, the definition of autism has been revised and broadened and is considered one of the pervasive developmental disorders (PDDs). Autistic disorder (AD) is classified in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) as one of five related PDDs (APA 2000). The remaining four types are PDD-not otherwise specified (PDD-NOS), Asperger syndrome (AS), childhood disintegrative disorder and Rett disorder. Autism spectrum disorder (ASD) is an umbrella term that is used to represent a broad heterogeneous disorder by collectively grouping AD, AS and PDD-NOS.

A fifth edition of the DSM (DSM-5) (APA 2010) is the currently being developed and is due for publication in 2013. In the DSM-5, ASD will likely incorporate the current diagnoses of AD, childhood disintegrative disorder and PDD-NOS while AS will be eliminated as a separate disorder. It is proposed that ‘dimensional assessments’ will be added to diagnostic evaluations of mental disorders thereby allowing clinicians to evaluate the severity of symptoms and to, take into account ‘crosscutting’ symptoms. Additionally, it is proposed merging the communication and social interaction into a ‘social affect’

description (Gotham *et al.* 2007). Under the proposed new classification, clinicians would rate the severity of clinical presentation of ASD as severe, moderate or mild.

The prevalence of ASD in the 1960s was reported to be only 1 in 2000 (Lotter 1966). More recently, epidemiological studies, however, estimate prevalence to be 4 per 1000 for AD and 12-16 per 1000 for the broader spectrum disorder (Baird *et al.* 2006; Baron-Cohen *et al.* 2009). The Centers for Disease Control and Prevention's most recent data show that between 1 in 80 and 1 in 240 children at 8 years of age have ASD in multiple communities in the United States (CDC, 2010). A report commissioned by the Australian Advisory Board for ASD estimated a 1 in 160 prevalence of ASD across Australia among 6-12 year old children in 2007 (MacDermott *et al.* 2007).

The dramatically increased prevalence of ASD in recent years may be due to several reasons. Firstly, the changing definition of ASD appears to play a key role in the rising prevalence. Although autism was first described in 1943, the formal diagnostic criteria for ASD (e.g. DSM-III) were initially published in 1980 (APA 1980). Thus, diagnoses made prior to this publication may be based on the different criteria. In addition, differences were existed in the diagnostic criteria between the DSM and the International Classification of Disease (ICD) series, another popular classification system at beginning. The various DSM versions (Rice *et al*. 2011) and the diagnostic criteria in the DSM-5 (APA 2010) may also influence the estimated prevalence. Secondly, prevalence estimates are derived in several ways, such as using registry' data, telephonic interviews and questionnaire surveys which may influence how representative the study sample is. Thirdly, environmental factors, such as heavy metal exposure, have been proposed, as an important factor, contributing to the incidence of ASD, however, further studies are required to confirm this. Finally, there is now increased awareness of ASD in the general population which may lead parents to have their children assessed when previously it may have remained undiagnosed.

1.1.2 Diagnosis

Early and proper diagnosis of ASD may facilitate the clinicians and the caregivers of children with ASD to employ appropriate therapeutic (nutritional, pharmacological, environmental) interventions. At present, diagnosis is based on clinical difficulties in communication, socialisation and restricted or repetitive behaviour, using questionnaires and observation-based rating scales, such as the Childhood Autism Rating Scale (Schopler

et al. 1980), DSM-IVRT (APA 2000) or ICD-10 (WHO 1992). These procedures require experienced psychologists and may be quite time-consuming in some cases. Input from a range of professionals in a multi-disciplinary team is essential for confirming a diagnosis of ASD (CCD 2001). The lack of a validated laboratory analytical procedure is a constraint.

A spectrum of symptom severity exists for individuals with ASD, ranging from mild with minimal impact on daily functioning, to severe with significant disruption (Johnson and Myers 2007). Symptoms are also classified under positive symptoms (i.e., the presence of atypical behaviours); negative symptoms (i.e. the absence of developmentally appropriate behaviours); developmental delays (i.e. behaviours that are immature) and splinter skills that are discordant with the overall level of functioning (Volkmar *et al.* 2005).

ASD is associated with a range of co-morbidities. Between 6% and 10% of children have a medical condition that might have lead to AD (e.g. fragile X syndrome, tuberous sclerosis and neurofibromatosis) leaving 90% of the cases to be idiopathic (Fombonne 2005). Moreover, as many as 35% of individuals with AD have epilepsy (Turk *et al.* 2009) and approximately 50% of individuals with ASD have an intelligence quotient in the intellectual disability range (Charman 2008).

The varying symptoms, the inconsistent pathological findings and the unpredictability in response to treatment in ASD indicate the presence of subgroups within the disorder. Each subgroup is likely to have a difference in terms of the aetiology and/or pathophysiology which in turn impacts on an aspect of neurological functioning that leads to the triad of difficulties that characterise ASD i.e. communication, socialisation and behaviour. A number of investigators have proposed that ASD may be more definitively analysed by defining endophenotypes, i.e. determining if subgroups of individuals exist that show predominance of specific autistic symptoms (Viding and Blakemore 2007). However, such strict quantitative measures of impairment are often difficult to obtain in behavioural phenotypes (Smith *et al.* 2009).

Subtyping includes categorical and dimensional systems. Categorical systems are intended to produce homogeneous subgroups that are distinct from other subgroups in terms of common aetiology, symptom presentation and cause. Dimensional systems view particular

subgroups ‘as falling along a continuous spectrum of severity’ (Beglinger and Smith 2001; Beglinger and Smith 2005).

In terms of ASD, the degree of symptom expression is observed to vary over chronological age and developmental level. Even within the same age group, individuals with ASD are also observed to express symptoms of varying characteristics and severity. Thus, the categorical approaches are potentially able to identify distinct, discrete aetiologies within the population of individuals with ASD. By contrast, dimensional systems view AD as one subtype falling along a continuum from normal to impaired functioning individuals (Beglinger and Smith 2001; Beglinger and Smith 2005). The ability to identify more homogenous, discrete subgroups or phenotypes of children with ASD is of benefit if such phenotypes are associated with specific clinical features such as differences in aetiology, treatment response or a predictable prognosis. Furthermore, narrowing down the phenotype in children with ASD may highlight on the underlying aetiology resulting in the observed symptoms, and allow identification of more efficacious treatments targeted at specific subtypes (Tager-Flusberg and Joseph 2003).

Diagnosis of ASD is problematic, as there is no single characteristic developmental deficit or behaviour and many of the features are also present in children with other disorders (Volkmar *et al.* 2005). Recent studies report using Magnetic Resonance Imaging (MRI) to diagnose ASD in adults (n = 20) (Ecker *et al.* 2010) or high-functioning autism (n = 30) (Lange *et al.* 2010) with short time procedure and high accuracy. This diagnosis could help to alleviate the need for the emotional and time consuming diagnostic process of ASD. However, there are concerns regarding the cost of using such a technique and whether it is suitable for children. Furthermore, comparisons of groups of individuals with developmental disorders other than high-functioning autism are needed to determine the specificity of this method for overall ASD diagnosis.

Future investigations that strike a scientifically effective balance between whole-brain exploratory approaches and metabolites analysis in body fluids will result in higher classification ability, reliability, and predictive power. If an earlier diagnosis was possible or a diagnostic marker could confirm specific pathology of an autistic subtype, targeted intervention could improve prognosis.

1.1.3 Neurobiology

Neurological research has revealed numerous abnormalities in the brain of individuals with ASD (Gerlai and Gerlai 2003; Wegiel *et al.* 2010). Neuroimaging approaches such as diffusion tensor imaging and functional MRI have demonstrated disruption of white matter tracts and disconnection between brain regions in ASD (Ecker *et al.* 2010; Lange *et al.* 2010). Post-mortem neuropathological studies show disturbances in neuronal and cortical organization in autistic patients which support the involvement of neuroimmune responses in the pathogenesis of ASD (Pardo and Eberhart 2007).

Stereotypical behaviour, disruption of communication and social behaviour common in ASD indicate that crucial neuroanatomic structures and neurodevelopmental pathways are affected during brain development. However, the exact mechanisms are still unclear. Clinical assessment, neuroimaging and neuropathological studies have been used to assess the structural and morphological brain abnormalities in ASD. One consistent finding in ASD is altered brain growth (Pardo and Eberhart 2007). A sudden and excessive increased head size between 1-2 months and 6-14 months of age (Courchesne 2004; Courchesne *et al.* 2004) is unique to ASD. Most neurodevelopmental disorders, including mental retardation syndromes, are associated with a reduced brain volume (Nicolson and Szatmari 2003). Perry and colleges (2001) reported a three fold increase in the level of brain-derived growth factor in the forebrain of ASD which suggested a potential for intervention based on cholinergic receptor modulation in ASD.

Moreover, the diverse range of symptoms expressed by individuals with ASD suggests that the disorder affects multiple sites or mechanisms within the brain and central nervous system (CNS). Specific areas in the brain that have shown abnormalities in neuropathological and neuroimaging findings in individuals with ASD include the cerebellum, brain stem and cortex (Piek and Dyck 2004). Increased cell density and smaller neuronal size in the hippocampus and amygdala and decreased Purkinje cells throughout the cerebellum have been associated with ASD (Palmen *et al.* 2004). Purkinje cells are responsible for sending outgoing signals to areas within the cerebellum, which may lead to processing difficulties (Kern *et al.* 2006). The cerebellum also serves a role in sensory modulation and the integration of activity within the cerebral cortex (Kern *et al.* 2006). Other researchers have suggested that a lack of connectivity between brain regions,

possibly due to an undersized corpus callosum which connects the brain hemispheres, may result in problems with integration of activity for individuals with ASD (Piek and Dyck 2004).

Two dysfunctional neurotransmitter systems, the serotonergic and glutamatergic system, have also been implicated in ASD (Gerlai and Gerlai 2003; McDougle *et al.* 2005). The serotonergic system is associated with appetite, mood and arousal and the glutamatergic system plays a crucial role in information processing and memory formation (Pardo and Eberhart 2007; Zafeiriou *et al.* 2009).

These neurobiological alterations likely affect social behaviour and communication and also appear to be influenced by both genetic and environmental factors. It is hypothesised that exposure to neurotoxins (Bolte 1998) and potential environmental pollutants (Adams *et al.* 2009) may disrupt neurodevelopmental pathways resulting in alterations of behaviours such as those that occur in ASD. Future studies will need to examine the cerebral regions which may be intimately involved in the development of social, communication and motor abilities that are impaired in ASD. Longitudinal studies will aid in understanding the pattern of brain development in ASD.

1.1.4 Genetics

Various studies including family and twins studies suggested high heritability in ASD, but the forms of the genetic mutations contributing to ASD and autistic susceptibility are very heterogeneous (Trottier *et al.* 1999; Nicolson and Szatmari 2003; Muhle *et al.* 2004; Rutter 2005; Gupta and State 2006; Bacchelli and Maestrini 2006; Herbert *et al.* 2006). Although, a recent study on the proband concordance in twin pairs with ASD by Hallmayer *et al* (2011) suggested genetic factors are of substantially lower significance than estimates from prior twin studies of autism. Eventual identification of the responsible genes will not only help the further understanding of the biologic pathways and mechanisms leading to ASD, but will also inform molecular diagnostics and novel therapeutic interventions. The typical approaches used to understand the genetics of ASD include linkage, association, cytogenetic and copy number variants (CNVs) studies.

Genetic linkage studies are designed to identify regions of the genome that are shared between affected family members. The genetic markers that are used may cover the entire

set of chromosomes or correspond to specific chromosomal regions or single genes. Several studies have successfully identified significant linkage on Xp22.23 in ASD (Laumonnier *et al.* 2004) and additional ASD loci, including the methyl CpG binding protein 2 (MECP2) (Moretti and Zoghbi 2006) and contactin-associated protein-like 2 (CNTNAP2) (Strauss *et al.* 2006) genes.

Other studies are suggestive to significant evidence of linkage on almost all chromosomes. However, only a few loci (i.e. 2q24-2q31, 7q and 17q11-17q21) have been replicated (Abrahams and Geschwind 2008). The Autism Genome Project Consortium (AGPC) investigated about 1160 multiplex ASD families using a genome-wide screen with 10000 markers and found suggestive evidence of linkage at 11p12-p13 (Szatmari *et al.* 2007).

Association studies examine differences in allele or genotype frequencies between two groups. The case-control association compares affected individuals with healthy controls from the general population. Family-based association utilise unaffected parents as controls for their affected offspring which avoids the potential confounding effects of population stratification. The selected genetic markers for association analyses correspond to small chromosomal regions or single genes which may be in a significant ASD linkage region, or involved in a metabolic pathway of ASD. Alternatively, it may relate to brain development (Pardo and Eberhart 2007). A number of candidate genes have been tested for association including homeobox protein engrailed-2 (EN2) (Benayed *et al.* 2005), Reelin (Serajee *et al.* 2006) and neuronal cell adhesion molecule (NRCAM) (Bonora *et al.* 2005), but findings were inconclusive.

Traditional cytogenetic analysis using G-banding techniques and fluorescent *in situ* hybridisation can be used to identify the underlying genetic causes of neurodevelopmental disorders. Several review papers have summarised the published studies examining cytogenetic analyses and reported that the overall rate of chromosomal abnormalities in ASD is approximately 3-7% (Veenstra-Vanderweele *et al.* 2004; Xu *et al.* 2004; Vorstman *et al.* 2006). Some common chromosomal abnormalities included:15q11-13 (Hogart *et al.* 2010), 2q37 deletion (Stein *et al.* 1992; Aldred *et al.* 2004; Casas *et al.* 2004) and 22q11 deletion (Niklasson *et al.* 2009). Moreover, the large chromosomal abnormalities have been mentioned in ASD include duplication 7q11, 17q11 deletion, trisomy 21 and

monosomy X (Zafeiriou *et al.* 2007).The Autism Chromosome Rearrangement Database, catalogues known chromosomal abnormalities in ASD and can be accessed.

CNVs have been associated with specific chromosomal rearrangements and genomic disorders. Since known CNVs lead to over 15% of the assembled human genome sequence, CNVs may account for a significant proportion of normal phenotypic variation (Freeman *et al.* 2006; Estivill and Armengol 2007). Recent reports have suggested CNVs are strongly correlate with ASD (Jacquemont *et al.* 2006; Marshall *et al.* 2008; Cusco *et al.* 2009). A specific CNV at 16p11.2 was found in about 1% of individuals with ASD (Ballif *et al.* 2007; Ghebranious *et al.* 2007; Weiss *et al.* 2008). The Autism Genome Project Consortium identified 624 CNVs in ASD, including 222 deletions and 402 duplications (Szatmari *et al.* 2007). Furthermore, one study demonstrated that submicroscopic to microscopic CNVs with *de novo* germline variants were found more often in ASD families (10% of cases) than in controls (1%) (Sebat *et al.* 2007) which was consistent with findings from cytogenetics and established *de novo* CNVs as a significant risk factor for ASD. In addition, two more recent papers confirmed several loci previously observed and reported some new candidate regions, adding substantially to the list of potential gene targets (Gai *et al.* 2011; Levy *et al.* 2011).

1.1.5 Metabolic aetiology

The lack of complete concordance between monozygotic twins with ASD provides compelling evidence that ASD does not have an exclusively genetic aetiology and suggests that both environmental and epigenetic factors impact on susceptibility to ASD (Persico and Bourgeron 2006; Hallmayer *et al*. 2011). Features such as gastrointestinal (GI) factors, immune dysfunction, oxidative stress, heavy metal toxicity and others have been implicated in the pathogenesis of subtypes of ASD.

The role of GI disturbance in the aetiology of ASD has recently attracted considerable attention although the prevalence described in published studies varies from 9% to 91% (Buie *et al.* 2010). Campbell *et al.* (2009) provided genetic evidence to strengthen the link between GI dysfunction and ASD. Altered GI microbiota (Finegold *et al.* 2002; Song *et al.* 2004; Parracho *et al.* 2005; Finegold *et al.* 2010) and their fermentation products (Yap *et al.* 2010; Altieri *et al.* 2011) have been reported in ASD compared with controls.

Many theories have been proposed to explain the link between impaired GI function and disturbance of neurological development and/or function resulting in ASD. Inflammatory and immune changes may cause increased intestinal permeability (i.e. leaky gut) (D'Eufemia *et al.* 1996; de Magistris *et al.* 2010). Excess dietary-derived opioid peptides may pass through the 'leaky gut' to enter systemic circulation and across blood-brain barrier into the brain and exert certain opioid effects which manifest in symptoms of ASD (Opioid Excess Theory, Reichelt *et al.* 1997). Also, GI overgrowth of neurotoxic bacteria (Bolte 1998; Finegold 2008) have been suggested to play a role in the aetiology of ASD. Since this is a major focus of the thesis, a thorough review of the available literature examining GI factors in ASD is presented in Chapter 2.

In addition to GI disturbance, there is growing evidence that abnormal immunological function, such as cytokines, immunoglobulins, inflammation, and cellular activation, is associated with ASD (Goines and Van de Water 2010). Systemic immunologic aberrations in ASD have been linked with both autoimmunity, antibodies reactive for CNS proteins with the potential for neuronal tissue destruction, and with dysfunctional immunity such as abnormalities or deficits of function in immune cell subsets. The disruption in immune response may lead to an inappropriate or ineffective immune response to pathogen challenge (Ashwood and Van de Water 2004).

Another factor emerging in recent years provides support that oxidative stress plays a vital role of in individuals with ASD, such as increased lipid peroxidation (Ming *et al.* 2005), altered antioxidant enzymes in plasma (Yorbik *et al.* 2002), mitochondrial dysfunction (Oliveira *et al.* 2005) and genetic factors (Cohen *et al.* 2003; Hovatta *et al.* 2005). Under normal conditions, a dynamic equilibrium exists between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cell. Oxidative stress occurs when ROS levels exceed the antioxidant capacity of a cell. The brain makes up about 2% of body mass but consumes 20% of metabolic oxygen. Thus, it is highly vulnerable to oxidative stress due to its limited antioxidant capacity and this may lead to neurologic deficits, such as ASD, in the early stage of childhood (Zecavati and Spence 2009).

Heavy metals, such as arsenic, lead, and mercury have been associated with a variety of neurologic deficits and disorders (Zecavati and Spence 2009). An impaired glutathione redox ratio observed in children with ASD (James *et al.* 2004) may be involved in the

aetiology of ASD by delaying the clearance of heavy metals from the body (Deth *et al.* 2008). However, the prevalence of an impaired glutathione redox ratio for individuals with ASD is uncertain. One study (James *et al.* 2009) found that in a sample of children with AD (n = 65), 75% had impaired *S*-adenosylmethionine: *S*-adenosylhomocysteine (SAM:SAH) or glutathione: oxidized glutathione (GSH: GSSG).

The association between heavy metal exposure and ASD, in particular mercury, has attracted considerable interest (Counter *et al.* 2002; Holmes *et al.* 2003; Palmer *et al.* 2006; Geier and Geier 2007). Several studies have suggested that mercury could disrupt neurotransmitter levels and biochemistry (Faustman *et al.* 2000; Redwood *et al.* 2001; Bernard *et al.* 2002) and impact on normal childhood development. Although there is no direct evidence linking mercury and ASD, mercury toxicity has been implicated in immune, sensory, neurological, motor, and behavioural dysfunction, all of which can also occur in ASD.

One remaining factor warranting mention is the possible link between ASD and vaccination. A controversial study by Wakefied *et al* (1998) (retracted on 2nd Feb 2010, Lancet) proposed the Measles-Mumps-Rubella (MMR) vaccine contributes to the aetiology of ASD. It was suggested that the MMR vaccine led to the measles virus lodging in children's intestines, releasing toxins and causing a 'leaky gut' in turn leading to ASD (Wakefield *et al.* 1998). This report provoked widespread debate and was ultimately shown to be without basis. Another hypothesis related to immunisation is that children with ASD have immune abnormalities that make them vulnerable to vaccine-induced immune-mediated CNS damage (Chez *et al.* 2004). However, an array of epidemiological studies conducted in multiple populations in Finland, Denmark, the USA, Japan, Sweden and Great Britain, do not support causality (Chez *et al.* 2004; Smeeth *et al*. 2004; Burgess *et al.* 2006; Richler *et al.* 2006).

Given the heterogeneity in the aetiology of ASD, there is great interest in identifying a biochemical marker or biomarker set enabling simple and objective diagnosis. All of the environmental and epigenetic factors described above result in impaired or modified metabolism. Therefore, a number of urinary chemicals have been studied for differences between children with and without ASD. A thorough literature review of the available literature examining potential urinary biomarkers in ASD is presented in Chapter 3.

1.1.6 Complementary and alternative medicines

Complementary and alternative medicines (CAMs) are defined by Ernst (2000) as "diagnosis, treatment and/or prevention which complements mainstream medicine by contributing to a common whole, by satisfying a demand not met by orthodoxy or by diversifying the conceptual frameworks of medicine". The use of CAMs is growing and is widespread in the general population, particularly in children with chronic illness or disability (Mamtani and Cimino 2002; Hyman and Levy 2005; Sinha and Efron 2005).

Although mechanistic evidence is not consistent or well developed at this time, a variety of CAMs are used in children with ASD with the intent to ameliorate the range of theorised biochemical abnormalities (Levy and Hyman 2005). CAMs are used in the hope of eliminating the cause or symptoms of ASD. Dietary modification and vitamin supplementation are the most frequent nutritional based therapies to be used in children with ASD (Brown *et al.* 2005). Several studies demonstrate that approximately 16-60% children with ASD have used dietary interventions and/or vitamin supplements at different stages (Levy and Hyman 2002; Levy and Hyman 2003; Witwer and Lecavalier 2005; Green *et al.* 2006). However, scientistic evidence is still lacking to support the wide range of interventions used. In particular, only limited evidence is available for the potential efficacy of popular diets such as the gluten- and casein-free diets (Millward *et al.* 2008; Whiteley *et al.* 2010), which largely remain in need of investigation. Additionally, most CAMs show the potential adverse effects. Therefore, it should be highlighted that implementation of CAMs is not risk-free although they are based on natural remedies or products (Hyman and Levy 2005).

1.2 Metabolomics

Metabolomics is an emerging field of 'omics' research that is widely applied in areas of nutrition, endocrinology, genetics, biotechnology, cytology, neurology, paediatric gastroenterology, chemistry, biochemistry, public health, risk assessment, agriculture and ecology (Krastanov 2010). Metabolomics focuses on exhaustive profiling of metabolites in organisms and is expected to be a powerful tool for functional analysis which could define the phenotype and bridge the genotype to phenotype gap (Krastanov 2010). Metabolomic

analysis methods such as targeted metabolite analysis, metabolic profiling and metabolite fingerprinting have the potential to improve the diagnosis and prognosis of diseases (Lindon *et al.* 2004; German *et al.* 2005; Nordstrom and Lewensohn 2010; Yap *et al.* 2010).

Targeted metabolite analysis refers to the detection and precise quantification of a single or small set of targeted compounds. It is known that abnormal gene expression and environmental stressors cause changes in biological systems which are reflected as altered metabolite profiles in body fluids. Ideally, the specific metabolites will leak or are excreted into body fluids, such as urine, where they can be quantified as potential biomarkers (Nordstrom and Lewensohn 2010).

A metabolite profile is a set of metabolites or derivatives related to a specific metabolic pathway(s) (Villas-Boas *et al.* 2005; Krastanov 2010). Metabolite profiling has been widely used to understand and predict diseases and the response to chemicals. Although, metabolite profiles are globally robust, they are also locally variable owing to environmental factors. For example, metabolite profiles might differ from species to species due to the species-specific metabolic network of biological organism (Goodacre *et al.* 2004). In contrast to metabolite profile analysis, metabolite fingerprinting is used for complete metabolome comparison without the need for compound identification (Krastanov 2010). Both of the two methods look for differences between cases and controls to discover the involvement of unexpected substances, provide new targets for drug treatment, guide nutritional approaches in health care, define a healthy phenotype as well as guide metabolic balance (German *et al.* 2002; German *et al.* 2005; Gibney *et al.* 2005; Clayton *et al.* 2006).

Interest in exploring abnormal metabolite profiles in biofluids, mostly blood and urine, of individuals with ASD have been increasing in recent years. Many studies have reported abnormal individual metabolites in children with ASD compared with controls. Examples include, *tran*-indolyl-3-acryloylglycine (Bull *et al.* 2003), neopterin (Messahel *et al.* 1998), methionine (James *et al.* 2004), or metabolite profiles (Geier and Geier 2006; Nataf *et al.* 2006; Yap *et al.* 2010). These altered metabolites are potential biomarkers to identify the phenotype of ASD, particularly to help ASD subtyping and assist in early diagnosis and intervention.

Metabolomics is a modern approach for studying *in vivo* metabolic profiles. It provides information on disease aetiology and facilitates investigation of the functional consequences of genetic variation. However, several factors like age, gender, nutritional status, and time of sampling may affect the metabolite composition of chosen biofluids. Changes in the physiologically normal metabolic patterns could potentially mask molecular changes caused by the disease(s). The heterogeneity of many diseases also poses a problem and choosing different control samples may give inconsistent results (Nordstrom and Lewensohn 2010). Future work on metabolomics should include and consider more background information. Since, metabolomics typically uses nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry-based techniques, the obtained data are multivariate and hence complex. Summarising, capturing and validating these data are difficult but essential.

1.3 Hypotheses and Aims

1.3.1 Hypotheses

- That specific urinary metabolites, faecal large bowel fermentation products as well as selected GI bacteria are altered in children with ASD compared with controls.
- Particular metabolite(s) or bacteria exist that are potential biomarkers indicating GI pathology involved in the aetiology of ASD.

1.3.2 Aims

- To review and summarise available literature examining GI disturbance in ASD.
- To review and summarise available literature where urinary metabolites have been determined in ASD and compared with controls, and identify the most promising candidates for ASD metabotyping.
- To develop and subsequently utilise methods such as liquid chromatography with tandem mass spectrometry (LC-MS/MS) to compare the concentrations of targeted urinary compounds between children with ASD, their typically developing siblings (SIB) and community controls (CON).

- To analyse and compare large bowel fermentation products, including: short chain fatty acids (SCFA), phenol, total *p*-cresol and ammonia levels in faecal samples from ASD, SIB and CON participants.

- To investigate targeted GI bacteria in children with and without ASD and determine if an association exists between the presence of functional GI disorders (FGID) in participants and presentation and abundance of GI bacteria.

- To compare the frequency and nature of GI disturbance between children with and without ASD and determine whether the presence of GI disturbance correlates with levels of targeted urinary and faecal metabolites.

2 A Literature Review of Gastrointestinal Factors in Autism Spectrum Disorder

2.1 Introduction

Neurological diseases and syndromes are widely associated with a variety of GI symptoms (Altaf and Sood 2008) and abnormal behaviours are frequently reported in patients with GI disorders (Drossman *et al.* 1999).The enteric nervous system is located within the wall of the GI tract. It contains as many as one billion neurons, which has many features in common with the CNS and can be considered as a 'mini-brain' or 'brain' in the gut (Van Oudenhove *et al.* 2004; Altaf and Sood 2008; Sullivan 2008). Research using modern brain imaging techniques has shown a considerable overlap between regions involved in the processing of visceral sensation and regions important for emotional regulation (Larsen *et al.* 2010), suggesting emotional state has an important influence on the function of the GI tract and *vice versa* (Van Oudenhove *et al.* 2004).

A clinical study observed a 49% prevalence of GI disorders in individuals with PDD (age range 19-72 years) compared with 8% in non-PDD individuals (age range 19-62 years) (Galli-Carminati *et al.* 2006). Although neurological symptoms are the most striking features in ASD, individuals with ASD also often carry biological co-morbidities related to the GI system, such as functional gastrointestinal disorders (FGIDs), food intolerance/allergies, vitamin deficiencies, and impaired ability to detoxify or vulnerability to toxic exposure. An emerging literature suggests that individuals with ASD who experienced GI symptoms may be at higher risk for behavioural problem than those with ASD but do not have GI symptoms (Buie *et al.* 2010).

Although a high frequency of GI disturbance occurring in individuals with ASD was first reported 40 years ago (Goodwin *et al.* 1971; Walker-Smith and Andrews 1972), data are conflicting. High rates of FGIDs in individuals with ASD have been reported in several studies including abdominal pain, constipation, diarrhoea, diarrhoea and alternating constipation and GI inflammation (Horvath and Perman 2002). A prospective study by Valicenti-McDermott *et al.* (2006) reported an increased prevalence of GI conditions in children with ASD (n = 50, 70%) compared with matched controls that included both neurotypical children (n = 50, 28%) and those with non-ASD developmental disorders (n = 50, 42%). However, a recent well-designed population-based study by Ibrahim *et al.* (2009) found that the overall incidence of GI symptoms did not differ between cases of autism and controls. Buie and colleagues (2010) have reviewed available literature and

produced consensus guidelines for evaluation and treatment of common GI problems in children with ASD, but general diagnostic criteria of GI symptoms for individuals with ASD are not yet available.

Many studies have tried to explain the link between impaired GI function and neurological development in ASD. Besides the 'Opioid Excess Theory' and overgrowth of GI bacteria, another mechanism suggests that GI disturbance can precipitate underlying malabsorption of essential nutrients and decreased digestive enzyme activity. This may result in brain malfunction, which potentially manifests as a developmental disorder (Genuis and Bouchard 2010). Levitsky and Strupp (1995) indicated either pre- or post-natal malnutrition could lead to abnormal brain concentrations of monoamines which were commonly found in individuals with ASD. Wakefield *et al* (1998) had advised that the MMR vaccine could increase the risk of ASD in children and postulated a GI-based mechanism. However, multiple other studies have discounted the link between vaccination and ASD (Fombonne and Chakrabarti 2001; Taylor *et al.* 2002).

2.2 Common Functional Gastrointestinal Disorders

2.2.1 Constipation

Constipation is a relatively common problem which presents more often in children with neurodevelopmental disabilities (Elawad and Sullivan 2001). This may be because of the inherent problems encountered in toilet training which may also appear in children with ASD. Other factors, including abnormal bowel motility, certain neurological lesions, and low fibre or fluid diets may lead to constipation. Further, the use of anticonvulsant, opioid, antispasmodic, antihistamine or aluminium antacid medications in children may predispose them to constipation (Sullivan 2008).

Previous studies suggested that the prevalence of constipation in children with ASD ranged from 9% to 45%, while the prevalence of constipation of age-matched control was from 3% to 38% (Fombonne and Chakrabarti 2001; Taylor *et al.* 2002; Afzal *et al.* 2003; Molloy and Manning-Courtney 2003; Loening-Baucke 2005; Parracho *et al.* 2005;

Valicenti-McDermott *et al*. 2006). Compared to the general population, a higher frequency of ASD children attended a Constipation Clinic (Pang and Croaker 2010). The authors claimed that children with ASD have an earlier onset of and a longer history of constipation which indicated that constipation was intrinsic rather than secondary to observed behavioural changes of ASD.

Afzal *et al* (2003) reported that constipation was a particularly common finding in children with ASD after examining abdominal radiographs of 103 children with ASD and suggested effective management of constipation would provide general benefit. Also, the authors claimed that milk intake was a primary predictor of constipation. Moreover, findings of colonic and duodenal immunopathology (Furlano *et al.* 2001; Torrente *et al.* 2002) indicates constipation is a more specific disturbance of GI motility in children with regressive autism.

A recent population-based study confirmed a significantly higher incidence of constipation in children with ASD than controls but the authors attributed these to a neurobehavioral basis rather than GI pathology as constipation has a behavioural aetiology (Ibrahim *et al.* 2009). Whether constipation in ASD is a primary feature, a result of a poor diet or just toilet training problems requires more investigation.

2.2.2 Diarrhoea, abdominal pain and gastro-oesophageal reflux

Other than constipation, other FGIDs, such as diarrhoea, abdominal pain and gastro-oesophageal reflux (GOR) have been reported in individuals with ASD. A consensus report revealed the prevalence of diarrhoea or unformed stool in individuals with ASD ranged from 3 to 76% based on multiple studies (Buie *et al.* 2010). It was suggested that the causes of diarrhoea in children with ASD are likely to be the same as in the general population and may be due to functional disorders, malabsorption syndromes, inflammatory bowel disease or chronic infections (Haas *et al.* 2007).

Chronic abdominal pain is defined as intermittent or constant abdominal pain that exceeds 1 or 2 months in duration. Studies have reported the incidence of abdominal pain in ASD to be between 2% and 47% (Fombonne and Chakrabarti 2001; Lightdale *et al*. 2001; Horvath and Perman 2002; Molloy and Manning-Courtney 2003; Parracho *et al*. 2010).

Children with ASD who have limited communication skills may express abdominal pain in an atypical manner, which may partially explain the variability is prevalence reported.

Evidence indicates that children with neurodevelopmental disorders may be predisposed to gastro-oesophageal disease (GORD) (Harrington *et al*. 2004) and one study has revealed a high prevalence of GORD (25/36) in children with AD (Horvath *et al*. 1999). It was suggested that GORD results from abnormal GI motility, which may be caused by malabsorption, food allergies or intolerance (Staiano *et al*. 2004), which are all reported in ASD.

2.2.3 Increased gut permeability

Few studies have directly examined the GI permeability of individuals with ASD. One study demonstrated a significantly higher mean lactulose recovery ($p < 0.001$) in a small group of children with AD (n = 21) compared with controls (n = 40) after the sugar intestinal permeability test (D'Eufemia *et al.* 1996). This is indicative of increased small intestine permeability. Moreover, a study by Boukthir *et al.* (2004) reported intestinal hyperpermeability as being present in some cases of autism. Recently, de Magistris *et al.* (2010) performed the lactulose test in children with ASD (n = 90), their first-degree relatives (n = 146) and healthy controls (64 children and 146 adults) to investigate the 'leaky gut' hypothesis. The authors confirmed a high percentage of children with ASD and their first-degree relatives had increased gut permeability compared with normal controls. However, Robertson and colleagues (2008) reported no abnormal permeability to be present in children with ASD. Although it is not clear, it is possible that they did not exclude children who were implementing the casein-/gluten-free dietary intervention and thus permeability changes had been resolved.

Several factors, including degree of malnutrition, have been proposed to lead to gut permeability in individuals with ASD. Evidence from animal studies revealed that both starvation and stress independently lead to deterioration of the intestinal barrier (Lochs 2005). Other factors which may damage and increase GI tract penetration, such as some GI bacteria, their toxic fermentation products and related immune reactions, will be discussed in later sections of this chapter.

2.3 Gut Microbiota

The human large intestine harbours a great number of microorganisms. A well-balanced GI microbacterial population has a profound influence on the nutritional (O'Keefe 2008), immunological (Sokol *et al.* 2008), and protective processes of the host (Berg 1996; Falk *et al.* 1998; Sartor 2008). Alterations in the GI microbiota could drive intestinal inflammation (McKay 1999), increase gut permeability (Bjarnason *et al.* 1995), cause food allergies (Ojetti *et al.* 2009) and change GI pH which in turn influences digestive enzyme production and action (Garvey 2002).

Previous GI microbiota studies (Finegold *et al.* 2002; Song *et al.* 2004; Parracho *et al.* 2005; Finegold *et al.* 2010) (Table 2.1) have revealed altered GI microbiota profiles in ASD compared with controls implicating altered GI microbiota in the pathophysiology of ASD. The following section discusses several GI bacteria or bacterial species that have previously been shown to be altered in children with ASD as well as others that are emerging as important markers of GI health but have not yet been measured in ASD.

2.3.1 *Clostridium spp.*

Clostridia is an extremely heterogeneous class of bacteria, with many species phylogenetically intermixed with other spore-forming and non-spore-forming genera. Within the class of *Clostridia* is the genera *Clostridium*. The genera comprise a large range of bacteria and they are therefore often referred to in their clusters, such as *Clostridium cluster I, XI* and *XIV*. The clusters are based on their phylogenetic taxonomy/clustering and therefore these clusters do not refer to functionality even though bacteria clustering together often have similar functions.

Table 2.1 GI microbiota reported to be altered in children with ASD

Participants	Methods	Results	Reference
ASD(n = 13) Controls (n = 8)	Culture and PCR sequencing	There were 23/25 species of *Clostridium* and 5/6 *Ruminococcal* species in faeces of children with regressive autism; 15/25 species of *Clostridium* and 5/6 *Ruminococcal* species were found in controls. There are 9 species of *Clostridium* only in children with regressive autism and 3 species just found in controls.	**(Finegold *et al.* 2002)**
ASD (n = 15) Controls (n = 8)	Real-time PCR	The cell count of *C.bolteae* ($(1.8 \pm 0.1) \times 10^5$ vs $(3.9 \pm 0.3) \times 10^3$, p = 0.01), *C.cluster I* ($(3.7 \pm 0.4) \times 10^6$ vs $(4.1 \pm 0.3) \times 10^5$, p = 0.014) and *C.cluster XI* ($(1.4 \pm 0.1) \times 10^7$ vs $(4.0 \pm 0.4) \times 10^6$, p = 0.004) were significantly higher in children with ASD compared with controls, but not for *C. cluster XIVab.*	**(Song *et al.* 2004)**
ASD (n = 48) Siblings (n = 12) Unrelated group (n= 10)	Fluorescence *in situ* hybridization	A higher incidence of the *C. histolyticum* group (*C.clusters I and II*) of children with ASD than unrelated healthy children and siblings ($p < 0.01$ & $p < 0.05$, respectively).	**(Parracho *et al.* 2005)**
ASD (n = 33) Siblings (n = 7) Controls (n = 8)	Pyrosequencing	*Bacteroidetes*, *Firmicutes*, *Actinobacterium* and *Proteobacterium* phyla percentages showed significant differences between severe ASD and controls (51.25 ± 7.04 vs 30.23 ± 16.41%, p = 0.001, 38.02 ± 13.77 vs 63.63 ± 17.59%, p = 0.001, 0.46 ± 0.60 vs 1.81 ± 1.68%, p = 0.012, 3.12 ± 2.58 vs 0.54 ± 0.43%, p = 0.011, respectively). Significantly higher frequency of *Bifidobacterium* genus was described in controls than severe autism (0.409 vs 0.258, p = 0.05), particularly higher levels of *B. longum* (0.636 ± 0.96 vs 0.084 ± 0.15, p = 0.037) and *B. angulatum* (0.046 ± 0.089 vs 0.00 ± 0.00, p = 0.05) were found in controls. *Desulfovibrio* genus showed significantly higher percentages in severe ASD than in controls (0.28 vs 0.03, p = 0.01).	**(Finegold *et al.* 2010)**

ASD, autism spectrum disorder

Significantly higher populations of several *Clostridium* species have been detected in children with ASD compared with controls (Finegold *et al.* 2002; Song *et al.* 2004; Parracho *et al.* 2005; Finegold *et al.* 2010). Altered urinary levels of total *p*-cresol (Altieri *et al.* 2011) or *p*-cresol sulfate (Yap *et al.* 2010) and 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) (Shaw 2010), have been observed in children with ASD compared to controls. These chemicals may be produced by *Clostridium spp.*. Bolte (1998) hypothesised that *C. tetani* colonising the GI tract could release neurotoxins that may contribute to behavioural deficits in some children with ASD. *C. tetani* produces tetanolysin, an oxygen-labile haemolysin, which promotes the growth of the organism by damaging healthy tissue (Bleck 1991) and thereby may increase gut permeability.

An open-label trial of oral vancomycin (antibiotic) conducted in 11 children with ASD resulted in short-term behavioural improvements which were attributed to the antibiotic temporarily eliminating the neurotoxin-producing pathogen (Sandler *et al.* 2000). However, use of antibiotics also disrupts the indigenous intestinal microbiota and allows colonisation of opportunistic pathogens. A history of multiple courses of antibiotic therapy is common in individuals with ASD, raising questions regarding the composition and stability of the GI microbiota (Parracho *et al.* 2005). Anecdotal data indicate high usage of antibiotics may be a trigger for autistic susceptibility. Relapse of autistic behaviours after cessation of oral vancomycin may have been due to the persistence of some *Clostridium spp*. after therapy (Sandler *et al.* 2000). Greater numbers of *Clostridium difficile* have frequently been found in hospital patients on antibiotics (Fekety *et al.* 1981). *C. difficile* belongs to the *C. cluster XI* (Collins *et al.* 1994) and significantly greater numbers of *C. cluster XI* have been demonstrated in children with ASD compared with controls (Song *et al.* 2004). This study did not indicate whether participants had received antibiotics during sample collection. The nature of recolonisation of the gut following acute and chronic antibiotic administration requires further investigation as limited information currently exists. Further clinical trials and follow-up studies examining bowel recolonisation following antibiotic treatment in the general population and specifically in individuals with ASD are required.

Some other *Clostridium spp.,* such as *Clostridium leptum* group and *Clostridium coccoides* group produce butyrate which has multiple functions thought beneficial for GI health (Lambert *et al.* 2002). Significantly decreased numbers of *C. leptum* and *C. coccoides*

groups were reported in faeces of patients with GI diseases, such as Crohn's disease and ulcerative colitis, compared with controls (Sokol *et al.* 2009). Similar faecal levels of *C. coccoides*, however, have been found in children with and without ASD (Song *et al.* 2004; Parracho *et al.* 2005). *Clostridium spp.* is extremely heterogeneous and includes many species with different structure and function. An altered *Clostridial* profile has been shown in ASD compared with controls. It is therefore important to be mindful that results may be misinterpreted when examining the *Clostridial* group as a whole, and only studies that examine specific *Clostridial* strains will be able to determine exactly where true differences (if any) lie.

2.3.2 *Bacteroides*

A recent paper using a pyrosequencing method reported significantly higher percentages of *Bacteroides* in a severely autistic group (Finegold *et al.* 2010) (Table 2.1). Despite *Bacteroides* having some beneficial effects on host health (Wexler 2007), including supplying energy to the host by facilitating fermentation (Hooper *et al.*, 2002), promoting T-cell-dependent immune responses (Mazmanian & Kasper, 2006), and limiting the colonisation of pathogens in the GI tract (Hopkins & Macfarlane, 2002), they are also pathogenic to humans through inflammatory and immunological manifestations such as fever, injury and neurological dysfunction (Gondalia *et al.* 2010).

Lipopolysaccharide (LPS) is found in the outer membrane of *Bacteroidetes* as an endotoxin that is responsible for activation of the innate immune system (Jyonouchi *et al*. 2005). LPS could damage tissue, and it has be proposed that it could increase the permeability of the brain-blood barrier (Gaillard *et al*. 2001; Xaio *et al*. 2001). It also decreases glutathione levels, potentially reducing ability to clear heavy metals (Zhu *et al*. 2007), which has led to the proposal that some children with ASD are vulnerable to heavy metal exposure (Counter *et al*. 2002; Holmes *et al*. 2003; Palmer *et al*. 2006; Geier and Geier 2007).

2.3.3 *Enterococcus* and *Streptococcus*

It has been noted that overgrowth of *Enterococcus* and *Streptococcus spp.* may affect the distribution of the anaerobic flora, the production and composition of primary bile acids in intestine (Salvioli *et al.* 1982). Thus, these species conceivably affect absorption of fats,

excretion of toxins and neurotransmitter activities. Raised *Streptococcal* titres were found to correlate with the severity of the attention deficit hyperactivity disorder (Peterson *et al.* 2000).

Some *Streptococcus* species may cause an autoimmune response. D8/17 antibody is a neuropsychiatric marker and increased antibody D8/17 has been observed in some individuals with AD correlating with the severity of the compulsive symptoms (Hollander *et al*. 1999). Paediatric Autoimmune Neuropsychiatric Disorders Associated with *Streptococcal* infection (PANDAS) is a 'psychiatric' illness caused by an autoimmune response to *Streptococcus* species (Dale *et al.* 2004). A case report (Cosford 2009) described a child originally diagnosed with autism and presenting features consistent with PANDAS, along with an overgrowth of *Streptococcal* and *Enterococcal* species. Overt *Streptococcal* infection is common in the history of children prior to the diagnosis of ASD, most commonly for otitis media (Cosford 2009). It has previously been noted that there is a high correlation between the prevalence of otitis media and ASD (Fallon 2005). An earlier age of onset of otitis media correlates with more severe ASD, and an increased incidence of otitis media also correlates with the more severe forms of ASD (Konstantareas and Homatidis 1987).

Amoxycillin is the treatment of choice for otitis media (commonly caused by *Streptococcus pneumoniae*) although in reality a combination of amoxicillin/clavulanate is frequently used. There is no strong evidence linking antibiotic usage and ASD incidence. However, a study by Fallon (2005) reported frequent chronic otitis media in children with autism under three years old and 36% of the antibiotic courses administered were amoxycillin/clavulanate. The author proposed a mechanism whereby the use of urea/ammonia in the production of clavulanate results in consumption of urea/ammonia. Fallon (2005) proposed a subgroup of children may at risk for neurotoxicity (manifesting as ASD) from the use of clavulanic acid in pharmaceutical preparations.

2.3.4 *Akkermansia muciniphila*

Mucin-degrading bacteria are able to ferment mucins (Miller and Hoskins 1981) and reported to potentially involve in the pathogenesis of intestinal diseases (Campieri and Gionchetti 2001). *Akkermansia muciniphila*, as a mucin-degrader, accounts for more than 1% of the total faecal bacteria in humans and numbers increase significantly from early life

to adulthood in healthy individuals (Collado *et al.* 2007). On the other hand, it is reduced many-fold in patients with Crohn's disease and ulcerative colitis (Png *et al.* 2010). A thinner mucous layer is present in patients with ulcerative colitis which indicate an alteration in numbers or activity of mucin-degrading bacteria in those patients (Campieri and Gionchetti 2001). Increased GI permeability has been reported in children with ASD (D'Eufemia *et al.* 1996; de Magistris *et al.* 2010), but none of these reports have examined the role of *A. muciniphila* in the permeability changes. The role of mucin-degrading bacteria in GI permeability changes in ASD deserves greater attention in the future.

2.3.5 *Bifidobacterium spp.*

Bifidobacterium spp. are some of the first bacteria to colonise the newborn human intestinal tract and are generally regarded as beneficial bacteria (Boesten and de Vos 2008). Alterations in *Bifidobacterium spp.* including decreased numbers or an atypical composition have been related to the development of GI diseases including allergies, irritable bowel syndrome and coeliac disease (Favier *et al.* 1997; Malinen *et al.* 2005; Collado *et al.* 2008). Similar levels of *Bifidobacterium spp.* were found in children with ASD compared with controls (Parracho *et al.* 2005), however, most participants in the study were using anti- and/or probiotics, which may confound analyses given that *Bifidobacteria* are usually used as probiotics.

In a small study, low numbers of *Bifidobacterium spp.* have been observed in individuals with GI problems and poor sleep patterns compared with controls (Ono *et al.* 2008). *Bifidobacterium spp.* supply muramyl peptides which may act as a somnogenic substance (Pabst *et al.* 1999) under typical conditions and contribute to normal sleep behaviours (Ono *et al.* 2008). Sleep problems are commonly reported in children with ASD but to date there is no research which correlates sleep issues with biological alterations in ASD.

2.3.6 *Lactobacillus*

Parracho *et al* (2010) recently conducted a probiotic study using *Lactobacillus plantarum WCFS1* to normalise the intestinal microbiota in children with ASD and found it had potential benefit in ASD with respect to improvements in stool consistency and some elements of behaviours. Certain probiotic strains can modulate various aspects of the microbiome-gut-brain axis (Gareau *et al.* 2010). A recent study reported a combination of

Lactobacillus helveticus and *Bifidobacterium longum* could reduce anxiety in animals and had beneficial psychological effects with a decrease in serum cortisol in humans (Messaoudi *et al.* 2010). Also, some probiotics have potential to lower inflammatory cytokines (O'Mahony *et al.* 2005; Brenner and Chey 2009), decrease oxidative stress, improve nutritional status (Logan and Katzman 2005) and detoxify heavy metals (Brudnak, 2002).

Many caregivers of children with ASD believed that using probiotics could alleviate GI symptoms and subsequently eliminate symptoms of ASD, there is currently insufficient high-quality evidence. Probiotics intervention is not routine practice given the lack of consistent evidence as to the specific microbial adjustment. Furthermore, it must be recognised that probiotics may not only have positive effects but also potentially lead into various other issues. Moreover, the specific 'good' bacteria used in probiotic formulations, the formulation itself (e.g. whether it is enteric coated) and storage conditions introduce variability in response. There is no specific guidelines yet for safety testing of probiotics, a battery of tests using *in vitro* and *in vivo* methods to determine the properties of the bacteria included in the probiotic is recommended.

Although altered GI microbiota profiles have been reported in children with ASD, whether these differences are responsible for the observed high incidence of GI disorders in children with ASD requires further investigation. A longitudinal study measuring a large range of bacteria, particularly those related to GI health, may supply important information and could potentially guide implementation of dietary interventions to improve GI health in individuals with ASD.

2.4 Large Bowel Fermentation Products

Carbohydrates and proteins are the main fermentative substrates of the large intestine (Macfarlane and Cummings 1991) and the majority of the fermentation products are derived from the large intestine (Roberfroid *et al.* 1995). The GI microbiota can impact on host health through these fermentation products but their effects are largely uncharacterised in children with ASD.

2.4.1 Short chain fatty acids

SCFA, primarily acetate, propionate and butyrate, are the predominant products of carbohydrate fermentation. These are generally regarded as beneficial to the host, especially the GI tract (Topping and Clifton 2001). Most of the acetate and propionate are metabolised by the liver and used in lipogenesis and gluconeogenesis (Hijova and Chmelarova 2007). Several studies have shown that intraventricular administration of propionate induces behaviours characteristic of ASD, e.g. repetitive dystonic behaviours, retropulsion, seizures, as well as social avoidance (MacFabe *et al.* 2007; MacFabe *et al.* 2008; Shultz *et al.* 2008; Shultz *et al.* 2009; Thomas *et al.* 2010). Also, elevated levels of propionate could result in neuroinflammation and induce oxidative stress in the CNS (MacFabe *et al.* 2008; Shultz *et al.* 2009). Butyrate is the primary source of energy for colonic epithelial cells (Roediger 1982) and can regulate colonic motility, increase colonic blood flow and may enhance colonic anastomosis healing (Velazquez *et al.* 1997). Increased levels of faecal SCFA levels in individuals with GI disorders have been reported (Tjellstrom *et al.* 2005), but to date no study has been conducted to analyse faecal SCFA, particularly propionate in children with ASD.

2.4.2 Phenols

In contrast to SCFA, protein fermentation products, such as phenols and ammonia are generally detrimental to the GI tract. Phenol, *p*-cresol and other derivatives are formed following bacterial degradation of the aromatic amino acids, such as phenylalanine, tyrosine and tryptophan (Hughes *et al.* 2000). Elevated levels of phenolic compounds have been implicated in colonic cancer (Nowak and Libudzisz 2006) and inflammatory bowel diseases (Ramakrishna *et al.* 1991). These compounds are absorbed from the colon into the systemic circulation, detoxified by the liver and then excreted in the urine or remain unabsorbed and excreted in the faeces (Ramakrishna *et al.* 1989). More recently, elevated urinary levels of total *p*-cresol have been found in children with ASD compared with controls (Altieri *et al.* 2011). Further, lower urinary levels of the detoxified form of phenols, *p*-cresol sulphate, have been reported in children with ASD compared with controls (Yap *et al.* 2010). However, there is no a clear correlation between urinary and faecal levels of phenolic compounds. Thus, urinary *p*-cresol and *p*-cresol sulphate

concentrations may not accurately reflect production in the colon and and thus measurement in faeces is important (Birkett *et al.* 1995).

2.4.3 Ammonia

Ammonia is produced from several tissues including small intestinal, kidneys and muscle. At physiological pH, ammonia is present as NH_3 and NH_4^+, both of which are capable of crossing membranes (Bosoi and Rose 2009). Most ammonia enters the systemic circulation from the GI tract and is rapidly converted to urea in the liver and subsequently excreted in the urine (Summerskill and Wolpert 1970). Ammonia is known to play a key role in hepatic encephalopathy and may act as a priming stimulus when the brain is inflamed (Wright and Jalan 2007). Accumulation of ammonia in the brain can disturb brain metabolism, cause extracellular potassium accumulation and hence may increase neuronal depolarization (Felipo and Butterworth 2002). It has been reported that high plasma levels of ammonia may increase permeability of the blood-gut and/or blood-brain barrier (Aldred *et al.* 2003) and cause neurophysiological impairment (Romero-Gomez *et al.* 2009). Several studies have described elevated plasma ammonia levels in children with ASD relative to controls (Clark-Taylor and Clark-Taylor 2004; Filipek *et al.* 2004) but none have reported analyses of faecal ammonia in children with ASD compared with controls.

Differences in levels of fermentation products in individuals with ASD compared with controls may reflect changes in GI microbiota composition and activities, as well as be indicative of GI disturbance. Given the significant role of fermentation products in GI health, it is important to know whether levels of these products are altered in children with ASD and if so whether alterations in fermentation processes contribute to GI dysfunction often found in ASD. All of these possibilities require further investigation.

2.5 Gastrointestinal Immune Function

The GI tract is the largest lymphoid organ containing up to 80% of immunoglobulin (Ig)-producing cells in the body (Agarwal and Mayer 2010). In the healthy condition, the intestinal immune system is tolerant against commensal luminal bacteria and food antigens. Once the barrier function is disturbed, however, a temporary or chronic activation

of the intestinal immune system will be observed (Lochs 2005). For instance, during GI infections, increased gut permeability can allow bacteria or antigens to adhere to and potentially invade the mucosa which can trigger the intestinal immune system and cause inflammatory reactions. Clinical and pathological studies have identified an apparently characteristic GI immunopathology in subgroups of children with ASD (Wakefield *et al.* 2000; Furlano *et al.* 2001; Torrente *et al.* 2002). Immunologic aberrations have been proposed to contribute to the aetiology of ASD.

Based on previous studies (Furlano *et al.* 2001; Jyonouchi *et al.* 2002; Torrente *et al.* 2002; Ashwood *et al.* 2003; DeFelice *et al.* 2003; Ashwood *et al.* 2004; Jyonouchi *et al.* 2005; Ashwood and Wakefield 2006), chronic ileo-colonic lymphoid nodular hyperplasia (LNH) is a key feature in ASD. The mucosal lesion consists of a panenteric lymphocytic infiltrate, with a variable degree of acute inflammation and eosinophillic infiltration (Furlano *et al.* 2001; Torrente *et al.* 2002). Moreover, immunohistochemical analyses in ASD have demonstrated qualitatively consistent abnormalities at different anatomical sites including the duodenum, ileum and colon (Furlano *et al.* 2001; Torrente *et al.* 2002; Ashwood *et al.* 2003), indicating a relatively homogenous mucosal lymphocyte infiltrate. Increased basement membrane thickness and abnormal patterns of epithelial glycosaminoglycans have been observed in children with ASD compared with controls and may be indicative of inflammatory degradation that may contribute to disruption of the intestinal barrier function (Furlano *et al.* 2001).

Furthermore, the inverse relationship between raised levels of tumour necrosis factor-alpha ($TNF\alpha^{+}$) and decreased levels of cytokine interleukin (IL)-10 in children with ASD with GI symptoms (Ashwood and Wakefield 2006) support the hypothesis that there is mucosal immune dysregulation with a proinflammatory lymphocyte cytokine profile in ASD. However, a sole study reported similar levels of several monokines in children with PDD and controls (DeFelice *et al.* 2003). The authors indicated there was no association between PDD and GI immune dysfunction (DeFelice *et al.* 2003). These data may suggest differential roles for activated lymphocytes and antigen presenting cells associated with some children with ASD. Given the small sample size, the results in this study require further investigation. A study by Jyonouchi *et al* (2002) also implied a possible link between GI and behavioural symptoms mediated by innate immune abnormalities due to

adverse reactions with dietary proteins. If confirmed this may lead to specific dietary intervention for ASD in the future.

2.6 Dietary and Nutrition Factors

Daily nutrient intake and absorption are essential to maintain the intestinal barrier, GI function and a balanced population of microorganisms. Also, proper nutrition helps the brain complete various physiological processes through a complex interaction of factors (Lochs 2005; Saito *et al.* 2005; Stewart and Latif, 2008). Deficiency of nutrients due to GI impairment could affect biological functions in the brain and may in turn manifest in signs and symptoms of a neurodevelopmental disorder such as ASD (Latif *et al.* 2002; Genuis and Bouchard 2010).

2.6.1 The evaluation of nutrient intake

A small number of studies have evaluated the dietary and nutritional intakes of individuals with ASD. Agreement exists that overall nutrient intake in children with ASD is adequate (Shearer *et al.* 1982; Raiten and Massaro 1986). Raiten *et al* (1986) reported children with ASD (n = 40) had a similar overall adequacy of diet to controls (n = 34) in a 7-day diet record. The authors claimed that the caregivers had a more positive attitude towards the importance of nutrition. However, the available evidence indicates that children with ASD tend to have increased food selectivity, such as selectivity by food category or texture (Ahearn *et al.* 2001; Field *et al.* 2003). A higher incidence of perceived pica, food cravings and eating problems were noted by the caregivers of children with ASD (Raiten and Massaro 1986). Shearer *et al* (1982) suggested that although children with ASD received more than 100% of the recommended daily amount of all nutrients, some other factors, such as food idiosyncracies, absorption and nutrient utilisation in children with ASD should be monitored and requires further investigation.

2.6.2 Food allergy and intolerance

It has been reported that children with ASD had significantly more positive pinprick reactions to at least one antigen, including casein, lactalbumin, beta-lactoglobulin, egg white, rice and soy (Lucarelli *et al*. 1995). Also, antibody levels including the total IgE,

IgA specific for casein, lactalbumin, beta-lactoglobulin and ovalbumin, IgM and IgG for casein and IgM for lactalbumin have been observed to be elevated in children with ASD (Lucarelli *et al.* 1995). It is notable that the traditional IgE-mediated hypersensitivity was no more common than in non-autism populations (Heuer *et al.* 2008) except perhaps in specific ASD subtypes (Magalhaes *et al.* 2009). Additionally, there is an association between the presence/amount of other classes of immunoglobulins and likelihood of ASD (Whiteley *et al.* 2010). Jyonouchi *et al.* (2002) demonstrated elevated Interferon-gamma (IFN-γ) and TNF-α response to dietary proteins in children with ASD that were akin to those observed in children with known dietary protein intolerance. It appears plausible that some children with ASD may have food intolerance of one form or another, however, the prevalence of such an intolerance within the autistic population is not clear and requires larger datasets to confirm.

2.6.3 Gluten- and/or casein- free dietary intervention

Despite the 'Opioid Excess Theory' in ASD not having been confirmed yet, gluten- and/or casein-free dietary interventions based on this theory are widely used in the treatment of ASD (Green *et al.* 2006). Several studies reported improvement of behaviours in children with ASD following dietary exclusion of gluten, casein or both (Knivsberg *et al.* 1990; Lucarelli *et al.* 1995; Adams *et al.* 1997; Whiteley *et al.* 1999; Cade *et al.* 2000; Knivsberg *et al.* 2002; Reichelt and Knivsberg 2009; Whiteley *et al.* 2010; Johnson *et al.* 2010). A few studies have suggested (Erickson *et al.* 2005; Barcia *et al.* 2008) that a link exists between coeliac disease and ASD, or at least in subgroups of ASD. Barcia *et al.* (2008) reported upwards of 3% of autism cases show diagnostic signs of coeliac disease. A case report described that a gluten-free diet resolved GI symptoms rapidly and abated autistic behaviours in a child with AD suffering from coeliac disease (Genuis and Bouchard 2010). However, other studies did not find any clear association between dietary intervention and behavioural improvement in ASD (Elder *et al.* 2006; Irvin 2006; Seung *et al.* 2007). Further, the gluten- and/or casein-free diet may increase the risk of nutritional deficiencies. Arnold *et al.* (2003) found the risk of nutritional deficiencies in children with ASD following gluten- and/or casein-free diet was significantly increased. This difference may be attributed to the lack of proteins specifically found in gluten and casein and/or the increase in food refusal which may follow the implementation of a gluten- and/or casein-free diet. There may be a subgroup of individuals with ASD who initially had gluten

(casein) allergy and/or intolerance that responded to a gluten- and/or casein-free dietary intervention. However, using such a diet as a primary treatment is not recommended as a general intervention in ASD until further evidence is forthcoming.

2.6.4 Other dietary factors

There is growing evidence identified that a deficiency in nutrients such as folate (Fletcher and Fairfield 2002), vitamin D (Genuis *et al.* 2009), or essential fatty acids (Genuis and Schwalfenberg 2006) could impair various biological processes required for normal metabolic and neurological functioning. Children with ASD who also have other neurologic impairments or metabolic diseases have been reported to have benefits from various dietary intervention. For example, early treatment of Phenylketonuria by appropriate dietary restrictions has greatly reduced the prevalence of ASD in affected children and may also improve the autistic phenotype in this population (Lowe *et al.* 1980). Alternatively, children with underlying mitochondrial disorders and ASD may benefit from coenzyme Q or carnitine supplements (Poling *et al.* 2006).

2.7 Other

2.7.1 Mesenchymal epithelial transition factor gene

Campbell *et al.* (2009) recently reported an association between variations of mesenchymal epithelial transition factor (MET) alleles in ASD and the presence of GI disturbance. It was suggested that disrupted MET signalling may contribute to increased risk for ASD that includes familial GI dysfunction. The functional variant in the promoter of the MET receptor tyrosine kinase has been reported to be associated with ASD in an earlier study (Campbell *et al.* 2006). The MET promoter variant is a common G-to-C single nucleotide polymorphism and the C allele is inherited by individuals with ASD more often than predicted by chance (Campbell *et al.* 2006). Furthermore, the same group demonstrated a significant decrease in both MET transcription and MET protein in the post-mortem temporal cortex of individuals with ASD compared with age- and gender- matched controls (Campbell *et al.* 2007). Given the studies demonstrating key roles for MET signalling in neural development (Ieraci *et al.* 2002; Powell *et al.* 2003) and function (Tyndall and Walikonis 2006), the genetic and expression data suggest that decreased

MET signalling may contribute to altered brain development that underlies disrupted behavioural and cognitive functions in ASD. As a pleiotropic receptor tyrosine kinase, MET also participates in immune function (Beilmann *et al.* 1997; Okunishi *et al.* 2005) and GI repair (Tahara *et al.* 2003; Arthur *et al.* 2004; Ido *et al.* 2005).

2.7.2 Mitochondrial dysfunction

Mitochondria are distinct cellular organelles that oxidise glucose and fatty acids to generate adenosine triphosphate (ATP). Mitochondrial disorders are heterogeneous but characterised by impaired energy production (Haas *et al.* 2007). Cells that require high-energy demands, such as muscle, brain and GI cells possess many mitochondria (Frye and Rossignol 2011). It has been suggested an association exists between ASD and mitochondrial dysfunction (Oliveira *et al.* 2005; Weissman *et al.* 2008). Carnitine is essential for the utilisation of fatty acids by the mitochondria. Carnitine deficiency has been observed in some individuals with ASD (Lombard 1998) and a recently published paper reported several significantly improved clinical measurements of ASD severity after 3 months of L-carnitine therapy (Geier *et al.* 2011). Several studies and/or case reports provided biochemical data indicating perturbation of mitochondrial energy metabolism in some individuals with ASD (Coleman and Blass 1985; Laszlo *et al.* 1994; Graf *et al.* 2000; Nissenkorn *et al.* 2000; Fillano *et al.* 2002; Filipek *et al.* 2003; Pons *et al.* 2004; Poling *et al.* 2006). GI impairment was the most common abnormality in these cases (Weissman *et al.* 2008) which may be due to the mitochondrial dysfunction in GI cells. On the other hand, abnormal mitochondrial function may be precipitated by many different environmental toxins that contribute to dysfunction in multiple systems, including GI disorders. Children with altered intestinal motility may have an underlying mitochondrial disease (Chitkara *et al.* 2003). Whether the GI dysfunction is a primary or secondary result of mitochondrial dysfunction in children with ASD requires further clarification.

2.8 Summary

The human GI tract is a complex ecosystem. Although it is widely accepted that GI factors are involved in the aetiology of ASD, epidemiologic studies investigating the relationship between ASD and FGIDs have been limited. Previous studies have described that GI symptoms are common in individuals with ASD, but resolution of key issues such as the

cause(s) of and the best treatment for these conditions are hampered by insufficient knowledge.

The metabolic activity of the GI microbiota has a functional impact on the host and can affect the host health, both positively and negatively (Davidson and Butler 2000). Despite the application of molecular and genetic techniques in recent years, yielding an increased body of information on the intestinal microbiota and its functions remains incompletely characterized. Altered levels of ammonia, *p*-cresol and HPHPA which are mainly produced by GI bacteria, such as *Clostridia*, have been found in plasma and urine samples in children with ASD. However, the link between abnormal levels of these metabolites in body fluids and the function of the GI microbiota in children with ASD, requires more extensive study. Mechanistic studies are needed to clarify the links between GI microbiota composition and GI symptoms, abnormal behaviour and personalised responses to pharmacological treatments.

Findings from most GI immune studies have illustrated a distinct lymphocytic colitis in ASD and suggest the involvement of the mucosal immune system in the aetiology of ASD (Chen *et al*. 2011). Accumulating evidence suggests that mucosal immune dysregulation may lead to secondary systemic and CNS immunopathology that may impact on neurodevelopment. The role of immune responses in the pathogenesis of GI disorders in individuals with ASD warrants additional investigation.

A better understanding of these abnormalities could potentially guide implementation of dietary interventions to improve GI health in individuals with ASD. Furthermore, linking these changes to CNS function will be helpful to develop preventive and therapeutic measures to ensure optimal cognitive development and avoid other medical complications in children with ASD.

3 A Literature Review of Potential Urinary Biomarkers in Children with Autism Spectrum Disorder

3.1 Introduction

ASD is a multifactorial disorder. Studies using whole-genome scanning methods, cytogenetics and genetic linkage/association analyses indicate ASD has a strong genetic basis (Geschwind and Levitt 2007; Szatmari *et al.* 2007; Christian *et al.* 2008; Morrow *et al.* 2008). The environmental factors, such as, GI factors (Horvath *et al.* 1999), immunological factors (Careaga *et al.* 2010), heavy metal toxicity (Grandjean and Landrigan 2006; Kern and Jones 2006; Slotkin *et al.* 2006), as well as metabolic abnormalities including dysfunctional neurotransmitter systems (McDougle *et al.* 2005; Zafeiriou *et al.* 2009) and oxidative stress (Main *et al.* 2010), all potentially contributing to the aetiology of ASD.

A common aberration is not consistently seen in all cases of ASD suggesting that ASD is a group of disorders with each probably having distinctive pathophysiology (Shattock *et al.* 1990). The combination of abnormal gene expression and environmental stressors can affect biological systems which in turn are reflected in metabolite profiles of body fluids (Nicholson *et al.* 2002). Interest in exploring abnormal metabolite profiles in biofluids, particularly blood and urine, of individuals with ASD has been increasing in recent years. Compared with blood, collection of urine samples is relatively simple, convenient and non-invasive and this chapter will focus on urinalysis. Identification of altered metabolite profiles in urine from individuals with ASD has the potential to elucidate the important underlying biologic defects thereby facilitating development of better targeted therapies, enable simple and objective diagnosis, identify ASD subtypes allowing for greater individualization of therapeutic decisions, and/or enable monitoring of ASD treatments (Adrien *et al.* 1989; Bailey and Ulrich 2004; Pardo and Eberhart 2007).

This chapter reviews a range of putative urinary biomarkers reported to be associated with ASD and is clustered under subheadings that aligned with factors implicated in ASD's aetiology: GI factors, immunological factors, heavy metal toxicity, altered neurotransmitters, oxidative stress and 'other'.

3.2 Gastrointestinal Factors

As mentioned in Chapter 2, GI disturbances are commonly reported in individuals with ASD, although the prevalence described in published studies varies substantially (Buie *et al.* 2010). Altered GI microbiota (Finegold *et al.* 2002; Song *et al.* 2004; Parracho *et al.* 2005; Finegold *et al.* 2010) and their fermentation products (Shaw 2010; Yap *et al.* 2010; Altieri *et al.* 2011) have been found in ASD compared with controls. Campbell *et al* (2009) recently provided genetic evidence to strengthen the link between GI dysfunction and ASD.

There are a number of theories proposed to explain the link between impaired GI function and disturbance of neurological development and/or function resulting in ASD. Inflammatory and immune changes may cause increased intestinal permeability (i.e. leaky gut) (D'Eufemia *et al.* 1996; de Magistris *et al.* 2010), which allow increased absorption of chemicals that are neuroactive (Reichelt *et al.* 1997). Overgrowth of bacteria in the gut capable of secreting neurotoxic chemicals has also been proposed. Moreover, it has been suggested that there may be dysfunction of secretin, a neurotransmitter and digestion control hormone, which has various behavioural effects, or its receptors (Horvath *et al.* 1999; Sandler *et al.* 2000; Shattock and Whiteley 2002; Molloy and Manning-Courtney 2003).

3.2.1 Peptides

The roles of the endogenous opioid system are diverse and include GI function, the sensory system, emotion and cognition (Akil *et al.* 1998) as well as development, such as neuronal pruning (Zagon and McLaughlin, 1991). Enhancement of the function of the endogenous opioid system or administration of opiates to infant animals can result in autistic-like symptoms (Sher 1997). It is hypothesised that exogenous opioid-like peptides, such as those derived from dietary casein and gluten (Reichelt *et al.* 1997) are implicated in ASD. Previous results of urinary opioid-like peptides in ASD are summarised in Table 3.1 (Israngkun *et al.* 1986; Shattock *et al.* 1990; Reichelt *et al.* 1997; Pedersen *et al.* 1999; Hunter *et al.* 2003; Dettmer *et al.* 2007; Cass *et al.* 2008).

Table 3.1 Results of studies examining urinary peptides in ASD compared with controls

Study	Participants (No.)	Results
(Israngkun *et al.* 1986)	ASD (n = 13) Control (n = 10)	The test was conducted using HPLC. Urinary patterns of individuals with ASD were different from controls.
(Le Couteur *et al.* 1988)	ASD (n = 26) Mentally handicapped (n = 17) Control (n = 26)	The test was conducted using UV absorbance. There were no significant differences and no consistent patterns of urinary chromatographic profiles between three groups.
(Shattock *et al.* 1990)	ASD (n = 25) Controls (n = 20)	The test was conducted using HPLC. Urinary patterns of individual with ASD were different from controls.
(Reichelt *et al.* 1997)	ASD (n = 64): 21 Italian, 6 American, 13 British, 12 Norwegians and 8 from Finland, Sweden & Germany. Controls (n = 8) with neuroleptics from Italian.	The test was conducted using HPLC. Altered urinary peptide pattern and large quantitative differences of urinary peptide profiles were found in ASD (peak area, $p < 0.001$) compared with controls.
(Pedersen *et al.* 1999)	ASD (n = 135) Controls (n = 126)	The test was conducted using HPLC. Significantly higher mean levels of the tripeptide (pyroGlu-Trp-GlyNH_2) peak area (10.3 vs 1.1 μm^2 at 215 nm trace, $p < 0.001$) in ASD than controls.
(Hunter *et al.* 2003)	ASD (n = 10) Siblings (n = 10)	The targeted peptides were not found in the urine from any participants using LC-UV-MS.
(Dettmer *et al.* 2007)	ASD (n = 54) Controls (n = 15)	The targeted peptides were not detected (the limit of detection is 0.25 ng/mL) in urine samples from any participants using HPLC tandem MS.
(Cass *et al.* 2008)	ASD (n = 65)	There were no significant differences of the urinary profiles between children

	Controls (n = 158)	with and without ASD using HPLC and MALDI-TOF MS.

ASD, autism spectrum disorder; HPLC, high pressure liquid chromatography; MS, mass spectrometry; MALDI-TOF MS, matrix assisted laser desorption ionisation-time of flight mass spectrometry.

Several early studies described different patterns of urinary peptides in individuals with ASD compared with controls (Israngkun *et al.* 1986; Shattock *et al.* 1990; Reichelt *et al.* 1997). However, a recent study did not find significant differences in urinary peptide profiles between children with and without ASD using HPLC-UV and checked the potential peaks using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) (Cass *et al.* 2008). The limitation of all these studies, however, was that the exact peptides were not isolated, identified or quantified. Thus it is unclear whether the specific peptides found to differ (or not) in individuals with ASD compared with controls in these studies were the same in all studies.

The first study to qualitatively investigate a specific urinary peptide, found that pyroGlu-Trp-GlyNH_2 was present in the urine of children with ASD (67%) much more commonly than controls (18%) (Pedersen *et al.* 1999). This study only recorded the area under the curve at 215 nm for each participant using HPLC-UV without referring to any calibration curve. Therefore, it did not allow for a comparison of quantitative results to match with other studies.

Two other studies (Hunter *et al.* 2003; Dettmer *et al.* 2007) used HLPC-mass spectrometry (HPLC-MS) based methods to detect several urinary peptides in children with and without ASD. However, none of these peptides were detected subsequently in test samples of children with ASD and controls (Hunter *et al.* 2003; Dettmer *et al.* 2007).

A highlighted issue to come out of this research is the importance of the analytical method. Urine is a very complex biological matrix and absorption of UV light is rather nonspecific for peptide detection. Hunter *et al.* (2003) claimed reference peptides spiked into urine could not be seen in UV-chromatograms but were clearly present in single ion chromatograms (mass spectrometry) and concluded that mass spectrometric technique has

greater sensitivity and specificity compared with UV-chromatograms when analysing peptides in urine.

3.2.2 Indolyl-3-acryloylglycine

Indolyl-3-acryloylglycine (IAG) is a regular constituent of human urine that is reported to be produced by specific GI bacteria. It is proposed that higher levels of urinary IAG are indicative of gut dysbiosis. Shattock & Whiteley (2002) have suggested the planar geometry of indolyl-3-acrylic acid (the acid precursor of IAG), disrupts membrane structures, and in turn increase GI permeability.

Table 3.2 Results of studies examining urinary IAG in ASD compared with controls.

Study	Participants (No.)	Results
(Bull *et al.* 2003)	ASD (n = 22) Controls (n = 18)	Increased median levels of *trans*-IAG were found in ASD compared with controls (942 vs 331 µV/mmol/L of creatinine, p = 0.0002).
(Whiteley and Shattock 2003)	PDD (n = 427) Controls (n = 83)	*Trans*-IAG was identified in more samples from the PDD group than controls (Fisher's exact test, two-side, p = 0.063).The HPLC results were affected by the epilepsy as a co-morbidity and/or medication use.
(Wright *et al.* 2005)	ASD (n = 56) including childhood autism (n = 33), atypical autism (n = 7) and AS (n = 16) Controls (n = 155)	No significant difference between the mean levels of IAG (*did not differentiate between isomers of IAG)* in controls and ASD (i.e., 0.0508 mmol/L vs 0.0511, p = 0.625). Also no difference between levels of IAG in controls and in childhood autism, 0.0433 mmol/L, atypical autism, 0.0637 mmol/L, AS, 0.0444 mmol/L, respectively.
(Whiteley *et al.* 1999)	Children with ASD implementing a gluten-free diet (n = 6) Children with ASD with a gluten	A mean reduction in *trans*-IAG excretion between baseline and post-diet samples in children who had implemented a gluten-free diet compared with two other groups but did not reach significant levels. No direct relationship was found between

	challenge (n = 4)	IAG and ASD behaviours.
	Controls (n = 3)	

ASD, autism spectrum disorder; PDD, pervasive developmental disorder; AS, Asperger syndrome; IAG, Indolyl-3-acryloylglycine.

Previous studies (Whiteley *et al.* 1999; Bull *et al.* 2003; Whiteley and Shattock 2003; Wright *et al.* 2005) examining urinary IAG in individuals with ASD (Table 3.2) have produced conflicting results which suggests that urinary IAG is not a suitable biomarker for overall ASD diagnosis, but may be useful for distinct subgroups of ASD, such as children with ASD and epilepsy (Whiteley and Shattock 2003).

It has been speculated that levels of urinary IAG may be reduced with a gluten-free diet. Whiteley *et al.* (1999) investigated urinary IAG levels over a 5 month period in children with ASD implementing a gluten-free diet, children with a gluten challenge and controls. They found participants on a gluten-free diet displayed an improvement in behavioural measures. However, there were no significant changes of urinary IAG between the three study groups and no direct correlation between IAG and autistic behaviours. From this preliminary data it appears that changes in IAG do not play a role in improvement resulting from a gluten-free diet. Gluten-free diet studies with larger samples and control are required to confirm the preliminary results.

3.2.3 Other urinary compounds of GI origin

A recent exploratory study by Yap *et al.* (2010) investigated urinary metabolic profiles in children with ASD (n = 39), their siblings (n = 28) and healthy controls (n = 34) using ^{1}H NMR spectroscopy. They identified a range of differences in the urinary metabolic profiles of children with ASD including higher levels of urinary dimethylamine, and lower levels of both urinary hippurate and 4-cresol sulphate. These compounds are produced by certain GI bacteria (e.g. *Bacteriodes, Clostridia*). Yap *et al.* (2010) attributed the alterations in the ASD group to an imbalance of GI microbiota.

Further, a study by Altieri *et al.* (2011) found elevated levels of total *p*-cresol in urine of young children with ASD compared with controls. The authors also reported a positive

correlation between urinary total *p*-cresol and ASD severity. *P-cresol* is a toxic metabolite of tyrosine catabolism by GI bacteria such as *Clostridium spp.* and *Pseudomonas stutzeri* (Altieri *et al.* 2011). Altieri *et al.* (2011) concluded follow up mechanistic studies will have to define the degree of overlap between GI microbiota composition and enhanced GI permeability in individuals with ASD as well as their potential relationship with GI symptoms, abnormal behaviour and personalised response to pharmacological treatments.

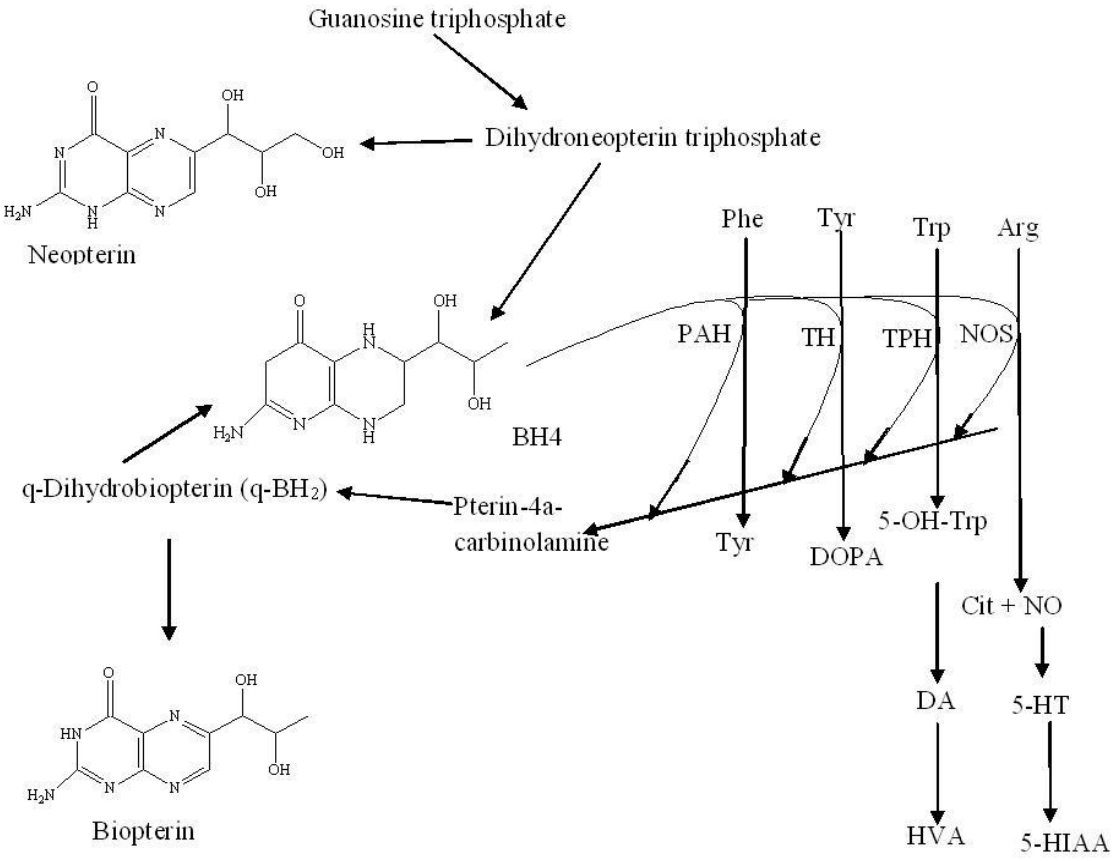
Shaw (2010) recent described a higher level of urinary HPHPA in children with ASD compared to controls. The source of HPHPA was reported to be multiple species of anaerobic bacteria of the *Clostridium* genus in the gut. The author speculated that HPHPA is a metabolite of m-tyrosine which depletes brain catecholamine and causes symptoms of ASD in experimental animals.

Many children with ASD have no or limited expressive language and may not be able to communicate GI discomfort. Thus, these children may react to pain by exhibiting behaviours not obviously related to the GI system (Weber and Newmark 2007). Routine analysis of the specific urinary compounds related to GI disturbance could facilitate early identification of whether children with ASD have or are at risk of GI disturbance and thereby enable appropriate interventions to be commenced which could potentially improve the quality of life of affected individuals.

3.3 Immune Dysfunction

Immunological anomalies involving cytokines, immunoglobulin, inflammation, and cellular activation have been reported in individuals with ASD (Goines and Van de Water 2010), strongly implicating immune dysfunction in the aetiology of some cases of ASD (Stigler *et al.* 2009). It has been established that increased levels of neopterin in body fluids often accompanies activation of the cellular immune system (Messahel *et al.* 1998). Determination of neopterin in body fluids of patients with diverse immune-related diseases has revealed that neopterin may play a key role in diagnostic and therapeutic decisions for such conditions (Fuchs *et al.* 1993).

A simplified pathway of the biosynthesis of pterins is shown in Scheme 3.1. Most of pterins occur in unstable reduced forms in biological systems while neopterin occurs as d-*erythro*-7,8-dihydroneopterin (Eto *et al.* 1992). This is important in terms of the analysis of neopterin in urinary samples, as it is possible to pre-treat urine samples to convert unstable reduced pterins to stable oxidised forms.



Scheme 3.1 Biosynthesis of pterins

Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; Arg, arginine; PAH, phenylalanine-4-hydroxylase; TH, tyrosine hydroxylase; TPH, typtophan hydroxylase; NOS, nitric oxide synthase; DOPA, 3,4-dihydroxyphenylacetic acid; Cit, citrulline; NO, nitric oxide; DA, dopamine; HVA, homovanillic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole acetic acid.

Three studies have been conducted analysing urinary levels of neopterin in children with ASD compared with controls (Eto *et al.* 1992; Harrison and Pheasant 1995; Messahel *et al.* 1998) (Table 3.3). Messahel *et al.* (1998) reported significantly higher levels of neopterin in urine samples (without oxidative pre-treatment) from children with ASD compared with controls. This study showed that the urinary neopterin: creatinine ratios in siblings were intermediate between ASD and unrelated control values. The raised levels of neopterin in siblings may indicate a common underlying cause within the families due to either genetic or environmental factors.

Table 3.3 Results of studies examining urinary neopterin in ASD compared with controls

Study	**Participants (No.)**	**Results**
(Eto *et al.* 1992)	ASD (n = 16) Controls (n = 12)	Decreased neopterin in ASD compared with controls (0.391 ± 0.041 vs 0.616 ± 0.132 mmol/mol creatinine, $p < 0.05$) when samples had oxidative pre-treatments.
(Harrison and Pheasant 1995)	ASD (n = 17) Controls (n = 17)	No significantly different levels of neopterin in children with ASD compared with controls (1359.9 ± 1102.5 vs 1283.1 ± 546.4 µmol/mol creatinine) when samples had oxidative pre-treatment. Higher neopterin levels in children with ASD than controls when the analyses were carried out on samples without oxidative pre-treatment in the same study (i.e. ASD vs controls, 1306.3 ± 786.3 vs 615.1 ± 373.4 µmol/mol creatinine, $p < 0.005$).
(Messahel *et al.* 1998)	AD (n = 14) Siblings (n = 21) Controls (n = 16)	Higher neopterin levels in the AD (no oxidative pre-treatment) versus the unrelated control group (3116 ± 686 vs 908 ± 201 µmol/mol creatinine, $p < 0.01$). Neopterin levels in sibling group (1490 ± 346 µmol/mol creatinine) were intermediate between children with ASD and controls.

AD, autistic disorder; ASD, autism spectrum disorder.

A second study, without oxidative sample pre-treatment, by Harrison and Pheasant (1995) also found that urinary neopterin levels were significantly increased in children with ASD compared with controls. With the use of oxidative pre-treatment, however, the same study found that the levels of urinary neopterin between children with ASD and controls were not significantly different. In contrast, a third study, in which oxidative pre-treatment was used, found significantly decreased urinary neopterin in children with ASD compared with controls (Eto *et al.* 1992).

The apparent discrepancies between these studies are likely due to different methodologies. The ratio between the reduced form, *d-erythro*-7, 8-dihydroneopterin, and oxidised form, neopterin, in collected samples was not reported. This ratio can change with time, unless all samples are collected and prepared simultaneously. Thus, fully oxidative pre-treatment of samples before neopterin testing is recommended. Neither study where samples were subjected to oxidative pre-treatment (Eto *et al.* 1992; Harrison and Pheasant 1995) appears to give evidence of elevated urinary neopterin levels in children with ASD compared with controls.

Another important issue highlighted is the potential importance of age when assessing neopterin levels. In Messahel *et al*' s (1998) study, high urinary levels of neopterin were found in children with ASD of less than 6 years old compared with those greater than 6 years, while the neopterin concentrations in controls did not change with age and resembled those of children with ASD greater than 6 years. Some inconsistent results reported in different studies may also be due to the various age ranges in the three studies described above i.e. 6-18 years (Eto *et al.* 1992), 3-21 years (Harrison and Pheasant 1995) and 3-5 years (Messahel *et al.* 1998).

3.4 Heavy Metal Toxicity

Heavy metals, such as arsenic, lead, and mercury have been associated with a variety of neurologic deficits and disorders, including lower intelligence quotient, Alzheimer's disease, and Parkinson's disease (Zecavati and Spence 2009). James *et al.* (2004) found a significantly reduced glutathione and oxidised glutathione ratio (GSH: GSSG) in children with ASD compared with controls. An impaired glutathione redox ratio is thought to play a

role in the aetiology of ASD by delaying the clearance of heavy metals from the body (Deth *et al.* 2008). The association between heavy metal exposure and ASD, in particular mercury, has attracted considerable interest (Counter *et al.* 2002; Holmes *et al.* 2003; Palmer *et al.* 2006; Geier and Geier 2007). Mercury has been implicated in immune, sensory, neurological, motor, and behavioural dysfunction resulting in clinical manifestations similar to those defining or associated with ASD. Some studies have suggested that mercury can disrupt neurotransmitter levels and biochemistry (Faustman *et al.* 2000; Redwood *et al.* 2001; Bernard *et al.* 2002) and impact on normal child development.

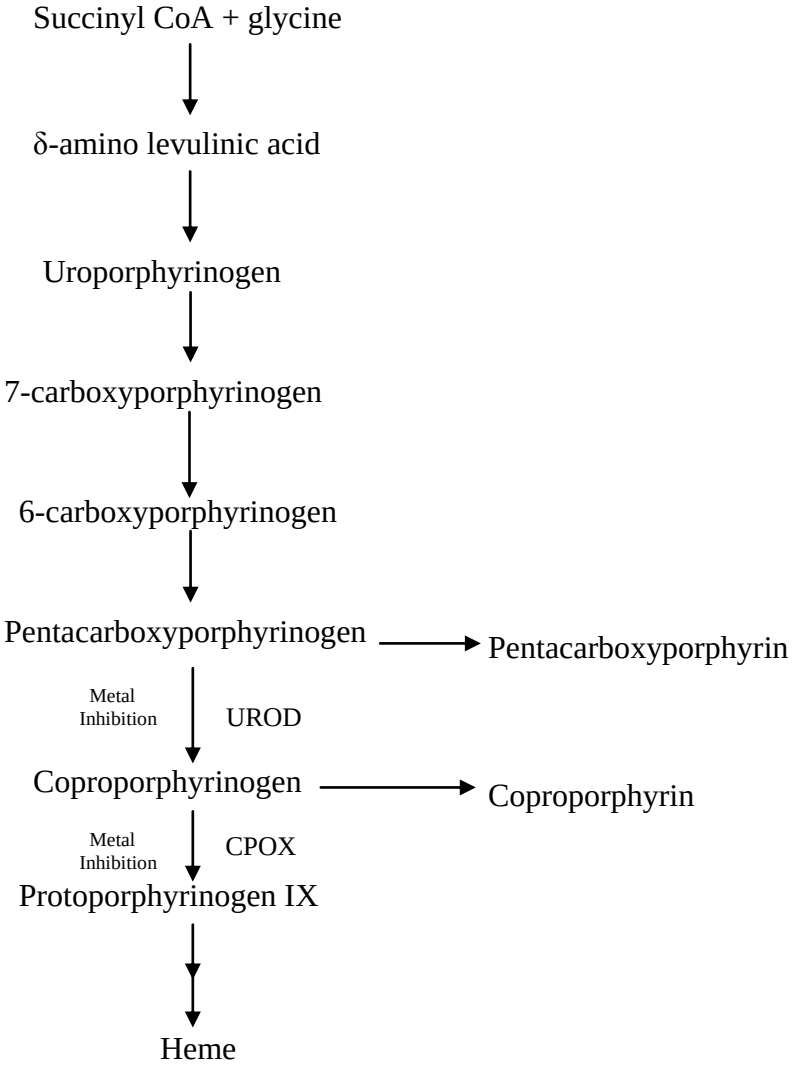
3.4.1 Heavy metals

It has been suggested that some children with ASD have an increased body-burden of mercury which may result from biochemical and genomic susceptibilities within detoxification pathways (Mutter *et al.* 2005). However, there have been few studies that have directly measured heavy metal burden in children with ASD in urine. Because urinary levels of heavy metals only show what is being excreted out of the body, urine studies are therefore not very useful to reflect the heavy metal burden in the body. A recent study reported an altered urinary heavy metal profile in children with ASD (n = 30) compared with healthy controls (n = 20). In particular, urinary chromium levels were significantly higher whereas cadmium and lead were significantly decreased in children with ASD compared with controls (Yorbik *et al.* 2009). The determinant of lower levels of urinary cadmium and lead in this study is unclear and the authors suggested these findings may be due to the children with ASD having poor heavy-metal-detoxifying mechanisms. This theory has been supported indirectly by studies that have shown decreased levels of heavy metal in the hair of children with ASD (Holmes *et al.* 2003). However, another study published by de Palma *et al.* (2011) suggested that there was no overall connection between levels of metallic elements analysed in hair and autism.

3.4.2 Porphyrins

Porphyrins, such as coproporphyrin, pentacarboxyporphyrin, uroporphyrin, and precoproporphyrin (Bozek *et al.* 2005) are involved in the formation of heme. Heme is essential for the proper function of many proteins including oxygen transport, energy production, and detoxification. The heme pathway is constantly changing and is active in

almost every cell of the body. Any disturbance in the pathway tends to cause rapid and relatively large accumulations of intermediates, such as porphyrins. Also, the enzymes of the pathway are widely distributed in human tissues, and are highly sensitive to the presence of various toxins, creating an accumulation of porphyrins in the pathway. Thus, the level of porphyrin production is useful for assessing the body's capacity to detoxify toxins (Brewster 1988). Heavy metals can inhibit the enzymes uroporphyrin decarboxylase (Woods and Kardish 1983) and coproporphyrinogen oxidase (Woods *et al.* 2005) (Scheme 3.2). Subsequent elevation of coproporphyrin and pentacarboxyporphyrin can be detected in urine as a marker of heavy metal levels.



Scheme 3.2 The heme synthesis pathway and major urinary metabolites: sites of heavy metal inhibition. UROD, Uroporphyrin decarboxylase; CPOX, coproporphyrinogen oxidase.

Elevated levels of porphyrins are found in urine from both laboratory animals and humans exposed to heavy metals (Bowers *et al.* 1992). Measurement of urinary porphyrins is an independent and non-invasive method to assess environmental toxicity in children with ASD. Studies that have examined and compared urinary porphyrins levels in children with and without ASD are summarised in Table 3.4 (Geier and Geier 2006; Nataf *et al.* 2006; Geier and Geier 2007; Austin and Shandley 2008; Nataf *et al.* 2008).

Table 3.4 Results of studies examining urinary porphyrin in ASD compared with controls

Study	Participants (No.)	Results
(Geier and Geier 2006)	ASD (n = 37) including subjects who received (n = 18) and did not received (n = 19, 6 AD and 13 PDD-NOS or AS) chelation therapy. Siblings (n = 7)	Increased median urinary COPRO levels were found in participants with AD (who were naive to chelation therapy, n = 6) compared with sibling controls (36 vs 16 µg/L, p <0.05). Decrease in the median levels of COPRO observed in chelated ASD participants compared with ASD participants who were not chelated (19 vs 32 µg/L, p < 0.05). Other urinary median levels were not significantly different between the total ASD participants and controls.
(Nataf *et al.* 2006)	AD (n = 106) AS (n = 12) Internal controls (n = 12) External controls (n = 107)	Elevations in urinary COPRO were found in children with AD compared with internal controls (p < 0.001). COPRO: URO was different between AD vs internal control (p <0.001). Similarly for AD vs AS (p = 0.009) Precoproporphyrin: URO was elevated in AD vs. controls (p <0.001). Urinary levels of pentacarboxyporphyrin and hexacarboxyporphyrinlevels were higher in ASD than controls (p <0.001 & p <0.002) A reduction in COPRO and precoproporphyrin was found following chelation treatment in AD (n=11) subgroup compared with their original porphyrin levels (p = 0.02).

(Geier and Geier 2007)	ASD (n = 63) including subjects who received (n = 26) and did not receive (n = 37) chelation therapy. Siblings (n = 9) General population controls (n = 5)	The mean levels of urinary pentacarboxyporphyrin and COPRO were elevated in unchelated ASD compared to chelated ASD (1.57 ± 1.04 vs 1 ± 0.85 µg/L, $p < 0.05$; 29.97 ± 19.1 vs 16.46 ± 9.51 µg/L, $p < 0.05$) and also compared to the combined chelated ASD plus siblings (1.57 ± 1.04 vs 1 ± 0.77 µg/L, $p < 0.05$; 29.97 ± 19.1 vs16.4 ± 8.42 µg/L, $p < 0.05$). The mean urinary precoproporphyrin: URO and COPRO: URO in the unchelated ASD participants were increased relative to the general population and were outside the lab reference ranges (26.66 ± 49.19 vs 5.56 ± 1.48 nmol/nmol, $p < 0.05$). Unchelated ASD and the general population controls had similar urinary heptacarboxyporphyrin: URO and within the lab reference range.
(Nataf *et al.* 2008)	AD (n = 100) AS (n = 11) Controls (n = 12)	Urinary COPRO levels in AD were higher compared with (AS + controls), $p = 0.02$. Similarly when COPRO was normalized to creatinine ($p = 0.01$).
(Austin and Shandley 2008)	ASD (n = 41)	A consistent trend in abnormal porphyrin levels was found compared with controls (Minder and Schneider-Yin, 1996; Nataf *et al.* 2006; Geier and Geier 2007) and laboratory reference (Laboratoire Philippe Auguste, Pairs, France) i.e. COPRO and pentacarboxyporphyrin levels were 289.6 ± 175.8 nmol/g creatinine, 5.2 ± 2.3 nmol/g creatinine, respectively).

ASD, autism spectrum disorder; AD, autistic disorder; AS, Asperger syndrome; COPRO, coproporphyrin; PDD-NOS, pervasive developmental disorder-not otherwise specified; URO, uroporphyrin.

Several studies reported atypical porphyrin profiles in children with ASD compared with controls, in particular, significantly higher levels of coproporphyrin were found in children with ASD (Geier and Geier 2006; Nataf *et al*. 2006; Austin and Shandley 2008). Also, increased urinary levels of pentacarboxyporphyrin in ASD were shown in Nataf *et al*' s

(2006) and Austin and Shandley's (2008) studies (Table 3.4). These studies demonstrated aberrant porphyrins in children with ASD. Nataf *et al*'s and Geier *et al*' studies have compared the urinary porphyrin levels in different ASD subtypes as well as children with ASD who had and hadn't received chelation while Austin *et al*'s did not. Since, abnormal levels of porphyrins were not found in individuals with AS, it is conceivable that different subtypes have different capacity to detoxify heavy metals. To date there is insufficient evidence available to conclude whether porphyrins are useful as biomarkers for diagnosis of overall ASD or for subtyping.

3.4.3 Chelation therapy

Chelating agents such as dimercaptosuccinic acid (DMSA) or 2,3-dimercapto-1-propanesulfonic acid (DMPS) are used as a treatment in ASD because they bind to heavy metals and increase their clearance from the body (Weber and Newmark 2007). Additionally, the levels of urinary heavy metals following chelation therapy may be useful to assess heavy metal levels.

Three studies have compared urinary heavy metals levels following chelation therapy between individuals with ASD and controls (Bradstreet *et al.* 2003; Geier and Geier 2007; Soden *et al.* 2007). Bradstreet and colleagues (2003) performed a retrospective urinary mercury excretion analysis in 221 autistic children compared with 18 controls after a three-day provocation with DMSA and demonstrated significantly higher levels of urinary mercury in children with ASD compared with controls. However, a limitation of this study is the large imbalance between the number of cases and controls. A case series has reported on 8 children with ASD that showed significantly elevated urinary levels of mercury and/or lead in urine after chelation therapy compared with before treatment (Geier and Geier 2007). It is difficult to interpret this report as this study had no control group(s). Soden *et al.* (2007) reported similar urinary heavy metal output in children with ASD (n = 17) compared with controls (n = 4), but it is likely that the small sample size limited the ability to detect a statistically significant difference.

There has been concern regarding the safety of chelation therapy to assess heavy metal burden and risks attached to chelation therapy has been reported (Baxter and Krenzelok 2008). Possible electrolyte imbalances can manifest from chelation therapy and it should

be done carefully under the direction of an experienced practitioner. Chelation therapy is therefore inappropriate for routine investigation of heavy metal burden in ASD.

3.5 Altered Neurotransmitters and Neurotransmitter Metabolites

Many studies have focused on neurotransmitter abnormalities in individuals with ASD (Garnier *et al.* 1986; Launay *et al.* 1987; Minderaa *et al.* 1987; Barthelemy *et al.* 1988; Garreau *et al.* 1988; Barthelemy *et al.* 1989; Minderaa *et al.* 1989; Martineau *et al.* 1991; Martineau *et al.* 1992; Herault *et al.* 1993; Martineau *et al.* 1994; Minderaa *et al.* 1994; Herault *et al.* 1996; Croonenberghs *et al.* 2000; Mulder *et al.* 2005; Mulder *et al.* 2009) (Table 3.5). Abnormalities in various neurotransmitters have been implicated in the development of ASD, including serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA), noradrenaline (NA), gamma-aminobutyric acid, glutamate and neuropeptides.

3.5.1 Serotonin and metabolites

5-HT is a monoamine neurotransmitter that plays an important role in the developing brain by directing both neuronal proliferation and maturation (McDougle *et al.* 2005). Central 5-HT activity has been involved in a range of physiological functions, such as sleep, sensory perception and appetite, which are often disrupted in ASD (Young *et al.* 1982). High levels of 5-HT during early development may cause a loss of 5-HT receptors and therefore impact on subsequent neuronal development (Whitaker-Azmitia 2001). Neuroimaging studies suggest altered developmental regulation of 5-HT synthesis may be associated with the pathogenesis of ASD (Chugani *et al.* 1999). Hyperserotonemia has been consistently reported in people with ASD in more than 25 published studies (Lam *et al.* 2006).

A number of studies have reported significantly higher urinary levels of 5-HT in ASD compared with controls (Barthelemy *et al.* 1989; Martineau *et al.* 1991; Herault *et al.* 1993; Herault *et al.* 1996). Moreover, a recent study revealed that urinary 5-HT excretion tended to be higher in hyperserotonemic children with ASD than the normoserotonemic group (Mulder *et al.* 2009). Barthelemy *et al.* (1989) found a significant decrease in urinary 5-HT levels in children with ASD after fenfluramine treatment. Fenfluramine is a 5-HT reducing agent which was used primarily as an appetite suppressant but also showed

some benefit in the management of ASD (Aman and Kern 1989). However, the drug was withdrawn from market in 1997 after reports of heart valve disease. In contrast, one other study (Spivak *et al.* 2004) reported significantly lower 'platelet-poor' plasma levels of 5-HT observed in autism compared with controls.

Metabolism of 5-HT is carried out primarily by monoamine oxidase to 5-hydroxyindole acetic acid (5-HIAA) which is excreted in urine (Grahame-Smith 1988). Most studies have shown no significant differences in urinary 5-HIAA between individuals with and without ASD (Minderaa *et al.* 1987; Herault *et al.* 1996; Mulder *et al.* 2005). One study reported a trend for higher 5-HIAA excretion in the urine of hyperserotonemic individuals with ASD than controls (Mulder *et al.* 2009). This study focused on the hyperserotonemic autistic subtype and therefore results do not necessarily conflict with other studies.

3.5.2 Dopamine and metabolites

DA is a catecholamine which acts as a major neurotransmitter in the brain. Generally, the dopaminergic system is thought to affect a wide range of functions, including cognition and attention (Nieoullon 2002), motor function (Niimi *et al.* 2009), predictive reward signal mechanisms (Schultz 1998) and immunity (Basu and Dasgupta 2000). Abnormal DA activity is thought to play a key role in the aetiology of schizophrenia (Davis *et al.* 1991). Some animal research has shown that stereotypes and hyperactivity can be induced by increasing dopaminergic functioning suggesting dopaminergic neurons may be overactive in ASD (Miller *et al.* 2010). Various DA antagonists are often used in the management of ASD (Lewis 1996). The atypical antipsychotic, risperidone, has reasonable evidence for efficacy with studies demonstrating it can alleviate some ASD related behaviours such as aggression and self-injury (Shea *et al.* 2004).

Levels of urinary DA have been investigated in several studies. Most studies have reported no significant differences in urinary DA levels between children with and without ASD (Launay *et al.* 1987; Minderaa *et al.* 1989; Martineau *et al.* 1992; Martineau *et al.* 1994; Croonenberghs *et al.* 2000) and only a single study found lower DA levels in ASD than controls (Barthelemy *et al.* 1988). Of particular interest are studies by Martineau *et al.* (1992, 1994) who found a significantly higher mean level of urinary DA in children with ASD who were not taking DA antagonists compared with those who were taking DA antagonists. A further study reported the various urinary levels of DA with different drug

treatments (i.e. phenothiazine antipsychotics, haloperidol and anticonvulsants) (Minderaa *et al.* 1989) which may be helpful for monitoring the effects of different drugs in ASD. Given various medications lead to alterations in urinary DA, since information relating to medication use was not identified in the majority of studies (Launay *et al.* 1987; Barthelemy *et al.* 1988; Minderaa *et al.* 1989; Martineau *et al.* 1992; Martineau *et al.* 1994; Croonenberghs *et al.* 2000). Thus, it cannot be concluded whether or not urinary DA is useful for ASD diagnosis.

The major metabolites of DA are homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC). Significantly increased level of urinary HVA in ASD compared with controls has been reported (Garnier *et al.* 1986; Barthelemy *et al.* 1988; Martineau *et al.* 1992; Martineau *et al.* 1994). However, as high levels of HVA are also reported in children with mental redaction (Martineau *et al.* 1992), it limits the value of urinary HVA as a biomarker for ASD diagnosis. There was no significant difference in urinary DOPAC levels between ASD and controls (Launay *et al.* 1987; Martineau *et al.* 1992; Martineau *et al.* 1994**)**, but one study reported a lower level of urinary DOPAC in children with ASD who had responded with fenfluramine treatment **(**Barthelemy *et al.* 1989).

3.5.3 Noradrenaline and adrenaline

Noradrenaline (NA) is synthesized from DA by dopamine β-hydroxylase and released from noradrenergic neurons as well as from the adrenal medulla into the bloodstream. NA plays a critical role in attention, the stress response (i.e. the 'fight or flight' response), anxiety, and memory (Amaral and Sinnamon 1977; Fitzgerald 2009), which are frequently observed to be impaired in individuals with ASD.

Previous studies have shown that measurements of NA (i.e. in plasma and urine) are generally well correlated with measurements in the CNS (Roy *et al.* 1988). Several neurochemical studies have attempted to examine excretion of urinary NA and/or adrenaline (A) in individuals with ASD compared with controls and have yielded inconsistent findings. Three studies found higher levels of NA and/or A in ASD compared to controls (Barthelemy *et al.* 1988; Herault *et al.* 1993; Martineau *et al.* 1994), while four studies found no differences (Launay *et al.* 1987; Martineau *et al.* 1992; Minderaa *et al.* 1994; Croonenberghs *et al.* 2000).

It was reported that urinary levels of NA and/or A significantly decreased with age (Martineau *et al.* 1992). Variations in the mean age of participants between studies indicated that age may have reduced the significant difference between individuals with and without ASD. For example the mean age was 6 years in one study which reported elevated levels of NA and/or A in ASD vs controls (Herault *et al.* 1993) while it was around 20 years in another study that reported no difference (Croonenberghs *et al.* 2000).

Table 3.5 Results of studies examining urinary neurotransmitters in ASD compared with controls

Study	**Participants (No.)** *(age range or mean)*	**Results**
(Herault *et al.* 1993)	ASD (n = 23) *2-11 years* Controls (n = 59) *2-11 years*	Higher levels of 5-HT (159.9 ± 17.5 vs 92.8 ± 5.1 nmol/mmol creatinine, p < 0.001) and NA+A (48.2 ± 5.5 vs 37.2 ± 2.6 nmol/mmol creatinine, p = 0.043) in ASD compared with controls.
(Herault *et al.* 1996)	ASD (n = 65) *2-19 years* Controls (n = 35) *NR*	Higher daily (9 am-4 pm) urinary levels of 5-HT in ASD than controls (137.2 ± 83.8 vs 95.8 ± 31.9 mmol/mmol creatinine, P < 0.001). No significant difference in urinary 5-HIAA levels between two groups (i.e. ASD vs controls, 4.9 ± 3.8 vs 4.9 ± 2.5 mmol/mmol creatinine).
(Martineau *et al.* 1992)	ASD (n = 156): 85 drug free and 71 had medicated[a] *2-12 years* Non-autistic participants who had mentally retarded (MR, n = 152): 111 drug free and 41 had medicated *2-12 years* Normal controls (n = 116) *2-12 years*	Higher daily (9 am-4 pm) urinary levels of DA were found in MR group than ASD or control groups (i.e., MR, ASD & normal, 382 ± 14, 309 ± 11 and 319 ± 12 nmol/mmol creatinine, p< 0.001). Daily levels of 3MT (i.e. ASD, MR & controls, 179 ± 12, 200 ± 13 & 140 ± 6 nmol/mmol creatinine, p< 0.005), HVA (7.9 ± 0.2, 8.1 ± 0.3 & 5.4 ± 0.2 µmol/mmol creatinine, p< 0.0001) and 5-HT (143 ± 6, 139 ± 6 & 95 ± 5, nmol/mmol creatinine, p<0.001) were higher in ASD and MR groups than controls. Urinary levels DA were lower in medicated participants with ASD compared to non-medicated participants with ASD (290 ± 17 vs 338 ± 14 nmol/mmol creatinine).

(Mulder *et al.* 2009)	Hyperserotoniemic ASD (n = 9) *(15.3 ± 4.4) years* Normoserotonimic ASD (n = 10) *(15.3 ± 4.0) years*	No significant different of 24 h urinary 5-HT and 5-HIAA levels in children with hyperserotonemics and normoserotonemics (63.2 ± 19.2 vs 60.1 ± 21.4 mmol/mol creatinine, 1.79 ± 0.75 vs 1.69 ± 0.86 mmol/mol creatinine, respectively)
(Barthelemy *et al.* 1989)	ASD (n = 13) with fenfluramine treatment: 6 had respond and 7 did not *3-10 years*	Daily (9am-4pm) urinary levels of 5-HT was lower in all participants after drug treatment. HVA were lower in responders compared with non-responders before treatment (6.6 ± 0.7 vs 10.4 ± 0.9 µmol/mmol creatinine, p<0.005). Increased HVA in responders after than before treatment (10.2 ± 1.8 vs 6.6 ± 0.7 µmol/mmol creatinine, p<0.05 Wilcoxon test) while levels returned to baseline later.
(Minderaa *et al.* 1987)	ASD (n = 36): 16 drug free and 20 had medicated[a] *8.9-28.7 years* Controls (n = 27) *9.1-36.1 years*	Two periods time (5pm -11pm and 11pm-8am) samples were collected. Higher overnight (5.12 ± 1.06 vs 3.44 ± 1.06 µg/mg creatinine, p< 0.01) and combined urinary levels (4.88 ± 0.87 vs 3.50 ± 1.07 µg/mg creatinine, p<0.05) of 5-HIAA were found in hyperserotonemic ASD compared with controls. There was a trend (p<0.1) to high of overnight (4.16 ± 1.70 vs 3.44 ± 1.06 µg/mg creatinine) and combined (4.07 ± 1.52 vs 3.50 ± 1.07 µg/mg creatinine) urinary 5-HIAA levels in children with unmedicated ASD compared with controls
(Mulder *et al.* 2005)	ASD (n = 20) *NR*	24 h urinary 5-HIAA levels (1.74 ± 0.79 mmol/mol creatinine) were similar as previously reported in healthy controls
(Launay *et al.* 1987)	Infantile ASD (n = 20) *5-16 years* Controls (n = 19) *5-16 years*	No significantly different in overnight urinary compounds were found between ASD and controls (DOPAC, 5.13 ± 3.34 vs 4.84 ± 4.37 umol/mol creatinine; MHPG, 3.79 ± 2.51 vs 5.06 ± 4.01 umol/mol creatinine; DA, 1.82 ± 1.40 vs 2.23 ± 1.35 10-7 mol/mol creatinine; NA, 1.06 ± 0.86 vs 1.39 ± 1.24 10-e mol/mol creatinine and A, 4.97 ± 5.34 vs 5.11 ± 3.92 nmol/nmol creatinine).
**(Minderaa *et*	ASD (n = 36): 16 drug free and 20	Complete evening (5pm-8am) urinary levels of HVA were higher in the medicated combined

***al.* 1989)**	had medicated[b] *(20.0 ± 4.4) years* Control (n=28) *(20.1 ± 6.8) years*	group than unmedicated children with ASD (4.70 ± 2.32 vs 3.33 ± 1.01 µg/mg creatinine, p<0.05). Complete evening urinary levels of DA were lower in ASD who had phenothiazines treatment compared with non-medicated ASD (125 ± 13.8 vs 192 ± 45.4 µg/g creatinine, p<0.01)
(Martineau *et al.* 1994)	ASD (n = 50) *2-16 years* Controls (n = 50) *Mean 8 years*	Daily (9am-4pm) urinary levels of DA were slightly lower in children with ASD compared with controls. High levels of HVA, 3MT, and NA+A in ASD compared with controls which confirm the previous study by Martineau *et al* (1992). No significant difference between urinary DOPAC between two groups.
(Garnier *et al.* 1986)	ASD (n = 19) *4-9 years* Controls (n = 15) who had not any psychological disorders *3-13 years*	Spot urine (9am-10am) sample collected. HVA levels were higher in ASD than controls (5.90 ± 0.41 vs 4.06 ± 0.33 nmol/µmol creatinine, p<0.02).
(Minderaa *et al.* 1994)	ASD (n = 36): 16 drug free and 20 had medicated[a] *9-29 years* Controls (n = 27) *9-36 years*	No significant differences of complete evening (5pm-8am) urinary compounds excretion found in all participants (i.e. controls, ASD with drug free, ASD with medicated) MHPG, 80.4 ± 46.7, 67.6 ± 20.5 & 70.9 ± 20.8 µg/hr; NA, 1.04 ± 0.48, 0.89 ± 0.26, 0.94 ± 0.47 µg/hr; A, 207 ± 167, 161 ± 65.9, 188 ± 95.0 ng/hr; VMA, 127 ± 38.5, 128 ± 44.6, *NR*.
(Barthelemy *et al.* 1988)	ASD (n = 8) *4.4-8.3 years* Controls (n = 8) *3-10 years*	Total levels of DA (577.35 ± 49.87 vs 1225.63 ± 143.88 ng/mg creatinine, p<0.01) and MHPG (1410 ± 100 vs 2040 ± 200 ng/mg, p<0.05) were lower in ASD, NA (159.25 ± 18.33 vs 93.00 ± 12.93 ng/mg creatinine, p<0.01) were higher in ASD.
(Croonenberghs *et al.* 2000)	AD (n = 13) *12-18 years* Controls (n = 13) *12-18 years*	No significantly different in 24 h urinary 5-HIAA, A, NA and DA between individuals with and without ASD.

(Martineau *et al.* 1991)	ASD (n = 145) *2-12 years* Nonautistic developmental disorders (NADD, n = 141) *2-12 years* Controls (n = 107) *2-12 years*	Daily (9am-4pm) urinary levels of 3MT (171 ± 13, 207 ± 15 & 139 ± 6 nmol/mmol creatinine, $p < 0.005$), total HVA (7.8 ± 0.2, 8.3 ± 0.3 & 5.4 ± 0.2 µmol/mol creatinine, $p < 0.001$) and 5-HT (141 ± 7, 144 ± 6 & 95 ± 5 nmol/mol creatinine, $p < 0.001$) were higher in children with ASD and NADD participants than controls.

ASD, autism spectrum disorder; AD, autistic disorder; 3MT, 3-methoxytyramine; 5-HIAA, 5-hydroxyindole acetic acid; 5-HT, Serotonin; A, adrenaline; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MHPG, methoxyhydroxy phenylglycol; NA, noradrenaline; VMA, vanillylmandelic acid; a , The autistic and/ or non autistic participants who had medicated with following drugs: anticonvulsants, haloperidol, phenothiazines, benzodiaze; b, The autistic participants who had mediated with flowing drugs : phenothiazines, haloperidol and anticonvulsant; *NR*, no record.

3.6 Oxidative Stress

A leading theory implicated in the aetiology of ASD is oxidative stress, which results from a complex interplay of genetic and environmental factors. Oxidative stress occurs when reactive oxygen species (ROS) levels exceed the antioxidant capacity of a cell. These ROS target lipids, proteins and nucleic acids (Chauhan and Chauhan 2006) resulting in a risk of neurologic deficits, especially during early life (Zecavati and Spence 2009). Evidence has emerged in recent years supporting the role of oxidative stress in the aetiology of ASD, such as increased lipid peroxidation (Ming *et al.* 2005), altered antioxidant enzymes in plasma (Yorbik *et al.* 2002), mitochondrial dysfunction (Oliveira *et al.* 2005) and genetic factors (Cohen *et al.* 2003; Hovatta *et al.* 2005).

3.6.1 Biomarkers for lipid peroxidation

A study by Chauhan *et al.* (2004) reported increased lipid peroxidation in children with ASD compared with their siblings and suggested that increased lipid peroxidation was associated with regressive ASD. Isoprostanes, including 8-isoprostane, are a family of eicosanoids of non-enzymatic origin produced by random oxidation of tissue phospholipids by oxygen radicals. Ming *et al.* (2005) demonstrated significantly higher urinary levels of

8-isoprostane in children with ASD (n = 33) compared with healthy controls (n = 29) and were in agreement with Chauhan *et al.* (2004)'s findings. However, the Ming *et al.* (2005) study found no significant correlation between the levels of 8-isoprostane and history of regressive ASD. More studies on urinary lipid peroxidation metabolites are required to confirm these preliminary results and reveal their usefulness as potential biomarkers for ASD diagnosis.

3.6.2 Biomarkers for nucleotide hydroxylation

Urinary 8-hydroxy-2-deoxyguanosine (8-OHdG) is the product of cellular DNA oxidation and hence a biomarker for oxidative damage to DNA (Awad *et al.* 1996). Ming *et al* (2005) also reported a trend for elevated urinary 8-OHdG in children with ASD compared with controls but it was not statistically significant.

3.6.3 Methylmalonic acid

Increased vulnerability to oxidative stress could impair vitamin B_{12} metabolism which can potentially cause severe and irreversible damage, particularly in the CNS and thus contribute to the development and clinical manifestation of ASD (James *et al.* 2004). Impaired vitamin B_{12} metabolism can lead to accumulation of methylmalonic acid in tissues and body fluids. A study by Wakefield *et al.* (1998) reported that urinary methylmalonic acid levels were significantly higher in children with ASD (n = 8) compared to age and gender matched normal controls (n = 14). However, the interpretation of the findings in relation to vitamin B_{12} deficiency has been challenged (Bhatt 1998) and no other studies to confirm these results to date.

Overall, there is limited evidence to substantiate the role of oxidative stress in studies where urinary metabolites have been examined. Most studies implicating oxidative stress in the aetiology of ASD have examined various metabolites in plasma or serum (Main *et al.* 2010). Preliminary results of these blood studies identify oxidative stress can lead to membrane lipid abnormalities, inflammation, an aberrant immune response and impaired energy metabolism, all of which can potentially contribute to the clinical manifestations of ASD. As such this area of study requires further investigation, specifically whether there are correlations between blood and urinary metabolites.

3.7 Other Compounds

3.7.1 Creatinine

Creatinine is primarily derived from muscle and is non-enzymatically converted at a near-constant rate from creatine. Creatinine passively diffuses out of the cells and is excreted by the kidneys into the urine and its excretion is relatively constant over time. Hence, when other urinary metabolites levels are reported they are usually normalised against urinary creatinine to correct the variations of urinary volume that occur from inter- and intra-day. The results of studies reporting urinary creatinine concentrations in children with ASD have been inconsistent. One previous study highlighted that median levels of urinary creatinine (weight adjusted) was lower in children with PDDs (n = 24) compared with controls (n = 50) (Whiteley *et al.* 2006). On the other hand, Nataf *et al.* (2008) reported no significant differences in urinary creatinine levels between children with ASD (n = 217) and 'controls' (n = 23) regardless of normalization for age and gender. However, Nataf *et al*'s findings should be interpreted with caution as they included children with AS i.e. 11/23 in their control group after they demonstrated that they had urinary creatinine levels that were not significant different to other controls i.e. 12/23 (Nataf *et al.* 2008).

3.7.2 Nicotinic acid metabolism

The previously mentioned urinary metabonomic study by Yap *et al.* (2010) reported urinary profiles in children with ASD that reflected altered nicotinic acid metabolism compared with controls. Specifically, they reported increased urinary levels of N-methyl-4-pyridone-3-carboxamide, N-methyl-nicotinic acid and N-methylnicotinamide and concluded the changes could be attributed to perturbation of the tryptophan-nicotinic acid metabolic pathway in children with ASD.

3.7.3 Amino acids

Yap *et al.* (2010) also reported altered urinary levels of some free amino acids, including lowered levels of glutamate and higher alanine, glycine and taurine in children with ASD compared to healthy controls. They suggested that the changes may be due to perturbation in sulphur dependent detoxification and amino acid metabolism in autistic children. A preliminary study (Alberti *et al* 1999) demonstrated that a low sulphation capacity was present in at least a subgroup of children with ASD. Alberti *et al.* (1999) speculated that, as

a result, these children were unable to effectively metabolise numerous compounds, particularly phenolic amines, such as, dopamine, tyramine, and serotonin which function as neurotransmitters.

3.8 Discussion

Given the heterogeneity in aetiology and/or pathophysiology in ASD, it is unlikely that a single urinary marker will be able to discriminate children with and without ASD. Thus, the putative urinary biomarkers examined in this chapter may only be useful for specific subgroups of individuals. The heterogeneity may also account for some of the variability in the results reported between studies. Moreover, the presence of specific co-morbidities such as epilepsy or ongoing GI disturbance, may also lead to altered urinary metabolite profiles. The overall effect is that significant differences in certain chemicals may only be evident if ASD specific subgroups are identified and investigated i.e. individuals with specific subgroups of individuals with ASD will show different urinary metabolite profiles. A consistent theme that is emerging in ASD research which have previously been stated by Buie *et al.* (2010), "Given the heterogeneity of persons with ASD and the many inconsistent research findings regarding ASD, it is imperative that the phenotype (biological, clinical, and behavioural features) of future study subjects should be well defined."

This chapter covers a range of studies that have different participant groups which can influence findings. Differences in ASD cases i.e whether participants have AD, AS or PDD-NOS may lead to inconsistent results. Similarly for control groups i.e. whether controls are siblings, unrelated healthy controls or have other developmental disorders. For instance, significant differences were found between children with ASD and community controls, but not in sibling controls (Messahel *et al.* 1998). Studies by Martineau *et al.* (1991, 1992) used children with non-autistic developmental disorder and neurotypical children as two different control groups. Significantly different levels of DA were found in children with non-autistic development disorders compared with controls or ASD. Thus, when a mix of such subgroups is analysed together this may cover up real differences and/or contribute to inconsistent results between studies that have different mixtures of these subgroups. Studies with large sample size and subjects that are well characterised

(age, gender, ASD characteristics, ASD subtype, co-morbidities) will be required to true understand which characteristics are important for each urinary biomarker. Furthermore, comparison against multiple different control groups can be useful to help identify how specific the urinary chemical is to ASD and thus addition of other control groups such as siblings and children with other related development disorders is suggested for future studies.

Previous studies reported a range of different urine sample collection periods including 24 h, daily (9 am-4 pm), evening (5 pm-8 am) or first morning urine samples. The collection periods may influence urinary volumes owing to more water intake and exercise during daytime and therefore urinary metabolites' concentrations. An important direction for future research will be to compare different collection periods and establish which is the most reliable and accurate method of urine collection for the purpose of identifying biomarkers.

It is clear that there is substantial variation in urine metabolite levels between days for the same individual due to environmental factors such as variation in diet. Fasting before sampling to avoid the changes caused by different dietary intake may be useful but is perhaps impractical for young children with ASD. An alternative would be to keep a food frequency questionnaire record so that nutrition influence may be subsequently investigated. Also, multi-sample collection per subject may be a viable option to minimize intra-individual variation and the influence of collection period.

Urine is a complex matrix and altered metabolite profiles relate to many factors, such as age, race and pubertal status. It has been reported that levels of many urinary metabolites are associated with the age of participants but there has been no study in the current literature describing metabolic trajectories of children with ASD. Thus, age should be considered as a potential confounder in the current studies. Options may include age restricting participants or adjusting for age during data analysis. The significant effects of pubertal status and race on various biochemical measurements have been established in recent studies (Lam *et al.* 2006).

A variety of different techniques and protocols have been used for sample collection, storage, and analysis which may also contribute to variation in results. For instance, IAG and neopterin are light sensitive, so samples need to be protected from light (Messahel *et*

al. 1998; Mills *et al.* 1998) and if this is not carefully undertaken it may significantly impact the study results. More specific and multi-analytical techniques are better for biomarker studies. For example, a study mentioned that combined HPLC-UV and mass spectrometric techniques may have greater sensitivity and specificity for sample analysis compared with the use of either technique separately (Hunter *et al.* 2003). In addition, differences in creatinine levels observed in children with and without ASD suggest it is important to use alternative normalising reference compounds.

Sample size is an issue for many studies which may reflect the difficulty in recruiting children into such studies. This does lead to difficulty in interpreting some studies, however, as small sample size can lead to low statistical power and decrease ability to represent the targeted population. The typical conclusion for many of the urinary markers that show promising results is that there is a need to independently replicate the results based on a well powered and valid study design before consideration of any clinical use.

A shortcoming of many of these studies is that the actual metabolic pathway is unknown or not investigated. For example, urinary IAG is believed to be produced by GI bacteria from a metabolite of the indole amino acid tryptophan (Marklova 1999) but the exact pathway has not been confirmed.' Many neurotransmitters have been suggested to relating with ASD but the correlation of between these compounds in CNS and in peripheral system is not clear, thus, it is unlikely to conclude that the changes in these urinary compounds totally reflect the changes in CNS. It would be valuable to establish a database containing clinical and biological data (including urine samples) to help direct further investigation.

In summary, despite a large number of studies focusing on urinary metabolites in ASD, there currently is no strong evidence for any single metabolite having significant clinical utility or providing conclusive insight into the aetiology or pathophysiology of ASD. A number of urinary metabolites are potentially useful and/or insightful but require further validation. Due to great potential of this line of research to aid ASD diagnosis, treatment development and selection, it is important that further studies are undertaken to confirm and expand current understanding of urinary metabolites in ASD.

4 Urinary Indolyl-3-acryloyglycine in Children with Autism Spectrum Disorder

4.1 Introduction

Growing evidence suggests that both genetic and environmental factors contribute to the aetiology of ASD. GI symptoms, such as abdominal pain, constipation and diarrhoea, and GI inflammation are reported to occur more frequently in individuals with ASD (Horvath and Perman 2002a, 2002b). Various theories such as the 'Opioid Excess Theory' (Reichelt *et al.* 1997), overgrowth of neurotoxic GI bacteria (Bolte 1998; Finegold 2008) and the 'leaky gut' theory (D'Eufemia *et al.* 1996; de Magistris *et al.* 2010) have been proposed to explain how impaired GI function and/or GI inflammation may disturb neurological development and/or function resulting in ASD.

Of particular interest in this chapter is the link between GI dysfunction and ASD. As mentioned in Chapter 3, abnormal levels of urinary metabolites in individuals with ASD compared with controls may help the understanding of the aetiology of ASD, but require further investigation. IAG is produced by gut microbiota and is suggested to be indicative of gut dysbioisis. Thus, urinary levels of IAG and IAG: creatinine ratio were analysed in this chapter.

4.1.1 Urinary indolyl-3-acryloylglycine

IAG (Figure 4.1) is a regular constituent of human urine and is produced by gut bacteria. Urinary IAG has been identified as being elevated in Hartnup's disease, polymorphous light dermatosis and phenylketonuria (Verhagen and Burbach 1966; Marklova *et al.* 1975; Jepson 1978). A study examining urinary IAG levels in children with ASD versus controls showed that the IAG: creatinine ratio was significantly higher in individuals with ASD (Bull *et al.* 2003). When a larger group of children with ASD were investigated and compared with matched controls, no significant difference was found (Wright *et al.* 2005). In addition, another study by Whiteley & Shattock (2003) found no overall association between IAG and ASD which they speculated may have been due the effects of anti-epileptic medication taken by participants on HPLC results.

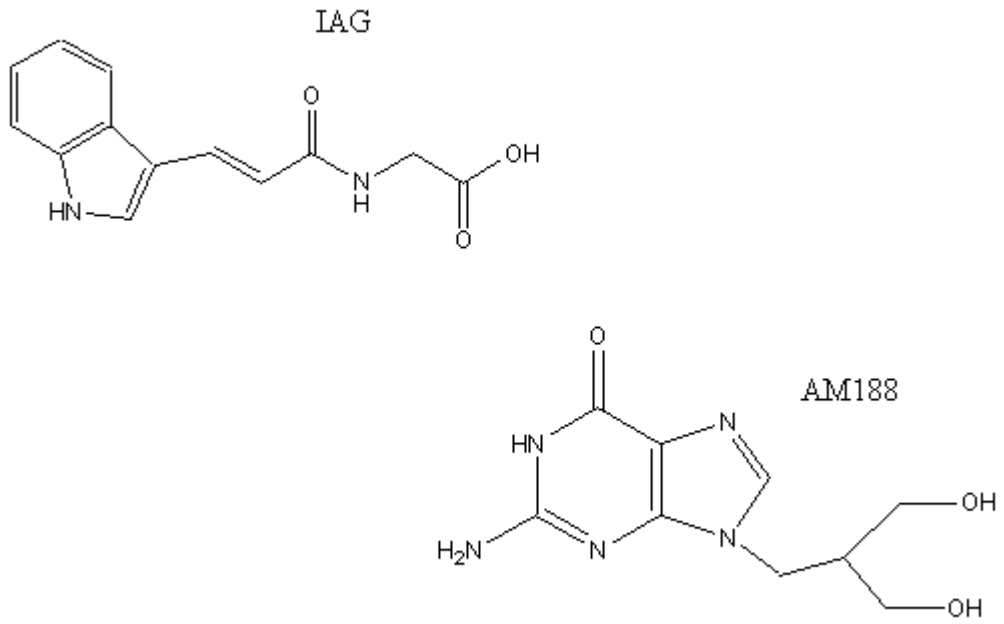


Figure 4.1 Structures of IAG and AM188

4.1.2 Urinary indolyl-3-acryloylglycine and gastrointestinal disturbance

It has been speculated that high levels of IAG in urine are indicative of gut dysbioisis. Shattock & Whiteley (2002) have suggested that because of its planar geometry, indolyl-3-acrylic acid (IAA), the acid precursor of IAG may disrupt membrane structures, and in turn increase the permeability of membranes. Then, excessive levels of endogenous opioid-like peptides as described by Reichelt *et al.* (1997) pass through the intestinal and blood-brain barrier into the brain, which increased social withdrawal and stereotypic behaviours and underpins the aetiology of ASD.

A preliminary study indicated a link may exist between IAG and the peptide gluten (Whiteley *et al.* 1999). Coeliac disease is a GI disorder triggered by gluten ingestion in genetically predisposed individuals. It has been suggested that a link exists between coeliac disease and ASD, at least in subgroups (Erickson *et al.* 2005; Barcia *et al.* 2008). The gluten-free diet is widely implemented in ASD and it has been theorised that is may ameliorate autistic behaviours as gluten/casein peptides have negative pharmacological

effects on attention, brain maturation, social interaction and learning. However, there have only been four randomized controlled trials and evidence for efficacy is conflicting (Millward *et al.* 2008; Johnson *et al.* 2010; Whiteley *et al.* 2010).

There were two major aims in this chapter. Firstly, to clarify whether any differences exists between ASD and control children regarding to urinary IAG levels. The second aim was to determine whether within the ASD group urinary IAG levels are associated with symptoms of ongoing GI disturbance.

4.2 Methods

4.2.1 Participants

Samples were collected from children with ASD (n = 57) enrolled in the Early Intervention Research Program (EIRP) at Flinders University and Autism SA, including 45 with AD and 12 with AS. The inclusion criteria for the ASD group were that a diagnosis of AD or AS had been made by a multi-disciplinary team using the Childhood Autism Rating Scale (Schopler *et al.* 1980) and/or the DSM-IVTR (APA 2000). This is a rigorous process that occurs when individuals with ASD are diagnosed in South Australia and is a requirement to receive services through Autism SA, the key autism service provider in the state. Children who met the criteria for AD or AS but who presented with a co-morbid diagnosis of a chromosomal abnormality, were excluded from the study. Participants who had a primary diagnosis of AD and who also had intellectual disability as a co-morbidity were not excluded. Three of the 57 children with ASD had epilepsy as a co-morbidity. There were 50 typically developing siblings of the ASD cohort as well as 56 age-matched unrelated community controls without a family history of ASD were recruited too. The CON participants were eligible to participate if they did not have a sibling or a first cousin with ASD. ASD was excluded in participants of both the SIB and the CON control groups by administering the Autism Spectrum Screening Questionnaire (ASSQ) (Ehlers *et al.* 1999). The current study did not involve restricting the participants' daily dietary intake or require them to keep daily diet records during the sample collection. The gluten-free diet was implemented for 14 children with ASD and 3 siblings. All families were sent a letter describing the study and invited to express an interest in participating. Written parental

consent was obtained. Two early morning urine samples (first production) were collected on non-consecutive days and stored at -80 °C until analysis.

4.2.2 Clinical data collection

Diagnostic data were obtained from participants' EIRP or Autism SA case records. In addition self-administered surveys were mailed to caregivers. To determine whether their child experienced chronic/ongoing gastrointestinal issues they were asked the following question. "Does your child have any chronic/ongoing GI issues i.e. bloating, diarrhoea, constipation, excessive flatulence, abdominal pain?"

4.2.3 Reagents

HPLC grade acetonitrile was obtained from Biolab (Adelaide, Australia). IAG was purchased from Biosynth AG (Staad, Switzerland). Creatinine and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). AM188 (98% assay purity) (Figure 4.1) was used as an internal standard (IS) (Wang *et al.* 2006) and was purchased from Amrad Corporation Limited (Victoria, Australia). Water (conductivity > 18MΩ at 25 °C) was purified for HPLC analysis using a Milli-RQ ultrapure Water System (Millipore, MA, USA).

4.2.4 Urinary analysis

Urine samples were diluted with HPLC mobile phase (5% acetonitrile in 5 mM aqueous ammonium acetate buffer) and analysed for IAG (1: 100) and creatinine (1: 1000) using LC-MS/MS.

The HPLC system consisted of two Shimadzu LC-10ADVP pumps, a DGU-14A degasser and SIL-HTC auto sampler (Shimadzu, Japan) kept chilled at 10 °C. The separative system consisted of a HPLC Gemini column (C18, 2.0 × 50 mm, 3 µm, Phenomenex, Australia) with a matching guard column (2.0 × 4 mm, 3 µm). The HPLC system was interfaced via an electrospray ionisation (ESI) source with a triple stage quadrupole mass spectrometer (API 3000, Applied Biosystems, Ontario, Canada). Optimal declustering potential (DP), collision energy potentials (CE), collision exit potentials (CXP) and focusing potential (FP) were determined based on the relative intensities of selected product ions (Table 4.1) for each analyte. The needle voltage of the turbo ion spray was optimized at 4500 eV and -4500 eV for positive and negative ion mode with source temperature of 200 °C,

respectively. Nitrogen used as the curtain (CUR) , nebuliser (NEB) and collision (CAD) gas was also shown on Table 4.1.

A gradient mobile phase were using to analyse IAG ranging from 5% acetonitrile in 5mM ammonium (aq) acetate buffer (A) to 95% acetonitrile (B) was used. The gradient proceeded from 0% B at time 0 to 1min, a linear increase to 30% B at 1.1 min until 4 min and then a linear decrease from 30% to 0% B within 0.1min. The total solvent flow rate was 0.2 mL/min. Peaks corresponding to both the *cis*- and *trans*- isomers of IAG were quantified although signals were not assigned specifically to either. The total run-time is 9 minutes per sample and retention times of the two isomers of IAG were 4.6 and 4.9 min. In contrast, a mobile phase of 5% acetonitrile in 5 mM ammonium (aq) acetate buffer was used for creatinine analysis with IS. The total run-time is 4 minutes with retention time of creatinine being 1.2 min and no solvent inference was observed.

4.2.5 Method validation

All samples were randomly analysed within stability parameters established during the method validation. Since IAG and creatinine are endogenous metabolites, mobile phase was used to prepare the standard curve. The validity of this method was tested by studying the matrix effect of urine compared to mobile phase. Urine gave no statistically different result. Six repetitions of quality controls (QCs) analyses gave a variation of less than 6%. The calibration curve was constructed by applying a weighting of y^{-2} of total peak area ratio (IAG/creatinine: IS) versus concentration of IAG/creatinine. All coefficients of correlation (r^2) of the calibration lines (n = 6) were better than 0.995 for IAG and creatinine.

Stability tests of the analytes were performed on six replicates of QCs' concentration after (a) three freeze (-80 °C)-and-thaw cycles in three days, (b) keeping 24 hours at 10 °C in auto-injector and (c) stored at -80 °C for a year, respectively. Concentrations of IAG and creatinine in the stored samples were compared with freshly prepared samples at the same nominal concentration. The difference between the tested samples and freshly prepared samples at three QC concentrations was less than 8%.

Table 4.1 The optimised settings for measurement of IAG, creatinine and AM188 (IS) in tandem mass spectrometry

Analyte	MRM ions (m/z)	DP (eV)	CE (eV)	CXP (eV)	FP (eV)	NEB (L/min)	CUR (L/min)	CAD (L/min)
IAG	243.1→141.9	-37	-28	-10	-200	12	8	6
AM188 (IS)	238.1→149.9	-58	-26	-11	-230	12	8	6
(negative ion pairs)	238.1→220.0	-56	-22	-18	-300	12	8	6
Creatinine	113.9→86.1	28	16	16	200	8	8	4
	113.9→72.2	34	23	13	200	8	8	4
AM188(IS) **(positive ion pairs)**	240.1→152.1	64	30	14	220	8	8	4

DP, declustering potential; CE, collision energy potentials; CXP, collision exit potentials: FP, focusing potential, CUR, curtain; NEB, nebuliser and CAD, collision; IS, internal standard.

4.2.6 Statistical analysis

IAG and IAG: creatinine ratio raw data were skewed to the left. In order to easily make comparisons with the data of Wright *et al.* (2005) study, the data of IAG and IAG: creatinine ratio were normalised by natural logarithm transformation. Analysis of covariance was primarily used to determine the association between either IAG or IAG: creatinine ratio and various subgroups, controlling for any small differences in age between the subgroups by including age as a covariate. A p-value of less than 0.05 was considered statistically significant. Differences between subgroups were visually displayed using a boxplot with the line across the inside of the box representing the median value, the protruding lines (whiskers) from the box go out to the variable's smallest and largest values and the small circles and stars on the graphs are outliers which extend more than 1.5 and 3 times from the edge of the box, respectively. The IAG: creatinine ratio values were age adjusted prior to plotting by using the residuals of the regression model between IAG: creatinine ratio and subject age. Statistical analyses and tests were conducted using SPSS for Windows™ (version 17, SPSS Inc., Chicago, IL, USA, 2008) and the R statistical language.

4.2.7 Ethics approval

Ethics approval for this study was granted by the Human Research Ethics Committees of the University of South Australia and The Flinders University of South Australia in accordance with the Declaration of Helsinki.

4.3 Results

4.3.1 Characteristics of participants

IAG and creatinine were measured in the urine of all participants. The sample size, age, gender of distribution and category of ASD diagnosis of the participants are summarised in Table 4.2. Most participants supplied two morning urine samples on non-consecutive days.

Table 4.2 Characteristics of participants

Participants	Total No.	No. of children who have gluten-free dietary	Age (months) Mean ± S.E.M	Gender Male / Female	Diagnosis (AD /AS)
ASD	57	17	82.7 ± 6.1	48 / 9	45 / 12
SIB	50	2	104.0 ± 8.9	28 / 22	--
CON	56	0	102.0 ± 6.3	28 / 28	--

ASD, autism spectrum disorder; AD, autistic disorder; AS, Asperger syndrome; SIB, typically developing siblings; CON, community controls; S.E.M, standard error of the mean.

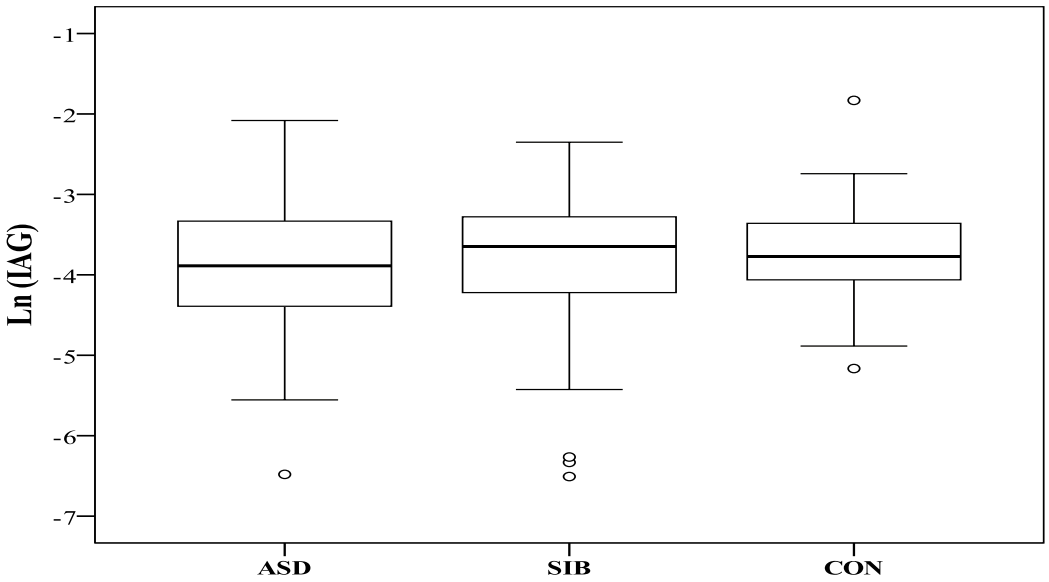
4.3.2 Urinary creatinine and indolyl-3-acryloylglycine

Urinary creatinine concentrations in the CON group were significantly higher than all other groups when compared separately ($p < 0.001$).

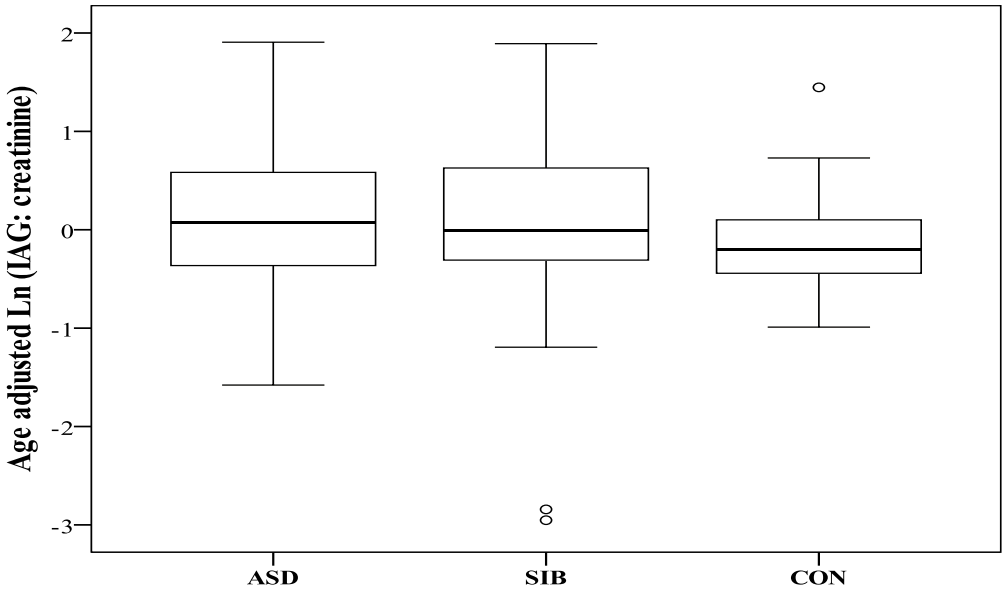
As per Wright *et al*'s (2005) data, the distribution of IAG: creatinine ratio data was skewed to the left and therefore normalised by natural logarithm transformation. Similarly, the natural logarithms of raw IAG values were obtained. The transformed values were used in all subsequent analyses.

Across all study participants there was no statistically significant association between either urinary IAG or the IAG: creatinine ratios and participants' gender ($p = 0.641$ and $p = 0.724$, respectively). However, there was a statistically significant association between the IAG: creatinine ratio and the age of the participants ($r = -0.34$, $p < 0.001$). This was predominantly due to a correlation between age and urinary creatinine concentration ($r = 0.48$, $p < 0.001$). In order to avoid confounding issues due to the small differences in age between the groups, the IAG: creatinine was adjusted for age in subsequent analyses.

The distribution of the resulting urinary IAG and IAG: creatinine ratios (age adjusted) for the ASD, SIB and CON groups is displayed in Figure 4.2 a and 4.2 b, respectively. An analysis of covariance including age as a covariate did not detect a statistically significant difference between the mean IAG and IAG: creatinine in the three groups ($p = 0.664$ and $p = 0.138$, respectively).



(4.2a)



(4.2b)

Figure 4.2 Boxplots displaying the distribution of (a) IAG and (b) age adjusted logarithm of IAG: creatinine in children with ASD, their siblings and community controls.

ASD, autism spectrum disorder; SIB, typically developing siblings; CON, community controls.

The caregiver questionnaire revealed that 46% of the ASD participants had 'GI disturbance' i.e. ASD-GI. Caregivers reported that a further 46% did not experience ongoing GI issues i.e. ASD-NGI. For the remaining 8%, caregivers reported they were 'unsure' whether their child experienced ongoing GI issues. 26% of caregivers of control participants of the SIB group and 5% of the CON group reported chronic GI disturbance (Figure 4.3). In subsequent analyses, individuals unsure about GI status were excluded.

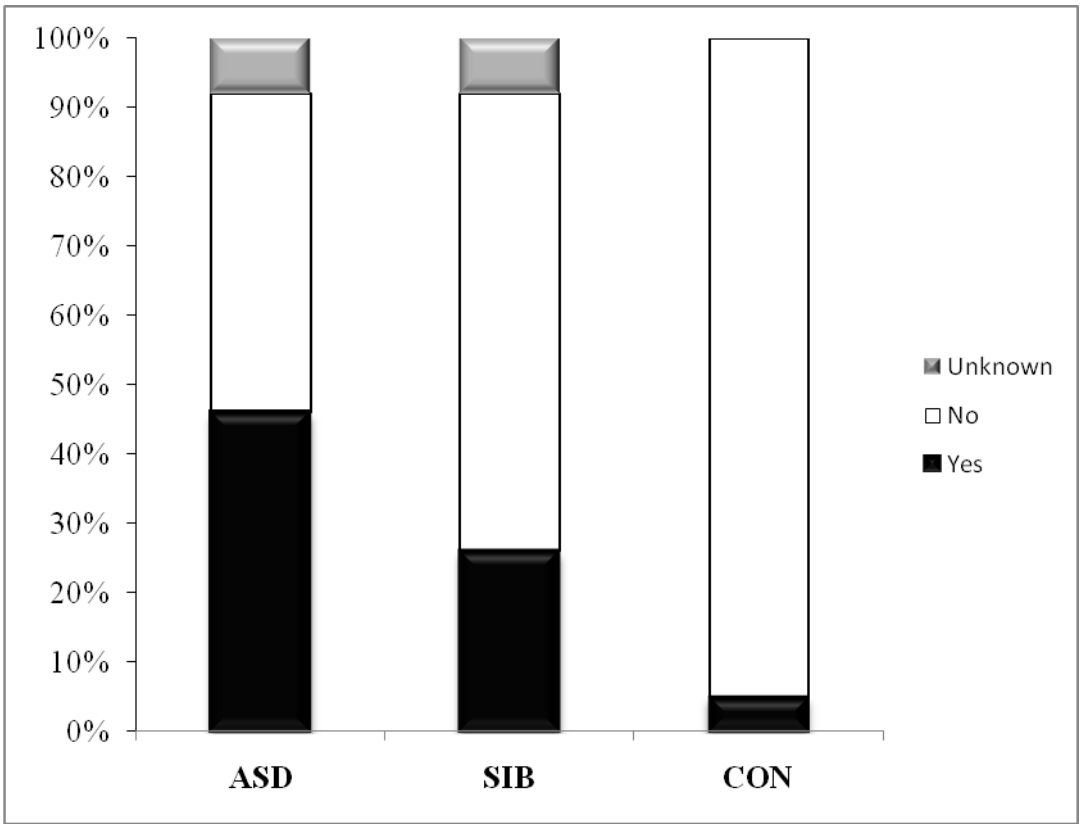


Figure 4.3 The proportion of participants' caregivers reporting GI disturbance in each of the three study groups.

ASD, autism spectrum disorder; SIB, typically developing siblings; CON, community controls.

An analysis of covariance including age as a covariate found that there were statistically significant higher urinary IAG levels and IAG: creatinine ratio in the ASD participants with ongoing GI problems compared to ASD participants without GI problems ($p = 0.006$ and $p = 0.001$, respectively) (Figure 4.4). However, there was no statistically significant

association between either urinary IAG or IAG: creatinine ratio and the presence of GI disturbance in the combined control cohort (p = 0.283 and p = 0.364, respectively). Both the IAG levels and IAG: creatinine ratios were statistically significantly higher in ASD individuals with GI symptoms than the control (combined SIB and CON groups) individuals with GI symptoms.

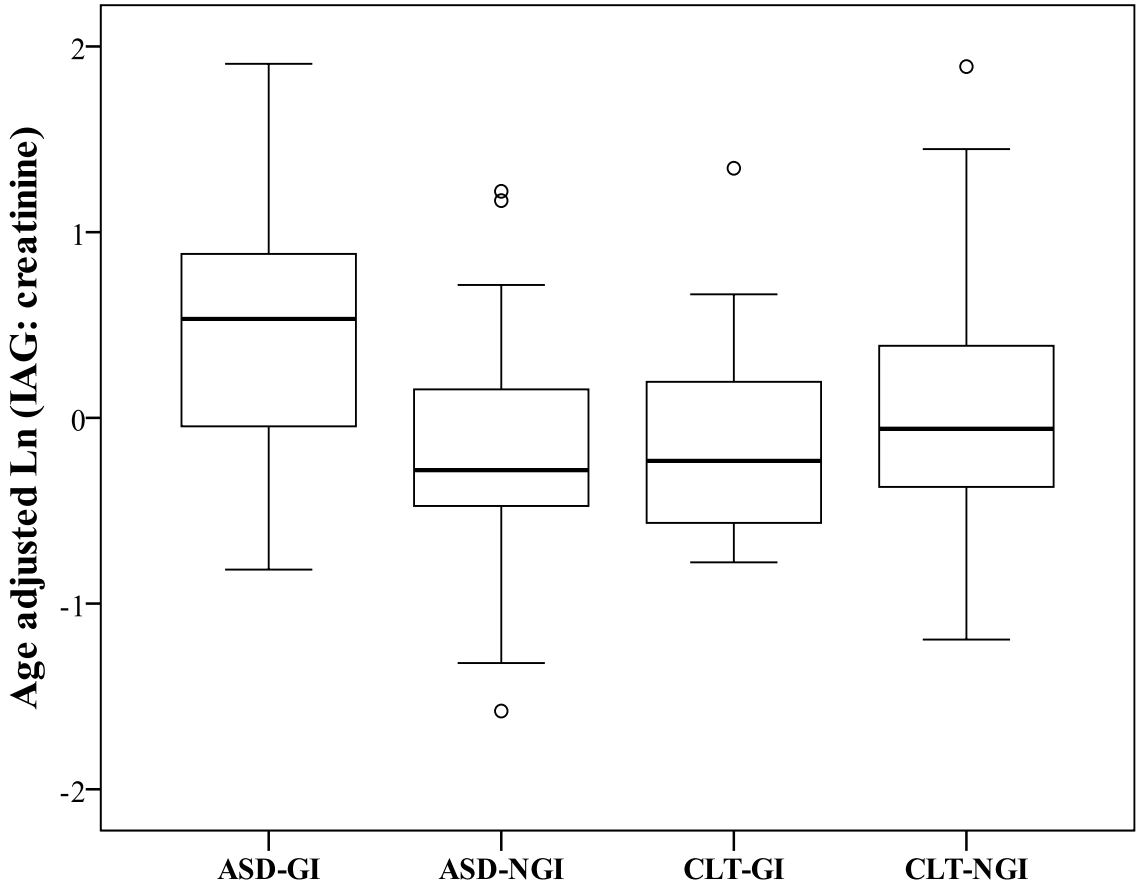


Figure 4.4 Boxplots of the distribution of IAG (age adjusted natural logarithm of IAG: creatinine ratio) in the ASD and combined control groups (SIB and CON) with and without ongoing GI disturbance.

Where: ASD-GI, children with autism spectrum disorder (ASD) with GI disturbance; ASD-NGI, children with ASD without GI disturbance; CLT-GI, combined control groups (SIB+CON) with GI disturbance; CLT-NGI, combined control groups (SIB+CON) without GI disturbance.

4.4 Discussion

The key finding of this chapter was that there were significantly higher urinary IAG levels and IAG: creatinine ratios in children with ASD whose caregivers reported the presence of GI disturbance compared with ASD children whose caregivers reported the absence of ongoing GI issues. It is not clear whether this is a consequence of the GI pathology or a factor that is contributing to the GI symptoms. There is also an issue of whether raised IAG levels are specific to ASD with ongoing GI problems. IAG did not differ between controls (SIB and CON groups) with and without ongoing GI problems. Additionally, ASD individuals with ongoing GI problems had statistically significantly higher IAG levels than control individuals with ongoing GI problems. This suggests that the raised IAG levels may be specific to ASD, but further research is warranted to reproduce this finding and specifically assess whether the IAG plays a causative role in GI disturbance in ASD.

Given the heterogeneity of the aetiology and pathophysiology of ASD it is therefore plausible that the anomalies that are evident with respect to IAG and the IAG: creatinine ratio may provide insights into the aetiology and pathophysiology of specific ASD subtypes. Therefore, while IAG is not useful as a diagnostic biomarker *per se*, it may be a useful biomarker for ASD with associated GI disturbance. This may assist with the selection of targeted interventions that normalise GI disturbance such as probiotics, prebiotics and/or antibiotics. Such interventions may in turn have the potential to mitigate the manifestations of ASD. It is also possible that IAG may be a useful biomarker to monitor response to such interventions.

As with previous studies (Horvath and Perman 2002a, 2002b), the proportion of children with ongoing GI issues was greater in the ASD group compared with control groups. Additionally, the frequency of GI issues in the sibling controls was intermediate between the ASD and the community controls, which is concordant with a previous study (Horvath and Perman 2002a). It is clear that ASD has a genetic basis from twin studies whereby 60-90% of cases have concordant expression of ASD or a related disorder (Veenstra-VanderWeele and Cook 2004). The rate of sibling recurrence of ASD is up to 8% (Muhle *et al.* 2004). It is therefore conceivable that the intermediate frequency of GI disturbance that was observed in the SIB group compared with the ASD and the CON groups is genetically pre-determined.

There was no statistically significant difference between the mean IAG and IAG: creatinine in the three groups, although there was a trend towards the IAG: creatinine ratio being higher in the ASD group compared to the two control groups. This is concordant with the study by Wright *et al.* (2005). The mean urinary IAG concentration in this study was very similar to Wright *et al*'s (2005) results. When IAG: creatinine results were transformed using the same approach as Wright *et al.* (2005) without age adjustment, the urinary IAG: creatinine (mean ± standard deviation) for the ASD and combined controls were -5.38 ± 0.61 and -5.62 ± 0.50 respectively compared to the report of -5.29 ± 0.62 and -5.25 ± 0.48 for an ASD and control cohort, respectively (Wright *et al.* 2005). However, as Bull *et al.* (2003) reported IAG levels as the area under the peak rather than an absolute concentration, it is not possible to directly compare the levels between studies.

A previous study examining age variations of IAG excretion vs. urinary creatinine levels in healthy children showed that the ratio of urinary IAG (mg) : creatinine was much higher in children aged 2 to 6 but decreased to lower levels and remained low until advanced age (Marklova and Hais 1978). In this chapter, urinary IAG: creatinine had a statistically significant negative correlation with the age of the participants due to the strong association between age and urinary creatinine concentration. Furthermore, a trend towards a decline in urinary IAG: creatinine with age in ASD children was also found by Wright *et al.* (2005), but it was not statistically significant. Bull *et al.* (2003) found no relationship with age, but their cohort did not include very young children. Due to the potential confounding nature of age, age was included as a covariate in the analysis of covariance tests, as was the case with the study by Wright *et al.* (2005).

It is generally accepted that the excretion of creatinine is relatively fixed over time and for that reason urinary metabolites are reported as a ratio of creatinine. Creatinine was elected to normalise urinary IAG in this study thus enabling differences in urinary dilution to be corrected. However, it was notable that the urinary creatinine concentrations in the CON group were much higher than all other groups when compared separately. These findings are congruent with a previous study where urinary creatinine was reported to be lower in children with PDD compared with controls (Whiteley *et al.* 2006). Impaired growth or significant differences in muscle mass were not prevalent in children with ASD as compared to those without ASD. Thus, neither condition was a likely explanation for the reduced urinary creatinine excretion seen in the ASD group (Adams-Chapman and Stoll

2006). In the current chapter, it was noticed that creatinine had a strong association with age which is expected as muscle mass increases with age in childhood. Due to the potential confounding nature of age, age was included as a covariate in the analysis of covariance tests. Thus although it seems that use of creatinine to normalise urinary IAG concentrations is problematic, it is the standard method used and the results from this study are consistent whether urinary IAG concentration or urinary IAG: creatinine ratio is used.

Of the 26 children with ASD who had GI disturbance, ten were implementing a gluten-free diet. There was no clear correlation between urinary IAG levels and whether the ASD-GI participants implemented a gluten-free diet or not. This finding is consistent with an earlier study by Whiteley *et al.* (1999) who found no significant changes in urinary IAG levels in participants before and after a period of gluten-free dietary intervention, although a downward trend of urinary levels of IAG was shown for individuals on the diet in Figure 3b. Given the samll sample size, it may be concluded that participants' IAG levels in the current study were unlikely to be affected by implementation of a gluten-free diet.

There was no statistically significant difference in the urinary IAG or IAG: creatinine ratio between males and females in this study. Differences in urinary IAG excretion between genders have not previously been reported in the literature. Thus, although the gender distribution of the ASD sample was 5:1 (male: female) compared with 1:1 amongst controls, the current study sample's gender ratio, was consistent with literature reports of the ASD gender ratio (Whiteley *et al.* 2010). It is therefore unlikely that this has significantly biased the comparison.

It should be noted that in this study, both typically developing siblings and unrelated community children were selected as distinct control groups. Previously, other studies stated their controls were children without ASD but it is not clear whether the controls were unrelated to the ASD participants or ASD participants' siblings, or a mixed cohort (Bull *et al.* 2003; Wright *et al.* 2005). Given the genetic basis of ASD, it is important that sibling controls and unrelated controls are considered as distinct comparator groups.

A limitation of this study is the observational cross-sectional study design that does not allow determination of whether IAG levels proceed and influence GI disturbance in ASD or are only a marker of the disturbance. Similarly, it would be useful for future studies to utilise more objective measures of illness severity in functional GI disorders. Study of IAG

in other groups with GI disturbance will reveal greater insight into whether raised IAG levels are specific to GI disturbance in ASD. Due to the small number of individuals with AS it was not possible to determine whether IAG relationship with GI disturbance differed between individuals with AD and AS.

4.5 Conclusion

This study reports for the first time that a significantly higher urinary IAG levels and IAG: creatinine ratio in children with ASD whose caregivers reported the presence of GI disturbance compared with children with ASD without ongoing GI issues. Additionally, the IAG levels were higher in children with ASD and ongoing GI issues than in control children with ongoing GI issues, suggesting that the raised levels may be specific to ASD. Further research is required to replicate these findings, directly study the specificity of IAG to GI disturbance in ASD, and to determine whether IAG plays a causal role in GI disturbance of ASD.

5 Urinary Creatine and Guanidinoacetate in Children with Autism Spectrum Disorder

5.1 Introduction

In addition to GI disturbance as a co-morbidity in ASD, several metabolic defects have been associated with autistic symptoms with a rate higher than that found in the general population. Inborn errors of metabolism account for less than 5% of individuals with ASD (Manzi *et al.* 2008). Creatine deficiency syndrome (CDS) is an inborn error of metabolism and individuals with CDS share several clinical features with ASD. Abnormal levels of urinary creatine (CR) and guanidinoacetate (GAA) levels have been reported in CDS and as such form the basis of laboratory diagnostic criteria for individuals with CDS. This study sought to determine if differences exist in urinary CR and GAA between children with ASD and sibling and community controls.

5.1.1 Creatine deficiency syndrome and autism spectrum disorder

CDS is an inborn errors of metabolism that is currently not routinely screened for during standard ASD diagnostic processes. Individuals with CDS have dysfunctional creatine synthesis or creatine transport, which include arginine: glycine amidinotransferase (AGAT) deficiency, guanidinoacetate methyltransferase (GAMT) deficiency and creatine transporter (CRTR) deficiency (Schulze 2003; Stromberger *et al.* 2003).

All individuals with CDS have absent or very low levels of CR in the CNS. CR plays a key role in neurological development and functioning and is essential for growth cone migration as well as dendritic and axonal elongation in the thalamus and hippocampus (Kan *et al.* 2007). Further, CR plays a role in Na+/K+-ATPase activity, neurotransmitter release and maintenance of membrane potentials (Braissant and Henry 2008). CR has also been hypothesised to act as a central neuromodulator, and particularly as a co-transmitter on gamma aminobutyric acid postsynaptic receptors (Almeida *et al.* 2006).

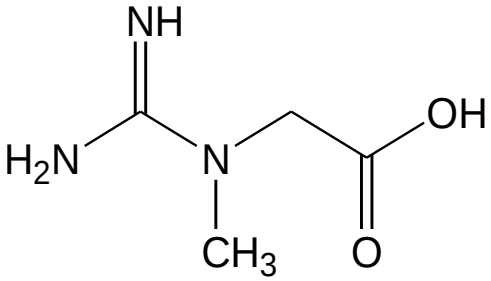
The common clinical features of CDS are developmental delay/regression, intellectual disability, epilepsy and severe disturbance in expressive and cognitive speech (Stockler *et al.* 2007). In comparison, as many as 35% of individuals with AD have epilepsy (Turk *et al.* 2009) and approximately 50% of individuals with ASD have an intelligence quotient in the intellectual disability range (Charman 2008). Language impairment is one of the three core deficits observed in ASD together with impairments in socialisation and repetitive and

restricted behaviours. A review of 20 studies by Schulze (2003) found 8 in 12 patients with CDS had features consistent with ASD.

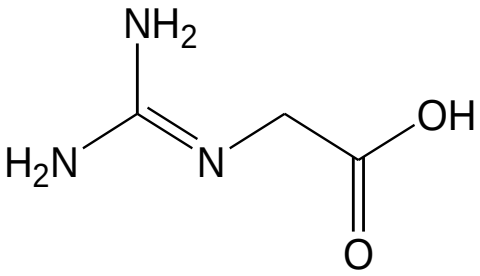
5.1.2 Biomarkers of creatine deficiency syndrome

A clinical diagnosis of CDS is based on determination of abnormal ratios of CR: creatinine and GAA: creatinine in urine (Stockler *et al.* 1994; Schulze *et al.* 1997; Schulze 2003). Clark *et al.* (2006) showed approximately 1% of males with intellectual disability of unknown origin had mutations in CR transporter genes and recommended that a CR: creatinine urine screen was warranted in this population. From the literature it is evident that patients with CRTR or GAMT deficiency have ratios of CR: creatinine and GAA: creatinine that are at least twice the normal population (Almeida *et al.* 2004).

Individuals with GAMT deficiency are unable to convert GAA to creatinine and can be treated with dietary restriction of arginine and supplementation of ornithine. This intervention prevents the endogenous synthesis of GAA and reduces its build because it cannot be converted to CR in GAMT deficiency. Further, early supplementation with CR may effectively prevent the neurological sequelae in AGAT deficiency patients (Sykut-Cegielska *et al.* 2004). In contrast, CRTR deficiency does not respond to dietary modification or supplementation as it is a transporter dysfunction.



Creatine



GAA

Figure 5.1 Structure of creatine and GAA

It is conceivable that inborn errors of metabolism that are not routinely screened for may contribute to the aetiology of ASD. Thus, it is hypothesised that CR and GAA (Figure 5.1)

may also be present in abnormal levels in the urine of children with ASD and screening may assist with diagnosis and/or subtyping of children with ASD. If present, they may be able to be used as biomarkers as well as assist with identification of targeted nutritional interventions for ASD management. In the current chapter, whether differences existed in urinary CR: creatinine and GAA: creatinine ratios between children with ASD, sibling and community controls was investigated.

5.2 Methods

5.2.1 Participants

Two morning urine samples were collected on non-consecutive days over a week from 57 children with ASD , 49 typically developing siblings and 49 unrelated community controls as described previously (please see section 4.2.1). Samples were stored at -80 °C until analysis.

5.2.2 Reagents

HPLC grade acetonitrile and tetrahydrofuran were obtained from Biolab (Adelaide, Australia). CR, GAA, creatinine and ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO, USA). AM188 (98% assay purity) was used as an internal standard (IS) (Wang *et al.* 2006) and purchased from Amrad Corporation Limited (Victoria, Australia). Water was purified for HPLC analysis using a Milli-RQ ultrapure Water System (Millipore, MA, USA).

5.2.3 Urinary analysis

All urine samples tested for CR and GAA were diluted 100-fold with mobile phase (50% acetonitrile, 1% tetrahydrofuran in 10 mM ammonium formate buffer) and analysed in a randomised fashion using LC-MS/MS. The HPLC system consisted of two Shimadzu LC-10ADVP pumps, a DGU-14A degasser and SIL-HTC auto sampler (Shimadzu, Japan) kept chilled at 10 °C.

5.2.3.1 Creatine and guanidinoacetate

10 µL of samples were injected onto a phenyl column (3.9 × 150 mm, Waters, USA) with a matching guard column (3.0 × 4 mm, Phenomenex, Australia). Samples were delivered at a flow rate of 0.2 mL/min. The HPLC system was interfaced with a triple stage quadruple mass spectrometer (API 3000, Applied Biosystems, Ontario, Canada) via an electrospray source. The DP, CE, CXP and FP were determined based on the relative intensities of selected product ions (Table 5.1) for each analyte. The needle voltage of the turbo ion spray was optimised at 5500 eV for positive ion mode with source temperature of 200 °C. Nitrogen used as the curtain , nebuliser and collision gas was also shown on Table 5.1. Instrument control, data acquisition and data analysis were performed with Analyst software V14 (Applied Biosystem, Ontario, Canada).

5.2.3.2 Creatinine

Urinary creatinine was determined as described previously (please see section 4.2.4).

5.2.4 Method validation

All samples were randomly analysed within stability parameters established during the method validation. Since CR and GAA are endogenous metabolites, mobile phase was used to prepare the standard curve. The validity of this method was tested by studying the matrix effect of urine compared to mobile phase. Urine gave no statistically different result. Six repetitions of QCs analyses gave a variation of less than 6%. The calibration curve was constructed by applying a weighting of y^{-2} of total peak area ratio (CR/GAA: IS) versus concentration of CR/GAA. All coefficients of correlation (r^2) of the calibration lines (n = 6) were better than 0.996 for CR and GAA.

Table 5.1 The optimised settings for measurement of creatine, guanidinoacetate, creatinine and AM188 (IS) in tandem mass spectrometry

Analyte	MRM ions (m/z)	DP (eV)	CE (eV)	CXP (eV)	FP (eV)	NEB (L/min)	CUR (L/min)	CAD (L/min)
Creatine	132.1→90.1	37	18	15	210	12	8	6
GAA	118.1→101.1	25	15	9	200	12	8	6
	118.1→76.1	29	18	13	190	12	8	6
Creatinine	113.9→86.1	28	16	16	200	8	8	4
	113.9→72.2	34	23	13	200	8	8	4
AM188(IS)	240.1→152.1	64	30	14	220	8	8	4

DP, declustering potential; CE, collision energy potentials; CXP, collision exit potentials: FP, focusing potential, CUR, curtain; NEB, nebuliser and CAD, collision; GAA, guanidinoacetate; IS, internal standard.

Analyte stability tests were performed on six replicates of QCs' concentration after (a) three freeze (-80 °C)-and-thaw cycles, (b) keeping 24 hours at 10 °C in auto-injector and (c) stored at -80 °C for a year, respectively. Concentrations of CR and GAA in stored samples were compared with fresh samples at the same concentrations. The differences between the stored and fresh samples at three QC concentrations were less than 8%.

5.2.5 Statistical analysis

As the distributions of CR: creatinine and GAA: creatinine were skewed to the left, data were normalised by natural logarithm transformation. Transformed values were used in subsequent analyses. A p-value of less than 0.05 was considered statistically significant. Differences between subgroups were visually displayed using a boxplot with the line across the inside of the box representing the median value, the protruding lines (whiskers) from the box go out to the variable's smallest and largest values and the small circles and stars on the graphs are outliers which extend more than 1.5 and 3 times from the edge of the box, respectively. The CR: creatinine and GAA: creatinine values were age adjusted prior to plotting by using the residuals of the regression model between CR/GAA: creatinine and subject age. Statistical analyses and tests were conducted using SPSS for Windows™ (version 17, SPSS Inc., Chicago, IL, USA, 2008) and the R statistical language.

5.2.6 Ethics approval

Ethics approval was granted by the University of South Australia's and The Flinders University of South Australia's ethics committees in accordance with the Declaration of Helsinki.

5.3 Results

5.3.1 Characteristics of participants

Participants' gender, age and diagnosis are summarised in Table 5.2. Although the recruitment of participants was opportunistic, the mean ages of the three groups were similar. The gender ratio between males and females in the ASD and control groups was 5:1 and 1:1, respectively.

Table 5.2 Characteristics of participants

Participants	No.	Age (month) Mean ± S.E.M	Gender Male / Female	Diagnosis (AD / AS)	Epilepsy
ASD	57	82.7 ± 6.1	46 / 11	45 / 12	3 / 57
SIB	49	106.1 ± 9.0	28 / 21	--	--
CON	49	102.2 ± 6.8	28 / 21	---	--

ASD, autism spectrum disorder; SIB, typically developing siblings; CON, community controls; AD, Autistic Disorder; AS, Asperger Syndrome; S.E.M, standard error of the mean.

5.3.2 Urinary creatine and guanidinoacetate

Urinary CR, GAA and creatinine levels were measured in the two urine samples taken from all participants. Excretion of creatinine is considered to be relatively fixed over time and thus urinary CR and GAA are reported as a ratio of creatinine to enable differences in urinary dilution to be corrected. The average values for CR: creatinine and GAA: creatinine were determined to compensate for day-to-day variation and are shown in Table 5.3.

Table 5.3 Average of urinary CR: creatinine and GAA: creatinine (mmol/mol) in two days' samples in ASD, SIB and CON participants

Participants	CR: creatinine (mmol/mol) Mean ± S.E.M	GAA: creatinine (mmol/mol) Mean ± S.E.M
ASD	415.5 ± 67.1	51.6 ± 3.3
SIB	443.7 ± 84.3	57.9 ± 6.8
CON	261.5 ± 39.4	45.7 ± 4.0

ASD, autism spectrum disorder; SIB, typically developing siblings; CON, community controls; S.E.M, standard error of the mean.

Urinary GAA: creatinine for all the participants were within the same ranges as described previously (Almeida *et al.* 2004; Valongo *et al.* 2004). The 5-95th ranges of urinary CR: creatinine were 0.01-1.63, 0.01-1.89 and 0.01-1.35 for the ASD, SIB and CON, respectively, which were also within the same ranges as described previously for controls (Almeida *et al.* 2004; Valongo *et al.* 2004; Mercimek-Mahmutoglu *et al.* 2009). However, there were nine participants including six with ASD and three siblings, where on one sampling day their urinary CR: creatinine was increased up to 2-fold when compared to literature control values (Almeida *et al.* 2004; Valongo *et al.* 2004). There was a statistically significant association between the CR: creatinine and GAA: creatinine and the age of the participants ($r = -0.56$, $p < 0.01$ and $r = -0.39$, $p < 0.01$) therefore ratios were age adjusted in subsequent analyses as displayed in Figures 5.2 & 5.3. There were no statistically significant differences between CR: creatinine and GAA: creatinine within the ASD, SIB and CON groups ($p = 0.180$ and $p = 0.222$, respectively).

In addition, there were no statistically significant differences between genders in urinary CR: creatinine and urinary GAA: creatinine within the ASD ($p = 0.486$ and $p = 0.185$, respectively), or SIB ($p = 0.719$ and $p = 0.664$, respectively) and CON groups ($p = 0.866$ and $p = 0.641$, respectively).

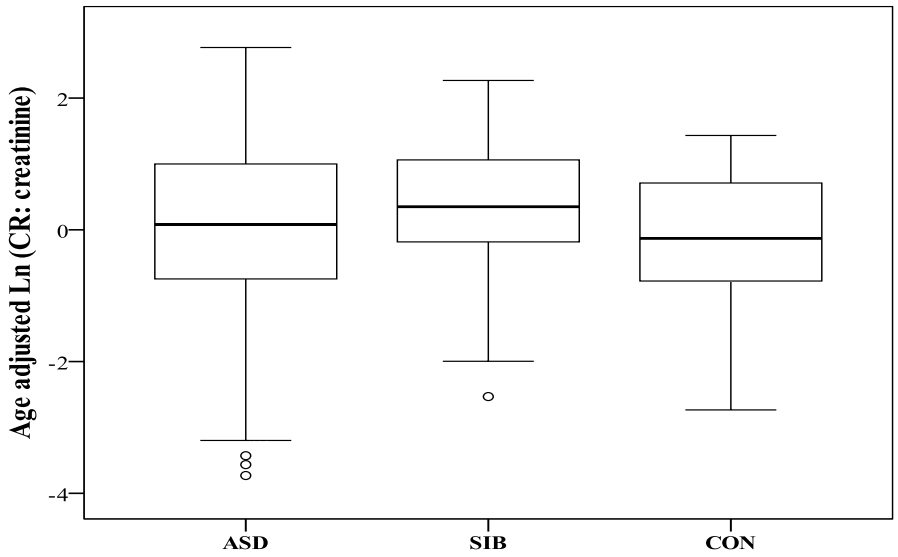


Figure 5.2 Boxplots displaying the distribution of CR (age adjusted natural logarithm of CR: creatinine) in ASD, SIB and CON participants.

ASD, autism spectrum disorder; SIB, typically developing siblings; CON, community control.

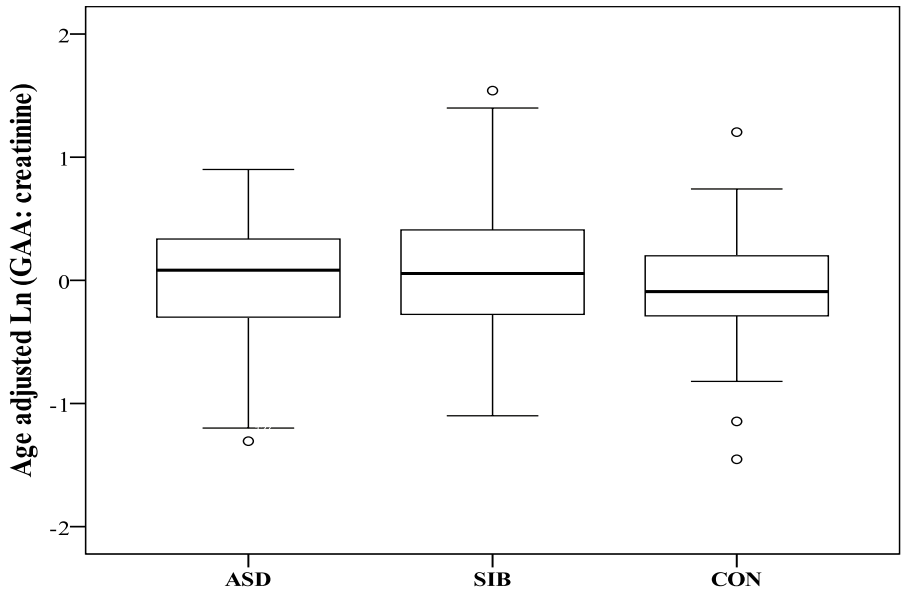


Figure 5.3 Boxplots of the distribution of GAA (age adjusted natural logarithm of GAA: creatinine) in the ASD, SIB and CON participants.

ASD, autism spectrum disorder; SIB, typically developing siblings; CON, community control.

5.4 Discussion

It has been suggested that learning disability, autistic features, and severe language disorders are due to depletion of brain CR, whereas epilepsy and neurological deterioration is a result of neurotoxic effects of GAA (Manzi *et al.* 2008). However, in the current study, no significant differences in urinary CR: creatinine and GAA: creatinine were found between ASD, SIB and CON participants indicating that inborn errors of creatine synthesis and creatine transport are unlikely to play a role in the pathogenesis of the majority of ASD cases. Although the mean CR: creatinine ratio in the CON participants appeared lower than the ASD and SIB participants, it was not statistically significant.

Although the participants were not exactly age matched, the impact of the small differences in age were adjusted for in the multivariate analysis. Therefore, age is unlikely to confound results. These findings regarding the influence of age and the CR: creatinine and GAA: creatinine ratios were consistent with Valongo *et al*'s study (2004) which showed these ratios in children no more than 4 years were significantly higher than children more than 4 years.

There was no statistically significant difference in the urinary CR: creatinine or GAA: creatinine between males and females in this study. Differences in urinary CR or GAA excretion between genders have not previously been reported in the literature. Thus, although the gender distribution of the current ASD sample was 5:1 (male: female) compared with 1:1 amongst controls, it is unlikely that this significantly biased the comparison.

A previous study by Arias *et al.* (2007) suggested 23 out of 33 cases of urinary CR: creatinine tests were false positives and could be attributed to prior consumption of a meal based on beef or oily fish as compared to eggs, pasta or salad. We found nine participants had a higher urinary CR: creatinine compared to literature values (Almeida *et al.* 2004; Valongo *et al.* 2004). This finding occurred on only one collection day but not the other. The resulting means of CR: creatinine ratio for all participants had large standard deviations. These variations may be due to participants' consumption of varying amounts of dietary protein. This also highlights the potential for confounding results if urinary GAA and CR are used as biomarkers for CDS (Arias *et al.* 2007). Therefore, sampling on

at least two different days and restricting protein consumption prior to sampling is an approach that will minimise intra-individual variation.

5.5 Conclusion

Routinely measuring urinary CR: creatinine or GAA: creatinine in diagnostic protocols for ASD is unlikely to yield many positive cases of CDS. However, as both markers are adequately sensitive to detect patients with CDS and measurement of CR and GAA is inexpensive and non-invasive, routine screening for GAMT or CRTR deficiency during the diagnostic work up process should be considered for all cases of suspected ASD. Early detection of CDS cases guides implementation of dietary interventions which can dramatically improve outcomes for affected individuals.

6 Faecal Large Bowel Fermentation Products in Children with Autism Spectrum Disorder

6.1 Introduction

A higher frequency of GI disturbance in children with ASD was described in Chapter 4. Specifically, abnormal levels of urinary IAG were reported between children with ASD with ongoing GI disturbance compared with those children without GI disturbance. The same urine samples as those used for the IAG, CR and GAA studies described in Chapters 4 and 5 were reanalysed by the Biomolecular Medicine, Division of Surgery and Cancer, Faculty of Medicine, Imperial College, London for broad metabolomic screening using ^{1}H NMR. An altered urinary metabolite profile was found in children with ASD. Specifically, higher levels of urinary dimethylamine, and lower levels of both urinary hippurate and *p*-cresol sulphate in children with ASD compared with controls were reported. It was concluded that the altered urinary metabolite profile was likely to be indicative of perturbation of GI bacteria (Yap *et al.* 2010). These findings highlighted that underlying GI factors, particularly levels of gut fermentation products in ASD required further investigation.

Large bowel fermentation products have beneficial or detrimental effects on GI health but are largely uncharacterised in children with ASD. In this Chapter, measurement of faecal levels of key fermentation products in children with and without ASD was investigated to examine whether there is an underlying disturbance in gut bacterial fermentation processes in ASD.

6.1.1 Short chain fatty acids

A well-balanced intestinal microbiota plays a crucial role in maintaining the GI tract and general health of the host. There is evidence that GI microbiota differ between children with and without ASD (Finegold *et al.* 2002; Song *et al.* 2004; Parracho *et al.* 2005). One way the large bowel microbial population can impact on health is through the fermentation of undigested food components and the subsequent production of a range of bioactive compounds. These include SCFA, primarily butyrate, acetate and propionate, which are the predominant products of carbohydrate fermentation and are generally regarded as beneficial to the host, especially the GI tract (Topping and Clifton 2001).

Butyrate is the primary source of energy for epithelial cells (Roediger 1982) and contributes to the maintenance of colonic tissue integrity through actions such as apoptosis,

cell differentiation and gene expression (Lambert *et al.* 2002). Butyrate can also regulate colonic motility, increase colonic blood flow and may enhance colonic anastomosis healing (Velazquez *et al.* 1997). A small proportion of the SCFA taken up by the colon reaches circulation and acetate and propionate in particular are further metabolised by the liver and used in lipogenesis and gluconeogenesis (Hijova and Chmelarova 2007). Furthermore, propionate can contribute to physiological and neurological processes including neurotransmitter release and synthesis, cell signalling, immune function and mitochondrial function (MacFabe *et al.* 2007).

6.1.2 Phenols and ammonia

Fermentation products other than SCFA, particularly protein fermentation by-products such as phenols, amines, ammonia and hydrogen sulphide, can be adverse to host health and toxic to the large bowel (Nowak and Libudzisz 2006; Meletis and Zabriskie 2008).

Phenolic compounds are formed following bacterial degradation of the aromatic amino acids including phenylalanine, tyrosine and tryptophan (Hughes *et al.* 2000). Faecal levels of phenols, particularly high levels of phenol and *p*-cresol may have been implicated in colonic cancer (Nowak and Libudzisz 2006) and inflammatory bowel disease (Ramakrishna *et al.* 1991). *In vitro* work has shown that phenols may enhance the N-nitrosation of dimethylamine by nitrite and the reaction between phenol and nitrite produces the mutagen diazoquinone (Kikugawa and Kato, 1988). Most phenolic compounds are absorbed from the colon into the systemic circulation, detoxified by liver and then excreted in the urine or remain unabsorbed and excreted in the faeces (Ramakrishna *et al.* 1989). More recently, high levels of urinary total *p*-cresol (Altieri *et al.* 2011) and lower urinary levels *p*-cresol sulphate (Yap *et al.* 2010) were reported in children with ASD compared with controls.

Similarly with phenols, ammonia, another protein fermentation product, enters the systemic circulation from the GI tract and is rapidly converted to urea in the liver and subsequently excreted in the urine (Summerskill and Wolpert 1970). High colonic levels of ammonia can damage the epithelial cells in the colon and increase gut permeability (Meletis and Zabriskie 2008). Accumulation of ammonia in the brain can disturb brain metabolism, cause extracellular potassium accumulation and hence may increase neuronal depolarization (Felipo and Butterworth 2002). Several studies have described elevated

plasma ammonia levels in children with ASD relative to controls (Clark-Taylor and Clark-Taylor 2004; Filipek *et al.* 2004) but faecal levels of ammonia have not been analysed in children with ASD compared with controls.

Given the significant role of fermentation products in GI health, it is important to know whether levels of these products are altered in the large bowel of children with ASD and if so whether alterations in fermentation processes contribute to GI dysfunction often found in ASD.

6.2 Methods

6.2.1 Participants and sample collection

Samples were collected from children with ASD (n = 23) recruited through Autism SA. The inclusion criteria for the ASD group were that a diagnosis of ASD had been made by a multi-disciplinary team using the Childhood Autism Rating Scale (Schopler *et al*. 1980) and/or the DSM-IVTR (APA 2000). Children who met the criteria for ASD but who presented with a co-morbid diagnosis of a chromosomal abnormality were excluded from the study. Participants who had a primary diagnosis of ASD and who also had intellectual disability as co-morbidity were not excluded. There were 22 typically developing siblings of the ASD cohort in one control group. Also, nine community controls without a family history of ASD were recruited via advertisements in community newspapers. The CON participants were eligible to participate if they did not have a sibling or a first cousin with an ASD. ASD was excluded in participants of both the SIB and the CON control groups by administering the ASSQ (Ehlers *et al*. 1999). All families were sent a letter describing the study and invited to express an interest in participating. Written parental consent was obtained. Participants' caregivers were required to complete three questionnaires. One questionnaire assessed the presence of functional GI disorders (Smith *et al.* 2009). Children with and without ASD who had severe GI conditions were excluded from participating. Another enquired about use of medications (conventional and complementary) in participants. The third was a dietary questionnaire (Cancer Council, Victoria), that was analysed by the Nutritional Assessment Office, Cancer Epidemiology Centre, Cancer Council Victoria, and enabled determination of participants' daily consumption of macronutrients and micronutrients.

Fresh faecal specimens were collected over a 48 h period. Each bowel movement was packaged separately and frozen directly after collection and stored at -20 °C. Specimens were transported frozen to the laboratory and stored at -80 °C until processing. Specimens were defrosted at room temperature and all processing was performed under anaerobic conditions (Bactron IV anaerobic chamber, Sheldon, USA). Each stool mass was recorded prior to combining the 48 h collection from each participant. The specimen was thoroughly mixed by hand homogenisation and faecal aliquots were taken for ammonia, SCFA, phenol and total *p*-cresol analyses. For dry matter determination, samples were weighted and then freeze-dried until the weight remained constant. The moisture content was determined from the difference between the initial and final weights.

6.2.2 Reagents

Ammonium chloride, phenol, sodium nitroprusside, sodium hydroxide, phosphoric acid, *o*-cresol, *p*-cresol, glacial acetic acid, acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, and heptanoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) and 96% ethanol were purchased from Merck (Whitehouse Station, NJ, USA) and sodium hypochlorite solution (12.5%) was obtained from Fronine Laboratory Supplies (Taren Point, NSW, Australia).

6.2.3 Measurement of ammonia

Ammonia concentrations in faecal specimens were determined using an indophenol blue colorimetric reaction according to a modified method of Chaney and Marbach (1962). Briefly, duplicate 0.50 - 0.55 g faecal specimens were diluted by adding 9 × volume of weight of Milli-Q water and mixed until homogenous. Samples were then centrifuged at 2000 × *g* for 10 min at 4 °C in a Beckman GPR centrifuge (Ramsey, MN, USA). Supernatant was transferred to 1.7 mL tubes and stored at -20 °C until analysis. The 96-well plates were set up as follows: standards (50 µL) (0.04, 0.08, 0.16, 0.24, 0.32 and 0.40 µmol/mL) in duplicate, QCs 0.20 µmol/mL in quadruplicate and samples in quadruplicate. The equation for the standard curve was Y= (2.5 ± 0.07) X + (0.08 ± 0.01). Samples were thawed and vortex mixed, then 5 µL of each sample extract were pipetted into wells to which 45 µL of Milli-Q water was added. An aliquot of 100 µL of Reagent A (10 g/L phenol and 50 mg/L sodium nitroprusside) was added to all wells followed by pipette 100 µL Reagent B (5 g/L sodium hydroxide and 400 mg/L sodium hypochlorite). The plate was

covered with a ThermalSeal and placed in the Plate Reader (Molecular Devices Spectra Max 250) with 1 h incubation at 30 °C. Thereafter, the Thermal Seal was removed and the plate read to determine the concentration of ammonia present in µmol/g.

6.2.4 Measurement of phenol and *p*-cresol

Faecal phenol and *p*-cresol concentrations were investigated according to a method of King *et al.* (2009). The glucuronide and sulphate forms of *p*-cresols that enter the large intestine in bile are hydrolysed to their free forms (King *et al.* 2009). Thus, faecal phenol and total *p*-cresol concentrations were measured in the current study. Briefly, duplicated samples (0.5 g) were added in 10 mL of internal standard (10 µg/mL *o*-cresol in water). Then, samples were mixed thoroughly by vortex and centrifuged (2000 × *g*) for 15 min at 10 °C. An aliquot of 150 µL was taken from each tube into a 5-mL Quick fit flask for vacuum distillation. The distillate was collected into a chilled receiving flask prior to injection. The HPLC system consisted of a Shimadzu LC-10AD instrument with an auto-sampler at 7 °C, an RF-10AXL fluorescence detector set at excitation 284 nm and emission 310 nm, an SIL-10A auto-injector, an LC-10AT pump, and a DGU-12A degasser (Shimadzu, Japan). The injection volume was 30 µL, and the flow rate was 1 mL/min. The instrument was controlled by Shimadzu Lab Solutions software (version 1.21). Separation was achieved with a column (C18, 250 × 4.6 mm, 5 µm, Varian, Victoria, Australia). The mobile phase contained 30% acetonitrile adjusted to pH 3.2 with glacial acetic acid. The limit of detection for faecal phenol and total *p*-cresol were 20 ng/g and 100 ng/g, respectively.

6.2.5 Measurement of short chain fatty acids

The measurement of faecal SCFA levels preformed on duplicate samples (1 g) which diluted by 3-fold with internal standard (1.68 mmol/L heptanoic acid). Samples were mixed well by vortex and pH values were determined, followed by centrifugation (3000 × *g*) samples for 15 min at 5 °C. An aliquot 150 µL of supernatant was acidified with 30 µL of 0.16 mol/L orthophosphoric acid and the mixture distilled under vacuum. Individual SCFA in the distillates were separated and quantified by capillary gas chromatography (GC; 5890 series II Hewlett Packard, NSW, Australia). The GC was equipped with a flame ionisation detector, split-less injector and a Zebron ZB-FFAP 30 m × 0.53 µm capillary column with 0.1 µm film thickness (Phenomenex, NSW, Australia). Injector and detector temperatures were both 210 °C held for 0.5 min and then raised at 30 °C/min to reach a

final column temperature of 190 °C. Helium was the carrier gas (head pressure 50 kPa) and an injection volume of 0.2 µL was used. Total SCFA concentration was calculated as the sum of acetic, propionic, butyric, isobtyric, caproic, isovaleric and valeric acid concentrations.

Faecal excretions of ammonia, SCFA and phenols were calculated by multiplying their concentrations by the wet weight of faeces collected over 48 h.

6.2.6 Statistical analysis

All data including ammonia, phenols, individual SCFA, and total SCFA are presented as the mean ± standard error of the mean (S.E.M). Data related to ammonia and phenols were analysed using SPSS for Windows™ (version 17, SPSS Inc., Chicago, IL, USA, 2008). Significant differences between groups were tested using an independent T-test. The SCFA data were analysed by a linear mixed model framework using the statistical package R (the R Foundation for Statistical Computing Version 2.11.0) with families as the random component and nutrient intake and child participant group as the fixed component. To reduce variability in the S.E.M, the modelling was carried out on mean-centred data. Statistical significance was deemed as $p < 0.05$ for all analyses.

6.2.7 Ethics approval

Ethics approval was granted by the University of South Australia's ethics committee in accordance with the Declaration of Helsinki.

6.3 Results

6.3.1 Characteristics of participants

The age, gender, dietary patterns, GI disturbance and anti-/probiotic usage of participants at the time of sample collection are summarised in Table 6.1. Of the 23 children with ASD, nine had functional GI disorders, and of those four had both constipation and diarrhoea, four had constipation only and one had diarrhoea only. Of the siblings, one had both constipation and diarrhoea, one had diarrhoea only and four had constipation only. One of the community controls was experiencing constipation.

Table 6.1 Characteristics of participants

Participants	Number	Age (months) Mean ± S.E.M *(Range)*	Males	GI disturbance	Gluten- and casein-free diet	Casein-free diet	Medicine/ Supplements	
							Antibiotic	Probiotic
ASD	23	123 ± 9 *(37-208)*	21	9	3	1	1	2
SIB	22	144 ± 12 *(55 -221)*	11	6	--	--	1	1
CON	9	114 ± 15 *(42-182)*	4	1	--	--	--	--

ASD, autism spectrum disorder; SIB, typically developing siblings; CON, unrelated community controls; S.E.M, standard error of the mean.

6.3.2 Nutrient intake, gluten-/ casein- free diets and anti-/ probiotic use

Caregivers' responses to the dietary questionnaire revealed there were no significant differences in daily consumption of protein, carbohydrate, sugars, starch or fibre between the three study groups. There were three participants who were implementing a gluten- and casein-free diet and one participant was implementing a casein-free diet (Table 6.1).

6.3.3 Faecal excretion pH and moisture

There were no significant differences in total faecal excretion over 48 h between study groups, nor were there differences in faecal pH and moisture (Table 6.2).

6.3.4 Ammonia

Higher concentrations of faecal ammonia were observed in children with ASD when compared with SIB ($p = 0.028$), but not CON participants. There was no significant difference in total ammonia excretion between three study groups (Table 6.2).

6.3.5 Phenol and total *p*-cresol

Faecal phenol concentrations tended to be higher in the ASD compared with CON participants ($p = 0.083$) but did not reach significance. Faecal levels of total *p*-cresol were not significantly different in the ASD, SIB and CON participants (Table 6.2).

6.3.6 Short chain fatty acids

Faecal concentrations and excretions of individual and total SCFA are presented in Table 6.3. Levels of total SCFA and propionate were higher in ASD compared with SIB participants ($p = 0.022$ and $p = 0.019$, respectively). They were also higher in the ASD compared with the CON participants ($p = 0.019$ and $p = 0.006$, respectively). Moreover, greater faecal levels of valeric and isobutyric acids were found in children with ASD compared with siblings ($p = 0.002$ and $p = 0.036$, respectively). Increased isovaleric acid levels were also found in the ASD compared with the SIB participants ($p = 0.053$). In addition, there were significantly elevated faecal levels of acetic acid in the ASD than the CON participants ($p = 0.036$).

Table 6.2 pH, moisture (%), 48 h faecal excretion, faecal concentrations and excretion of ammonia, total *p*-cresol and phenol in ASD, SIB and CON participants

	ASD	**SIB**	**CON**
	Mean ± S.E.M *(Range)*		
pH	**6.9 ± 0.1** *(5.8-7.6)*	**6.9 ± 0.1** *(5.8-7.9)*	**7.1 ± 0.1** *(6.7-7.4)*
Moisture (%)	**73.8 ± 1.4** *(50.2-85.3)*	**72.1 ± 1.2** *(63.0-83.4)*	**71.1 ± 1.6** *(65.7-82.8)*
Total faecal excretion (g) (48 h)	**171.0 ± 28.7** *(51.8-523.1)*	**157.5 ± 21.0** *(30.0-320.4)*	**146.2 ± 20.2** *(72.7-257.4)*
Concentration (mmol/kg wet weight)			
Ammonia	**42.7 ± 3.3*** *(12.9 - 81.6)*	**31.9 ± 2.3*** *(11.3 - 53.9)*	**33.4 ± 4.0** *(14.8 - 51.0)*
Total *p*-cresol	**0.8 ± 0.1** *(0.2 - 1.5)*	**0.7 ± 0.1** *(0.3 - 1.7)*	**1.0 ± 0.1** *(0.6 - 1.6)*
Phenol (nmol/g wet weight)	**13.9 ± 4.4** *(3.9 - 107.0)*	**9.6 ± 0.9** *(4.0 - 18.6)*	**6.2 ± 0.9** *(3.7 - 11.6)*
Faecal excretion (mmol)			
Ammonia	**7.5 ± 1.3** *(0.7 - 21.7)*	**5.3 ± 0.8** *(0.3 - 13.4)*	**5.1 ± 1.0** *(1.4 - 9.7)*
Total *p*-cresol	**0.1 ± 0.0** *(0.0 - 0.4)*	**0.1 ± 0.0** *(0.0 - 0.4)*	**0.1 ± 0.0** *(0.1 - 0.3)*
Phenol (µmol)	**2.1 ± 0.5** *(0.2 - 9.5)*	**1.6 ± 0.3** *(0.1 - 4.6)*	**0.9 ± 0.2** *(0.3 - 2.2)*

ASD, autism spectrum disorder; SIB, typically developing sibling; CON, community controls; S.E.M, standard error of the mean; *, statistically significant difference in ASD compared with SIB participants, $p < 0.05$.

Table 6.3 Faecal concentrations and excretion (48 h) of individual and total SCFA in ASD, SIB and CON participants

	ASD	**SIB**	**CON**
Concentration (mmol/kg wet weight)		**Mean ± S.E.M** *(Range)*	
Acetic acid	**74.8 ± 5.1**[*] *(31.5 - 116.9)*	**64.6 ± 4.9** *(31. - 112.5)*	**54.7 ± 6.3**[*] *(35.4 - 87.9)*
Propionic acid	**24.7 ± 1.8**[*,†] *(12.1 - 41.7)*	**21.3 ± 1.7**[†] *(7.4 - 35.0)*	**15.8 ± 1.7**[*] *(8.7 - 24.6)*
Butyric acid	**24.5 ± 1.8** *(12.3 - 41.2)*	**20.1 ± 2.0** *(7.8 - 45.5)*	**17.8 ± 2.3** *(9.6 - 27.0)*
Isobutyric acid	**3.0 ± 0.3**[†] *(0.9 - 5.6)*	**2.4 ± 0.2**[†] *(1.2 - 4.6)*	**2.8 ± 0.1** *(2.1 - 3.4)*
Valeric acid	**3.6 ± 0.3**[†] *(1.3 - 6.0)*	**2.8 ± 0.3**[†] *(0.3 - 4.9)*	**2.8 ± 0.2** *(1.9 - 3.4)*
Isovaleric acid	**4.7 ± 0.5** *(1.3 - 8.9)*	**3.6 ± 0.3** *(1.5 - 6.9)*	**4.4 ± 0.2** *(3.4 - 5.2)*
Caproic acid	**1.3 ± 0.3** *(0.1 - 5.5)*	**0.7 ± 0.2** *(0.1 - 1.7)*	**0.5 ± 0.2** *(0.1 - 1.4)*
Total SCFA	**136.6 ± 8.7**[*,†] *(65.7 - 202.4)*	**116.3 ± 8.2**[†] *(59.5 - 192.4)*	**98.5 ± 9.9**[*] *(69.4 - 145.3)*
Faecal excretion (mmol)			
Acetic acid	**13.6 ± 2.6** *(1.6 - 47.5)*	**11.2 ± 2.0** *(1.0 - 32.9)*	**8.4 ± 1.7** *(3.1 - 16.3)*
Propionic acid	**4.4 ± 0.8** *(0.6 - 15.1)*	**3.6 ± 0.6** *(0.3 - 10.5)*	**2.4 ± 0.5** *(0.5 - 5.0)*
Butyric acid	**4.4 ± 0.9** *(0.6 - 15.6)*	**3.5 ± 0.6** *(0.2 - 9.3)*	**2.9 ± 0.7** *(0.9 - 6.3)*
Isobutyric acid	**0.5 ± 0.1** *(0.1 - 2.1)*	**0.4 ± 0.1** *(0.1 - 1.1)*	**0.4 ± 0.1** *(0.2 - 0.7)*
Valeric acid	**0.6 ± 0.1** *(0.1 - 1.8)*	**0.5 ± 0.1** *(0.0 - 1.2)*	**0.4 ± 0.1** *(0.2 - 0.9)*
Isovaleric acid	**0.8 ± 0.2** *(0.2 - 3.3)*	**0.5 ± 0.1** *(0.1 - 1.6)*	**0.7 ± 0.1** *(0.4 - 1.3)*

Caproic acid	**0.2 ± 0.1** *(0.0 - 1.1)*	**0.1 ± 0.0** *(0.0 - 0.6)*	**0.1 ± 0.0** *(0.0 - 0.1)*
Total SCFA	**24.5 ± 5.6** *(3.4 - 81.5)*	**19.9 ± 3.3** *(1.8 - 57.0)*	**15.2 ± 3.0** *(3.8 - 28.8)*

ASD, autism spectrum disorder; SIB, typically developing sibling; CON, community controls; S.E.M, standard error of the mean; *, statistically significant difference in ASD compared with CON group, $p < 0.05$; †, statistically significant difference in ASD compared with SIB group, $p < 0.05$.

6.3.7 Influence of implementing gluten- and/or casein-free diet or anti-/probiotic usage

Further statistical analyses were conducted where participants implementing special diets or using antibiotics or probiotics were excluded from the analyses and the key findings remained unchanged.

6.3.8 Influence of gender

As shown in Table 6.1, there were a disproportionate number of males in the ASD group. As recruitment of controls was opportunistic, they were not gender matched. To ensure an adequate sample size of controls, we combined the sibling and community controls together and examined the influence of gender on fermentation metabolite results. There were no significant differences in levels of large bowel fermentation products between males and females except ammonia concentrations ($p = 0.025$) which were higher in female participants compared with males.

When further statistical analyses were conducted with females excluded, the key findings again remained unchanged. Specifically, ammonia concentrations remained significantly higher in males with ASD compared with males in combined group of SIB and CON participants ($p = 0.001$) (Table 6.4). Faecal phenol concentrations were not significantly different between male ASD participants and males in the combined control group (Table 4). Further, significantly higher concentrations of total SCFA ($p = 0.021$), propionate ($p = 0.018$), valeric acids ($p = 0.009$), isobutyric acid ($p = 0.038$), isovaleric acid ($p = 0.056$) and acetic acid ($p = 0.055$) were found in male participants with ASD compared with male

combined controls. Notably, butyrate concentration were significantly higher in males with ASD compared to combined male control (p = 0.024) (Table 6.4).

Table 6.4 Faecal concentrations and excretion (48 h) of large bowel fermentation products in male ASD participants and combined (SIB and CON) male controls

Concentration (mmol/kg wet weight)	**ASD (Male)**	**Combined group (Male) (SIB + CON)**
	Mean ± S.E.M *(Range)*	
Acetic acid	**75.5 ± 5.8** *(52.1 - 116.9)*	**64.6 ± 4.9** *(31.2 - 101.5)*
Propionic acid	**25.0 ± 1.9*** *(12.1 - 33.1)*	**18.5 ± 1.7** *(8.7 - 29.1)*
Butyric acid	**24.9 ± 1.9**† *(12.3 - 41.2)*	**18.1 ± 2.0** *(8.1 - 30.4)*
Isobutyric acid	**3.1 ± 0.3**‡ *(0.9 - 5.6)*	**2.2 ± 0.2** *(1.2 - 3.4)*
Valeric acid	**3.8 ± 0.3**§ *(1.3 - 6.0)*	**2.5 ± 0.3** *(0.3 - 4.9)*
Isovaleric acid	**4.8 ± 0.5**‖ *(1.3 - 8.9)*	**3.5 ± 0.3** *(1.5 - 5.5)*
Caproic acid	**1.4 ± 0.3**¶ *(0.1 - 5.5)*	**0.9 ± 0.3** *(0.2 - 4.1)*
Total SCFA	**138.3 ± 9.9**** *(65.7 - 188.6)*	**104.8 ± 9.0** *(69.4 - 167.5)*
Ammonia	**44.7 ± 3.6**†† *(22.0 - 59.5)*	**27.9 ± 2.6** *(12.7 - 47.4)*
Total *p*-cresol	**0.8 ± 0.1** *(0.2 - 1.5)*	**0.8 ± 0.1** *(0.4 - 1.7)*

Phenol (nmol/g wet weight)	**14.8 ± 5.3** *(3.9 - 107.0)*	**9.6 ± 1.1** *(4.5 - 12.4)*
Faecal excretion (mmol)		
Acetic acid	**14.6 ± 3.0** *(4.2 - 47.5)*	**11.5 ± 2.2** *(1.7 - 27.8)*
Propionic acid	**4.7 ± 0.9** *(1.0 - 15.1)*	**3.5 ± 0.6** *(0.6 - 6.2)*
Butyric acid	**4.8 ± 0.9** *(0.6 - 15.6)*	**3.5 ± 0.6** *(0.4 - 8.0)*
Isobutyric acid	**0.5 ± 0.1** *(0.1 - 2.1)*	**0.4 ± 0.0** *(0.1 - 0.7)*
Valeric acid	**0.6 ± 0.1** *(0.1 - 1.8)*	**0.5 ± 0.1** *(0.0 - 1.2)*
Isovaleric acid	**0.8 ± 0.2** *(0.2 - 3.3)*	**0.6 ± 0.1** *(0.1 - 1.1)*
Caproic acid	**0.3 ± 0.1** *(0.0 - 1.1)*	**0.1 ± 0.0** *(0.0 - 0.5)*
Total SCFA	**26.3 ± 5.1** *(7.4 - 81.5)*	**20.2 ± 3.7** *(4.7 - 45.7)*
Ammonia	**8.2 ± 1.5** *(1.2 - 8.4)*	**5.3 ± 0.9** *(0.5 - 4.6)*
Total *p*-cresol	**0.1 ± 0.0** *(0.0 - 0.4)*	**0.1 ± 0.0** *(0.0 - 0.4)*
Phenol (µmol)	**2.3 ± 0.6** *(0.2 - 6.0)*	**1.8 ± 0.4** *(0.5 - 4.6)*

ASD, autism spectrum disorder; combined controls, siblings plus community controls; S.E.M, standard error of the mean. *****, †, ‡, §, ‖, ¶, ** & ††, statistically significant differences between male ASD and combined control groups, $p < 0.05$.

6.4 Discussion

This chapter compared the faecal concentrations and total excretion of the major bacterial fermentation products: SCFA, ammonia, phenol and total *p*-cresol, in children with ASD, their siblings and community controls. Significantly higher faecal concentrations of propionate, acetate, total SCFA and ammonia are demonstrated for the first time in children with ASD compared with controls. The measured fermentation product levels represent what remains following the generation of these products from the fermentation of dietary sources by the microbiota and their subsequent removal by absorption and/or utilisation by other gut bacteria. Therefore, any of these factors could be contributing to the higher fermentation product levels observed. The majority of evidence points towards the source of the faecal fermentation products as being the large intestine (Roberfroid *et al.* 1995) but the small intestine cannot be excluded as a potential source. Given the potential impact that these fermentation products can have on GI health, and possibly the overall health of the host, these findings could shed light on how GI function is affected in children with ASD and could also lead to greater understanding of the aetiology of ASD.

For the three participant groups, the faecal concentrations of SCFA, ammonia and phenols were within the range of levels previously reported for studies involving healthy controls as shown in Table 6.5a & 6.5b (Birkett *et al.* 1996; Muir *et al.* 1998; Heavey *et al.* 2003; Samuelsson and Ludvigsson 2004; Sandin *et al.* 2009; Tiihonen *et al.* 2010). This is consistent with the observation that the colonic microbiota, and hence fermentation capacity, can be described as ‘adult-like’ after the age of two years (Hopkins *et al.* 2002). All participants in the current study were older than 2 years, with an average age of between 11 and 12 year old. The participants’ ages in each group were reasonably well matched and there was no significant association between age and the levels of any faecal fermentation product.

Table 6.5a Reference values of individual SCFA and total SCFA in healthy controls

Individual SCFA								
Acetic acid	**Propionic acid**	**Butyric acid**	**Caproic acid**	**Valeric acid**	**Isovaleric acid**	**Isobutyric acid**	**Total SCFA**	**Reference**
29.6 - 73	3.4 - 30.7	4.2 - 49.1	0 - 2.6	0.2 - 5.1	0.2 - 9.3	0.8 - 6.4	45-175.5	**(Samuelsson and Ludvigsson 2004)[a]**
12 - 126	4 - 45	4.3 - 61	<0.2 - 4.7	0.6 - 6.6	0.2 -10	0.3 - 6.8	--	**(Sandin *et al.* 2009)[b]**
42.1 ± 3.8	11.5 ± 1.2	11.3 ± 1.4	0.5 ± 0.1	1.8 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	67.3 ± 6.2	**(Tiihonen *et al.* 2010)[c]**
56.1 ± 4.3	15.0 ± 1.3	18.4 ± 2.3	--	--	--	--	98.0 ± 7.6	**(Muir *et al.* 1998)[d]**
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Table 6.5b Reference values of individual phenol, total *p*-cresol and ammonia in healthy controls

Phenol	*p*-cresol	Ammonia	Reference
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--	--	--	--
305.6 ± 61.6[h]		22.6 ± 2.2	**(Tiihonen *et al.* 2010)[c]**
68.5 ± 12.9[i]		450 ± 40	**(Muir *et al.* 1998)[d]**
2.8 ± 0.4	0.3 ± 0.1	37.9 ± 32.2	**(Heavey *et al.* 2003)[e]**
(2.5 - 3.1)	*(0.26 - 0.31)*	*(8.8 - 109.8)*	
4.0 ± 0.6	65.0 ± 8.0	397 ± 33	**(Birkett *et al.* 1996)[f]**
3.0 ± 0.5	36.0 ± 10.0	278 ± 49	**(Birkett *et al.* 1996)[g]**

SCFA, short chain fatty acids; [a] the data are presented in ranges as mmol/kg wet weight; [b] the data are presented as median and range as mmol/kg in four year old children; [c] the data are presented as mean ± S.E.M with mmol/kg fresh digesta weight; [d] the data are presented as mean ± S.E.M as mmol/l for SCFA and mg/L for ammonia and phenols with Australian diet; [e] the data are presented as mean ± SD and range as mmol/kg for phenol & *p*-cresol and µmol/g for ammonia; [f] the data are presented as mean ± S.E.M as µg/g faeces with low-resistant starch diet; [g] the data are presented as mean ± S.E.M as µg/g faeces with high-resistant starch diet; [h], the data are presented with mean ± S.E.M of phenolic acids as mg/kg fresh digesta weight; and [i] the data are presented as phenols.

One of the SCFA found to be significantly higher in children with ASD compared with controls was propionate. Generally, the majority of propionate is absorbed by the colon (Wong *et al.* 2006). Disorders of gut function or absorption have been involved in a range of neurological manifestations (Perkin and Murray-Lyon 1998). In this study, a higher frequency of GI disturbances was seen in children with ASD than controls, thus the raised concentrations of faecal propionate may reflect impaired utilization of propionate in the colon of children with ASD. Studies in rats have shown that intraventricular administration of propionate induces behaviours resembling ASD (e.g. repetitive dystonic behaviours, retropulsion, seizures and social avoidance) (MacFabe *et al.* 2007; MacFabe *et al.* 2008; Shultz *et al.* 2008; Shultz *et al.* 2009; Thomas *et al.* 2010). These studies also showed

intraventricular propionate reproduced the neuropathological changes that have previously been reported to occur in ASD, such as neuroinflammation, reactive astrogliosis and activated microglia as well as induced oxidative stress in the CNS (MacFabe *et al.* 2007; MacFabe *et al.* 2008; Shultz *et al.* 2008; Shultz *et al.* 2009; Thomas *et al.* 2010). However, only 10% of faecal propionate is transported into peripheral blood under normal conditions (Wong *et al.* 2006). Consequently, the magnitude of the increase in faecal propionate levels observed in children with ASD is unlikely to translate to large increases in propionate levels in blood and subsequently have neuropathological manifestations. Nevertheless, it will be important to measure the circulating levels of the propionate in future studies to examine this possibility.

Interestingly, increased faecal concentrations of acetic acid and total SCFA were found in children with ASD. These changes could contribute to, or reflect, altered GI function in ASD. High levels of total SCFA and acetic acid have been reported in children with coeliac disease (Tjellstrom *et al.* 2005), a GI disorder which has been linked with autistic subgroups. The changes in coeliac disease that have been observed are similar to the degree of alterations we have observed in children with ASD in the current study. Elevated luminal levels of acetic acid can produce local chronic inflammation in the gut with an accompanying increase in gut permeability (Tjellstrom *et al*. 2005). The 'leaky gut' has also been previously implicated in ASD (D'Eufemia *et al*. 1996; de Magistris *et al.* 2010). Hence, the degree of changes in faecal SCFA in the current study may be also clinically relevant in ASD.

Faecal concentrations of the minor SCFA, isobutyric, isovaleric and valeric acids were also elevated in children with ASD compared to controls. Levels of isobutyric and isovaleric acids have been reported to have a tendency to be higher in children with coeliac disease compared with controls (Tjellstrom *et al.* 2005). Another study showed that the GI microbiota from patients with inflammatory bowel disease produced more SCFA, especially the branched isobutyric and isovaleric acids, than the microbiota from healthy controls (van Nuenen *et al.* 2004). Although 42% (9/23), 31% (6/22) and 10% (1/9) of participants were reported as having GI symptoms by caregivers (e.g. constipation, diarrhoea and abdominal pain) in ASD, SIB and CON groups, respectively, there were no clear relationships between GI symptoms and the changes in bacterial fermentation products.

The level of faecal ammonia, a protein fermentation product, was significantly elevated in ASD compared with controls. At physiological pH, ammonia is present as both NH_3 and in the ionic form ($NH4^+$), both of which are capable of crossing membranes (Bosoi and Rose 2009). High colonic levels of ammonia can damage the epithelial cells in the colon and increase gut permeability (Meletis and Zabriskie 2008). Increased gut permeability has been reported in ASD (D'Eufemia *et al.* 1996; de Magistris *et al.* 2010) but to date the relationship between faecal levels of ammonia and gut permeability in ASD has not been examined. Accumulation of ammonia in the brain can disturb brain metabolism, cause extracellular potassium accumulation and hence may increase neuronal depolarization (Felipo and Butterworth 2002). The urea cycle disorder is a genetic disorder where high plasma levels of ammonia are found due to a deficiency of one of the enzymes in these patients. Several studies have described elevated plasma ammonia levels in children with ASD (Clark-Taylor and Clark-Taylor 2004; Filipek *et al.* 2004) which may be indicative of urea cycle dysfunction (Felipo and Butterworth 2002). However, in the current chapter, faecal urea levels were not measured and there was no direct evidence to suggest an association between children with ASD nad urea clycle disorder.

Most ammonia enters the circulation from the GI tract and is metabolised by the liver (Felipo and Butterworth 2002). The observed increase in faecal ammonia levels in children with ASD may not translate to clinically significant increases in ammonia levels in blood and brain. Consequently, it is difficult to see that the changes in the large bowel would lead to neuropathological effects within the CNS. It is plausible that the observed high level of faecal ammonia in the current ASD cohort is associated with elevated plasma ammonia levels but it was not determined in the current study.

The present study did not find significant differences in faecal levels of phenol or total *p*-cresol between children with and without ASD. In contrast, one recent study reported higher urinary total *p*-cresol concentrations (Altieri *et al.* 2011) and another study reported lower urinary *p*-cresol sulphate (Yap *et al.* 2010) concentrations in children with ASD compared with controls. Phenols are generally absorbed from the GI tract into the systemic circulation and detoxified by the liver. Although measuring urinary levels of phenol and *p*-cresol could reflect gut microbial changes (Clayton *et al.* 2009), it may not accurately reflect production in the colon and it has been suggested that these metabolites should be

measured in faeces (Birkett *et al.* 1995). The relationship between urinary and faecal levels of phenols in children with and without ASD requires further investigation.

Both faecal concentrations and excretion of SCFA, ammonia and phenols in children with and without ASD are reported in the current study. Notably, while differences between groups were found in faecal concentrations of bacterial fermentation products, there were no significant differences found in *overall faecal excretion* of these products between children with and without ASD. Faecal metabolite concentrations and excretions reveal different aspects of fermentation activity in the large bowel. Specifically, faecal concentrations can influence pH and other concentration-dependent biological activities in the gut tissues. Therefore, differences in faecal concentrations are likely to translate into altered biological potency within colorectal tissues even if the *overall faecal excretion* does not differ. While *overall faecal excretion* of fermentation products is an indicator of how much in total is being produced, it does not exactly reflect production as there can be absorption by the bowel tissues. Further, *overall faecal excretion* can be influenced by many factors including total food and fluid consumption which affects faecal weight and other large bowel health parameters. Currently, the optimal balance between production of bacterial fermentation products and maximal promotion of colonic health is unknown.

Daily nutrient intake relates to various bodily functions. Altered patterns of bacterial fermentation products not only reflect a deviant intestinal microbial profile but can also be indicative of variation in nutrient intake delivery to the colon. Studies by Birkett *et al.* (1996, 1997) identified that resistant starch diets can influence the concentrations of faecal SCFA, as well as decrease faecal phenols and ammonia in humans. However, the inconsistent findings from studies reporting the efficacy of dietary interventions in ASD (Levy and Hyman 2008) and the similar levels of macronutrient consumption within the three study groups in the current study suggests that dietary factors alone are unlikely to explain the observed differences in patterns of bacterial fermentation products between children with and without ASD. Some ASD participants were on casein-free and/or gluten-free diets, and these diets have the potential to influence levels of faecal fermentation products. However, current analyses indicate that exclusion of these children did not alter the observed significant differences between study groups.

The gender ratio (male to female) in ASD and control groups was 10:1 and 1:1, respectively and is consistent with literature reports of the ASD gender ratio (Whiteley *et al.* 2010). Gender imbalance in the ASD group reflects the higher incidence of ASD in males. The lack of a gender matched control group is a limitation of the current study. However, the gender did not influence the findings with respect to SCFA and phenols. Notably, increased ammonia levels were found in females compared with males in the combined control group. Current understanding of ASD and large bowel biochemistry and physiology does not provide any obvious explanation for the difference in faecal ammonia concentrations between genders.

Levels of fermentation products in the SIB group were intermediate between children with ASD and community controls, which indicates potential genetic and/or environmental influences. Intermediate effects in siblings of children with ASD compared with ASD and CON participants have previously been reported (Messahel *et al.* 1998). This highlights the importance of considering siblings as a distinct control group and including unrelated community controls when conducting ASD research.

Alterations in the populations or activities of microbiota in the GI tract, especially in the colon, could impact on the capacity of individuals to ferment dietary substrates and may explain the observed differences in faecal concentrations of SCFA and ammonia between children with and without ASD. Analyses of the faecal microbiota in the present and future studies of children with ASD and controls are needed to determine if this is the case. Some earlier studies have speculated that *Clostridia* play a role in the pathophysiology of ASD by producing toxins which gain access to the CNS (Bolte 1998; Finegold 2008). Notably, an open-label trial of short-term oral vancomycin (antibiotic) conducted in 11 children with ASD resulted in short-term behavioural improvement. The improvements were attributed to the antibiotic disrupting the colonization of neurotoxin-producing bacteria in the GI tracts (Sandler *et al.* 2000).

The demonstration that intraventricular injection of propionate into the brain can produce ASD characteristics in rodents and elevated faecal propionate concentrations in children with ASD in the current study would also suggest there may be changes in the number and activity of bacteria producing propionate in the large bowel. Consequently, these bacteria should be prime targets of future faecal analyses. Insight into alterations in the gut

microbiota in ASD will be important for developing strategies that might alleviate GI dysfunction in affected children which could include probiotic or prebiotic interventions.

6.5 Conclusion

In conclusion, the significantly higher faecal concentrations of SCFA and ammonia in children with ASD may contribute to their altered GI function and overall health status. Although, the underlying cause of the observed higher levels of SCFA and ammonia are unknown, it can be speculated to be due to differences in the uptake of these products from the large bowel, different enteric microbiota profiles or differences in dietary intakes. All of these possibilities require further investigation.

7 Faecal Microbiota in Children with Autism Spectrum Disorder

7.1 Introduction

Altered GI fermentation products were reported in Chapter 6, which suggested an altered GI microbiota profile in children with ASD compared with controls. Although, several studies have reported different microbiota profiles in children with ASD compared with controls (Finegold *et al.* 2002; Song *et al.* 2004; Parracho *et al.* 2005; Finegold *et al.* 2010), the nature of these alterations in ASD is poorly understood. In this chapter, the abundance of a range of faecal bacteria were measured and compared between children with and without ASD.

The GI tract harbours a great number of microbacteria which plays an important role in the general health of the host. If the symbiotic relationship between the commensal gut bacteria and the host is perturbed, dysbiosis can rapidly occur. An imbalance of GI microbiota, particularly a shift towards a pathogenic profile, may lead to the onset and/or progression of GI disturbance. Several studies have described specific GI bacteria, such as, *Clostridium* species, which are present in greater numbers in children with ASD experiencing GI disturbance compared with controls (Finegold *et al.* 2002; Song *et al.* 2004; Parracho *et al.* 2005). Some researchers have speculated that the differences in the GI microbiota could be contributing to the pathophysiology of ASD (Bolte 1998; Finegold *et al.* 2010). Indeed, the modulation of intestinal microbiota in children with ASD through the use of antibiotics (Sandler *et al.*, 2000) and probiotics (Parracho *et al.*, 2010) may result in improved behaviour and bowel health outcomes.

In chapter 6, the observed altered levels of large bowel fermentation products in children with ASD, especially elevated SCFA levels in children with ASD compared with controls, may have been indicative of an imbalance in GI microbiota in the current cohort. Confirmation of perturbations in the numbers of a range of GI bacteria in the current ASD cohort was required. Focussing on whether numbers of bacteria which are indicative of GI health differ within the ASD group, several common GI bacteria, , such as *Bifidobacterium spp*. (Mitsuoka 1990), *Faecalibacterium prausnitzii* (Sokol *et al.* 2008), *Akkermansia muciniphila* (Collado *et al.* 2007) and sulphate-reducing bacteria (SRB) (Gibson *et al.* 1991) were examined. Most of these bacteria had bot been analysed in children with ASD previously. Moreover, whether a correlation exists between GI microbial profiles and the

presence or absence of a FGID, such as constipation, diarrhea, abdominal pain, flatulence in children with ASD was investigated.

7.2 Methods

7.2.1 Participants and sample collection

The participants and sample collection were same with section 6.2.1

7.2.2 Reagents

The sodium chloride, tris base, ethylene diamine tetra acetic acid (EDTA), ammonium acetate, bovine serum albumin (BSA) and sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). SsoFast™ EvaGreen® Supermix was purchased from Bio-Rad (Hercules, CA, USA). Lysis buffer was prepared in-house and sterilised by filtration. QIAmp® DNA Mini Kits (Qiagen, Hilden, Germany) were used for faecal bacteria DNA extraction and purification.

7.2.3 DNA extraction and purification

Extraction of DNA from 0.25 g faecal samples was performed using the repeated bead beating plus column method. Briefly, 1mL of lysis buffer and 0.4 g sterile zirconia beads (0.3 g of 0.1mm and 0.1 g of 0.5 mm) were added to each sample and homogenised for 3 min at maximum speed on a Mini-Beadbeater. Then the mixture was incubated at 70 °C for 15 min with gentle hand shaking every 5 min. The mixtures were centrifuged at 16000 x *g* for 5 min at 4 °C and the supernatant transferred to a fresh 1.7 mL micro tube. Fresh lysis buffer (300 µL) was added to the lysis tube and the above steps repeated. The supernatants were collected and pooled and the tube discarded. Ammonium acetate (260 µL of 10M) was added to each tube of pooled supernatant (approx 1300 µL), mixed well and incubated on ice for 5 min. The mixtures were centrifuged at 4 °C for 10 min at 16000 x *g* and the supernatant transferred to 2 x 1.7 mL micro tubes. Then, 1 x volume of isopropanol (~100%) was added to each tube, mixed well and incubated on ice for 30 min to enable the DNA to precipitate. Then, the tubes were centrifuged at 4 °C for 15 min at 16000 x *g* and the supernatant decanted off. The nucleic acid pellet was then washed with 500 µL 70% ethanol and centrifuged at 16000 x *g* for 5 min and the supernatant aspirated. The nucleic

acid pellet was then dried under vacuum for 3 min and dissolved in 100 µL TE (pH 8) buffer and left over night in the fridge to dissolve pellet.

The two aliquots (the nucleic acid pellet in 100 µL TE buffer) of each sample were pooled on Day 2 and purified DNA following with a modified protocol in QlAamp® DNA Mini Kit Handbook. Briefly, 2 µL of DNase-free RNase (10mg/mL) was added to each sample and incubated at 37 °C for 15 min. After that, 15 µL of Proteinase K and 200 µL of AL buffer were added, mixed well and incubated at 70 °C for 10 min. Then, 200 µL of 100% ethanol was added and mixed well. The solution was transferred to a QIAmp column and centrifuged at 16000 x *g* for 1 min. After discarding the flow through, 500 µL of buffer AW1 was added and centrifuged at 16000 x *g* for 1 min at room temperature. Again the flow through was discarded and 500 µL of buffer AW2 added and centrifuged at 16000 x *g* for 1 min at room temperature. After discarding the flow through and collection tube, the filter was transferred to a new collection tube and the column was dried by centrifuging at 16000 x *g* at room temperature for 1 more min. The filter was transferred to a new 1.7 mL tube and 200 µL of buffer AE added and incubated at room temperature for 1 min to elute DNA. Then, it was centrifuged at 16000 x *g* for 1 min at room temperature to get the DNA solution. 2 µL was run on a 0.8% gel to check the DNA quality and quantify the concentration of DNA using the NanoDrop ND-1000 Spectrophotometer (Wilmington, DE, USA).

7.2.4 Primers and real-time quantitative PCR

The primers and optimised qPCR conditions used are summarised in Table 7.1. The qPCR was performed on a CFX 384TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and data was analysed with the Bio-Rad CFX Manager software (Version 1.5, Bio-Rad, Hercules, CA, USA). All qPCR setups were performed with epMotion® automated pipetting systems (Eppendorf, Hamburg, Germany) in triplicate with a volume of 10 µL, using 384-well optical grade PCR plates and a Microseal 'B' Adhesive Seal (Bio-Rad, Hercules, CA, USA). Each reaction consisted of: 4 µL (1 ng/µL) DNA template and 6 µL of PCR mixture containing 5 µL SsoFast™ EvaGreen® Supermix, 0.5 µL bovine serum albumin and 0.5 µL PCR-grade water (Sigma-Aldrich, St. Louis, MO, USA).

The qPCR cycling conditions were as follows: hot-start 98 °C for 3 min followed by 35 cycles of two-step PCR with denaturing at 98 °C for 15 s and annealing/elongation time

and temperature as given in Table 7.1. This was followed by fluorescence acquisition after each cycle and a final melt-curve analysis was performed after completion of all cycles with fluorescence acquired at 0.5 °C intervals between 55 °C and 95 °C. For each bacterial assay an 8-series of 10-fold dilutions of a plasmid construct containing the target amplicon was analysed in parallel with DNA samples for estimation of absolute abundance and PCR efficiency.

Table 7.1 Primers used and amplification conditions for real-time PCR assays

Target	Primers	Sequence (5'-3')	Conc. (nM)	Annealing Temp. (°C)	Annealing Time (Sec.)	Reference
A. muciniphila	AM1	*CAGCACGTGAAGGTGGGGAC*	350	63	30	**(Collado *et al.* 2007)**
	AM2	*CCTTGCGGTTGGCTTCAGAT*				
B. fragilis group	Bfr-F	*CTGAACCAGCCAAGTAGCG*	500	56	20	**(Liu *et al.* 2003)**
	Bfr-R	*CCGCAAACTTTCACAACTGACTTA*				
Bifidobacterium spp.	Bif-F	*TCGCGTC(C/T)GGTGTGAAAG*	600	56	20	**(Rinttila *et al.* 2004)**
	Bif-R	*CCACATCCAGC(A/G)TCCAC*				
C. coccoides group	g-Ccoc-F	*AAATGACGGTACCTGACTAA*	500	60	20	**(Matsuki *et al.* 2004)**
	g-Ccoc-R	*CTTTGAGTTTCATTCTTGCGA A*				
C. leptum group	Sg-Clept-F	*CTTTGAGTTTCATTCTTGCGAA*	500	56	20	**(Matsuki *et al.* 2004)**
	Sg-Clept-R	*GCACAAGCAGTGGAGT*				
C. cluster I	C.clu F	*TACCHRAGGAGGAAGCCAC*	500	63	20	**(Song *et al.* 2004)**
	C.clu R	*GTTCTTCCTAATCTCTACGCAT*				

Table 7.1 Cont.

C. difficile	C.diff F	*TTGAGCGATTTACTT CGGTAAAGA*	500	60	20	**(Penders *et al.* 2005)**
	C.diff R	*TGTACTGGCTCACCTTTGATATTCA*				
Enterocuccus spp.	Entero F	*CCCTTATTGTTAGTTGCCATCATT*	600	59	15	**(Rinttila *et al.* 2004)**
	Entero R	*ACTCGTTGTACTTCCCATTGT*				
E. coli	E.coli F	*CATGCCGCGTGTATGAAGAA*	300	60	20	**(Huijsdens *et al.* 2002)**
	E.coli R	*CGGGTAACGTCAATGAGCAAA*				
F. praunitzii	FPR-1F	*AGATGGCCTCGCGTCCGA*	500	60	20	**(Wang *et al.* 1996)**
	FPR-2R	*CCGAAGACCTTCTTCCTCC*				
Lactobacillus group	Lacto-F	*AGCAGTAGGGAATCTTCCA*	600	56	20	**(Rinttila *et al.* 2004)**
	Lacto-R	*CACCGCTACACATGGAG*				
Prevotella	PreF	*CACCAAGGCGACGATCA*	700	56	45	**(Larsen *et al.*)**
	PreR	*GGATAACGCCYGGACCT*				
SRB[1]_aps[2]	APS3F	*TGGCAGATCATGWTYAAYGG*	500	56	30	**Modified from (Deplancke *et al.* 2000)**
	APS2R	*GGGCCGTAACCRTCYTTRAA*				

Table 7.1 Cont.

***SRB_dsr*[3]**	DSR1F+	*ACSCACTGGAAGCACGGCGG*	500	63	15	**(Kondo *et al.* 2004)**
	DSR-R	*GTGGMRCCGTGCAKRTTGG*				
Total bacteria	UnivF	*TCCTACGGGAGGCAGCAGT*	500	60	45	**(Nadkarni *et al.* 2002)**
	UnivR	*GGACTACCAGGGTATCTATCCTGTT*				

[1], Sulphate-reducing bacteria; [2], Adenosine-5-phosphosulfate reduce gene; [3], Dissimilatory sulphate reduce gene; Conc., Concentration; Temp., temperature.

7.2.5 Statistical analysis

All qPCR reactions were performed in triplicate. For each assay, absolute quantities were analysed using Bio-Rad CFX Manager (version 1.5, Bio-Rad, Hercules, CA, USA) and converted to numbers of targeted bacteria per 1g of faeces (wet weight). Then, all data were log10 transformed to obtain homogeneity of variance. The estimation of relative abundance of bacterial groups results were analysed with qBase^{+} (Biogazelle, Ghent, Belgium) (Vandesompele *et al.* 2002; Hellemans *et al.* 2007) and expressed relative to the total number of bacteria. These data were also normalised by log10 transformation. Statistical analyses were conducted by SPSS for Windows™ (version 17, SPSS Inc., Chicago, IL, USA). Significant differences between groups were tested using an independent T-test. A p-value of less than 0.05 was considered statistically significant.

7.2.6 Ethics approval

Ethics approval was granted by the University of South Australia's ethics committee in accordance with the Declaration of Helsinki.

7.3 Results

7.3.1 Characteristics of participants

Information on the age, gender, anti-/probiotic usage and incidence of FGIDs for individuals of each study group is same as Table 6.1. A much higher incidence of FGIDs was observed in children with ASD compared with CON participants. Of note, the incidence of FGIDs in the SIB participants was intermediate between the ASD and CON participants.

7.3.2 *Bifidobacterium spp.*, *Akkermansia muciniphila* and *Bacteroides fragilis* group

Absolute and relative numbers of each targeted faecal bacteria in ASD, SIB and CON participants were calculated as mean ± S.E.M (Table 7.2). Significant differences between groups were found for *Bifidobacterium spp.* and *A. muciniphila* only. Overall, there were lower absolute and relative numbers of *Bifidobacterium spp.* in children with ASD

compared with both CON participants ($p = 0.016$ and $p = 0.006$, respectively) and SIB participants ($p = 0.023$ and $p = 0.032$, respectively). There were no differences in numbers of this bacterium between SIB and CON participants.

Moreover, decreased absolute and relative numbers of *A. muciniphila* were present in ASD children relative to those in CON participants ($p = 0.043$ and $p = 0.029$, respectively). The relative numbers of *A. muciniphila* were also lower in the SIB group than the CON group ($p = 0.031$). In addition, we compared the abundance of the targeted bacteria in ASD children with and without FGIDs. Elevated absolute numbers ($p = 0.059$) and relative numbers ($p = 0.019$) of the *B. fragilis* group were evident in children with ASD whose caregivers reported them as having FGIDs compared to those without a FGID (Table 7.3). Faecal numbers of other bacterial targets did not differ significantly between ASD individuals with and without FGIDs.

7.3.3 Others

None of the other bacterial assays were found to be significant different between any of the groups. Further statistical analyses were conducted where participants implementing special diets or using antibiotics or probiotics were excluded from the analyses and the key findings remained unchanged.

Table 7.2 The absolute and relative abundances of target bacteria

Target	Absolute numbers (per g wet faeces) Mean ± S.E.M			Relative numbers[4] Mean ± S.E.M		
	ASD	**SIB**	**CON**	**ASD**	**SIB**	**CON**
A. muciniphila	$(3.33 \pm 1.48) \times 10^{7a}$	$(2.02 \pm 0.61) \times 10^{7}$	$(3.98 \pm 1.14) \times 10^{7\,a}$	$5.07 \pm 1.54^{\,b}$	$4.67 \pm 1.72^{\,c}$	$14.18 \pm 6.21^{b,c}$
B. fragilis group	$(9.30 \pm 4.68) \times 10^{7}$	$(1.27 \pm 0.66) \times 10^{8}$	$(7.73 \pm 3.97) \times 10^{7}$	1.25 ± 0.32	1.75 ± 0.44	2.45 ± 1.82
Bifidobacterium spp.	$(2.23 \pm 0.62) \times 10^{7d,e}$	$(3.73 \pm 0.86) \times 10^{7e}$	$(6.27 \pm 2.57) \times 10^{7\,d}$	$0.78 \pm 0.18^{\,f,g}$	$1.37 \pm 0.27^{\,g}$	$1.96 \pm 0.48^{\,f}$
C. coccoides group	$(2.41 \pm 0.37) \times 10^{8}$	$(2.09 \pm 0.33) \times 10^{8}$	$(1.78 \pm 0.21) \times 10^{8}$	1.21 ± 0.17	1.01 ± 0.11	1.14 ± 0.16
C. leptum group	$(8.57 \pm 1.18) \times 10^{8}$	$(1.03 \pm 0.19) \times 10^{9}$	$(1.15 \pm 0.39) \times 10^{9}$	0.98 ± 0.12	0.99 ± 0.08	1.13 ± 0.16
C. cluster I	$(9.42 \pm 2.19) \times 10^{6}$	$(9.26 \pm 2.31) \times 10^{6}$	$(1.27 \pm 0.40) \times 10^{7}$	1.20 ± 0.28	0.89 ± 0.17	1.72 ± 0.49
C. difficile	$(7.69 \pm 7.33) \times 10^{4}$	$(4.73 \pm 0.85) \times 10^{3}$	$(2.57 \pm 0.43) \times 10^{3}$	3.23 ± 2.82	0.45 ± 0.07	0.32 ± 0.06
Enterococcus spp.	$(1.52 \pm 0.73) \times 10^{5}$	$(1.30 \pm 0.48) \times 10^{5}$	$(1.51 \pm 0.73) \times 10^{5}$	1.39 ± 0.76	0.71 ± 0.25	1.47 ± 0.80
E. coli	$(6.09 \pm 4.49) \times 10^{6}$	$(6.04 \pm 3.37) \times 10^{6}$	$(4.36 \pm 2.65) \times 10^{6}$	2.95 ± 1.48	7.40 ± 5.55	3.22 ± 1.21

Table 7.2 Cont.

Target	Absolute numbers (per g wet faeces) Mean ± S.E.M			Relative numbers[4] Mean ± S.E.M		
	ASD	**SIB**	**CON**	**ASD**	**SIB**	**CON**
F.praunitzii	$(5.40 \pm 0.92) \times 10^7$	$(6.01 \pm 2.00) \times 10^7$	$(4.50 \pm 2.10) \times 10^7$	1.18 ± 0.17	1.13 ± 0.13	0.73 ± 0.15
Lactobacillus group	$(9.97 \pm 3.39) \times 10^4$	$(1.67 \pm 0.96) \times 10^5$	$(1.53 \pm 1.08) \times 10^5$	0.81 ± 0.29	0.99 ± 0.43	0.72 ± 0.27
Prevotella group	$(6.03 \pm 2.53) \times 10^6$	$(7.06 \pm 2.22) \times 10^6$	$(1.28 \pm 0.31) \times 10^6$	1.04 ± 0.33	1.49 ± 0.43	0.44 ± 0.13
SRB[1]_aps[2]	$(1.24 \pm 0.46) \times 10^6$	$(1.82 \pm 0.68) \times 10^6$	$(6.75 \pm 3.50) \times 10^5$	0.57 ± 0.09	0.79 ± 0.19	0.33 ± 0.07
SRB_dsr[3]	$(1.28 \pm 0.49) \times 10^6$	$(1.23 \pm 0.37) \times 10^6$	$(1.16 \pm 0.52) \times 10^6$	0.59 ± 0.12	0.70 ± 0.19	0.70 ± 0.11
Total bacteria	$(1.20 \pm 0.21) \times 10^9$	$(1.36 \pm 0.30) \times 10^9$	$(1.26 \pm 0.16) \times 10^9$	--	--	--

[1], Sulphate-reducing bacteria; [2], Adenosine-5-phosphosulfate reduce gene; [3], Dissimilatory sulphate reduce gene; [4], calculated using qBase$^+$ software and expressed as targets relative to the total number of bacteria; [a, b] Significantly different absolute and relative numbers of *A. muciniphila* between ASD and CON, respectively; [c], Significantly different relative numbers of *A. muciniphila* between SIB and CON; [d,f,] Significantly different absolute and relative numbers of *Bifidobacterium spp.* in ASD compared with CON, respectively ; [e,g,] Significantly different absolute and relative numbers of *Bifidobacterium spp.* ASD compared with SIB, respectively; [a, b, c, d, e, f, g,] $P < 0.05$.

Table 7.3 The absolute and relative abundances of targeted bacteria in children with ASD with and without FGID

Target	Absolute numbers (per g wet faeces) Mean ± S.E.M		Relative numbers [4] Mean ± S.E.M	
	ASD^{GI} (n = 9)	**ASD^{NGI} (n = 14)**	**ASD^{GI}**	**ASD^{NGI}**
A muciniphila	$(3.46 \pm 1.53) \times 10^7$	$(3.24 \pm 2.28) \times 10^7$	5.83 ± 1.78	4.58 ± 2.31
B. fragilis	$(1.77 \pm 1.15) \times 10^8$	$(3.90 \pm 1.64) \times 10^7$	2.17 ± 0.66	0.65 ± 0.20*
Bifidobacteria	$(1.77 \pm 1.31) \times 10^7$	$(2.52 \pm 0.62) \times 10^7$	0.51 ± 0.26	0.96 ± 0.25
C. coccoides	$(2.48 \pm 0.76) \times 10^8$	$(2.36 \pm 0.38) \times 10^8$	1.21 ± 0.20	1.22 ± 0.14
C. leptum	$(9.29 \pm 1.66) \times 10^8$	$(8.11 \pm 1.65) \times 10^8$	1.10 ± 0.20	0.90 ± 0.14
C. cluster I	$(9.98 \pm 3.40) \times 10^6$	$(9.06 \pm 2.95) \times 10^6$	1.37 ± 0.44	1.11 ± 0.36
C. difficile	$(1.90 \pm 1.87) \times 10^5$	$(3.89 \pm 0.87) \times 10^3$	7.59 ± 7.22	0.42 ± 0.07
Enterococcus	$(2.24 \pm 1.60) \times 10^5$	$(1.05 \pm 0.67) \times 10^5$	2.25 ± 1.77	0.83 ± 0.54
E. coli	$(1.00 \pm 0.44) \times 10^6$	$(9.35 \pm 7.33) \times 10^6$	0.80 ± 0.34	4.19± 2.28
F. praunitzii	$(5.54 \pm 1.13) \times 10^7$	$(5.31 \pm 1.36) \times 10^7$	1.30 ± 0.34	1.09 ± 0.19
Lactobacillus	$(1.50 \pm 0.79) \times 10^5$	$(6.49 \pm 1.71) \times 10^4$	1.18 ± 0.64	0.53 ± 0.15
Prevotella	$(4.20 \pm 3.07) \times 10^6$	$(7.21 \pm 3.72) \times 10^6$	0.69 ± 0.45	1.26 ± 0.47
SRB[1]_aps[2]	$(1.92 \pm 1.10) \times 10^6$	$(8.09 \pm 2.88) \times 10^5$	0.78 ± 0.18	0.44 ± 0.08
SRB_dsr[3]	$(1.75 \pm 1.10) \times 10^6$	$(9.78 \pm 4.12) \times 10^5$	0.73 ± 0.28	0.52 ± 0.12
Total bacteria	$(1.38 \pm 0.45) \times 10^9$	$(1.08 \pm 0.19) \times 10^9$	--	--

ASD^{GI}, children with ASD who were experiencing FGIDs; ASD^{NGI}, children with ASD without FGIDs; *, significantly elevated relative numbers of *B. fragilis* in ASD^{GI} compared with ASD^{NGI}; [1], Sulphate-reducing bacteria; [2], Adenosine-5-phosphosulfate reduce gene; [3], Dissimilatory sulphate reduce gene; [4], calculated using qBase+ software and expressed as targets relative to the total number of bacteria.

7.4 Discussion

In the current chapter, the numbers of key human GI bacteria in the faeces of children with ASD have been estimated. Many species or groups were quantified for the first time in the ASD cohort. The abundance of *Bifidobacterium spp* and *A. muciniphila* were significantly lower in the faeces of children with ASD compared to CON participants, which are novel findings. However, it is not clear whether GI microbial differences determine whether or not children with ASD experience FGIDs. It is also unclear whether these differences were responsible for the observed higher incidence of GI disorders in children with ASD relative to CON children in this study. Only the abundance of bacteria belonging to the *B. fragilis* group differed between the ASD children with and without reported FGIDs. These findings add to the limited knowledge of changes that may be occurring in the GI tracts of individuals with ASD.

Bifidobacterium spp. is generally considered to be beneficial bacteria and a lower abundance has been associated with a range of GI disorders (Favier *et al.* 1997; Malinen *et al.* 2005; Collado *et al.* 2008). Consequently, the finding of lower faecal numbers in children with ASD may also be associated with a reduced robustness of the GI system in these individuals. However, numbers of these bacteria were not significantly different between ASD children with and without FGID. Therefore, it is not possible to make definitive conclusions regarding a role for *Bifidobacterium spp.* in the GI health status of children with ASD. Moreover, these findings are in contrast to a previous study where it was reported that there were no significant changes in *Bifidobacterium spp.* between children with and without ASD (Parracho *et al.* 2005). It should be mentioned in this previous study, 90% and 53% of ASD participants had taken antibiotics and probiotics during the recruitment respectively which is likely to have significantly impacted on the composition of the GI microbiota. Only one of ASD participants had received an antibiotic at the time of the study (immediately prior to the study) and two of ASD participants were taking probiotics during the sample collection period in the current cohort.

Significantly decreased numbers of *A. muciniphila* in the faeces of children with ASD compared with CON participants. *A. muciniphila* is a mucin-degrading bacterium present in abundance in the gut of healthy adults. Some studies have reported reduced counts in patients with Crohn's disease and ulcerative colitis or the elderly (Miller and Hoskins

1981; Collado *et al.* 2007; Png *et al.*). A thinner mucous layer is present in patients with ulcerative colitis compared with the controls (Campieri and Gionchetti 2001; Corazziari 2009) which properly indicated an alteration of mucin-degrading bacteria and hence lower numbers in the GI tract. Therefore, the finding of lower *A. muciniphila* numbers in the faeces of ASD children could indicate that the GI tract mucous barrier is thinner compared with controls. It will be important to examine whether this is true in future studies. A compromised mucous barrier could have significant consequences for the health of the GI system and the health of the host by increasing the likelihood of pathogenic bacteria breaching the barrier. Interestingly, increased gut permeability in ASD has been described in children with ASD and/or their first-degree relatives (D'Eufemia *et al.* 1996; de Magistris *et al.* 2010). However, like the *Bifidobacteria*, *A.muciniphila* numbers were not significantly different between ASD children with and without FGID, and therefore it is not possible to clearly relate changes in faecal *A. muciniphila* numbers to the reported FGIDs.

The Gram-negative *B. fragilis* group of bacteria are commonly isolated in the clinical laboratory and consists of 10 species including *B. distasonis, B. fragilis, B. thetaiotamicron,* and *B. vulgates*, but may not all be present in faeces (Craft 1999). Some species in *B. fragilis* group have beneficial effects on host health (Wexler 2007). Such effects include supplying energy to the host by facilitating fermentation (Hooper *et al.* 2002), promoting T-cell-dependent immune response (Mazmanian and Kasper 2006), limiting the colonization of pathogens in GI tract (Hopkins and Macfarlane 2002) and potentially reducing effects of obesity (Turnbaugh *et al.* 2006). However, some species in the *B. fragilis* group can be responsible for infections with significant morbidity and mortality (Wexler 2007). It is possible that some of these species are responsible for the finding that the numbers of *B. fragilis* group bacteria were higher in ASD children with reported FGIDs. Real-time PCR assays that target the members of the *B. fragilis* group in future studies will shed further light on the species involved.

A previous study showed significantly higher numbers of *C. cluster I* and *C. bolteae* in faecal specimens of children with ASD compared with controls (Song *et al.* 2004) but the finding differed from the current study. Song *et al.* (2004) did not provide much information about the participants or sample collection methodology, both of which are needed to understand points of difference with the current study. Another study reported a

higher abundance of *C. histolyticum group (C. cluster I* plus *C. cluster II)* in children with ASD (Parracho *et al.* 2005). In that study, most of the 58 participants with ASD were reported as experiencing GI disturbance (91%) including 76% with diarrhoea. Also, 66% of participants in that study were implementing gluten- and/or casein-free diets and the results were based on a single faecal sample from each participant. In contrast, 48 h samples were collection in the current study, which provide a better representation of the microbial population of each participant. Further, the most common issue of FGIDs in children with ASD in the current cohort was constipation, and only four ASD participants were implementing a gluten- and/or casein-free diet. Thus, the different findings regarding the abundance of *C. cluster I* may relate to different types of FGIDs and dietary intervention in the study cohorts.

In the current study bacterial functionality was measured via SCFA, phenol, *p*-cresol and ammonia production and this data can then be related to the quantities of bacteria measured. Altered SCFA profiles, especially increased levels of propionate in children with ASD compared to controls, have been described in Chapter 6. Despite this, there was no significant difference in the absolute and relative numbers of *Prevotella*, which are key propionate-producing bacteria, between the study groups of the present study. This suggests that other bacteria that have not targeted in this chapter may be responsible for the differences of propionate observed in chapter 6. It is possible that the activities of the bacteria responsible for SCFA production have been altered rather than bacterial numbers. RNA molecules play an active role in cells by catalysing biological reactions, controlling gene expression, or sensing and communicating responses to cellular signals. The microbial functionality also could be measured through bacterial RNA. The target genes in the current study were mainly the 16S rRNA gene and therefore to do further analysis of which bacterial genes are switched on in relation to fermentation, other genes specific for carbohydrate and protein fermentation should also be targeted.

While, other factors, such as differences in GI function that alter GI transit time in ASD, should also be considered. In addition, numbers of targeted bacteria such as the *F. prausnitzii, C. leptum* group and *C. coccoides* group, which cover many of the primary butyrate producers, did not significantly differ among the study groups although low counts of *F. prausnitzii* have been found in individuals with inflammatory bowel disease (Sokol *et al.* 2009). Furthermore, SRB did not differ between groups but a positive

correlation has been reported to between the total viable counts and the clinical GI disorder severity (Pitcher *et al.* 2000).

7.5 Conclusion

In summary, these findings add to the knowledge of the changes that occur in the GI tracts of children with ASD. A high frequency of FGIDs has been confirmed in children with ASD, but the association between the incidence of FGIDs and altered GI microbiota profile needs further investigation. A longitudinal study may supply more important information on the temporal influences on GI microbiota in ASD and could potentially guide implementation of dietary interventions that impact on the gut microbiota and improve GI health in individuals with ASD.

8 General Discussion and Future Direction

8.1 General Discussion

One of the aims of this thesis was to review and summarise published papers on GI studies on ASD. Reports on the GI factors in ASD to date have been diverse and often contradictory. The true incidence of GI disturbance in individuals with ASD is unknown, with estimations ranging from 9-70%. Altered GI microbiota in ASD compared with controls was described in several studies. Moreover, previous clinical trials in children with ASD using oral vancomycin (Sandler *et al.* 2000), non-absorbable antibiotics/antifungals (Whelan 2000) and probiotics (Parracho *et al.* 2010) have shown improvements of GI symptoms and cognitive functioning. Indeed, modulating an imbalanced GI microbiota in ASD could be an important step to reduce GI symptoms and improve behaviours in ASD. Thus, several bacteria, such as *A. muciniphila*, and large bowel fermentation products, such as SCFA, which related to GI health and suggested to be involved with the aetiology of ASD had more fully explored in this thesis.

It emerged that there is widespread consensus about the involvement of urinary biomarkers such as peptides, neopterin, porphyrins and monoamines which are associated with GI disruption, immune dysfunction, heavy metal exposure and altered neurotransmitters in ASD. This thesis reviewed a range of putative urinary biomarkers reported to be associated with ASD. Although there was no strong evidence for any single metabolite having significant clinical utility or providing conclusive insight into the aetiology or pathophysiology of ASD, these metabolites are potentially useful for distinguishing and/or supplying dietary intervention to subgroups of ASD. Further validation is required.

Based on the literature review, several urinary metabolites including IAG, CR, GAA and creatinine were identified as potential biomarkers in this study. The third aim of this thesis was to develop reliable LC-MS/MS methods to quantify the targeted chemicals between children with ASD, their siblings and community controls. Previous results of urinary IAG between children with and without ASD were inconsistent (Whiteley *et al.* 1999; Bull *et al.* 2003; Whiteley and Shattock 2003; Wright *et al.* 2005). Urinary levels of IAG and the GI disturbance were described in children with and without ASD in the current study. Significantly higher ratios of urinary IAG: creatinine were found in children with ASD experiencing GI disturbance compared with those children who did not suffer from this condition. However, IAG: creatinine did not differ significantly between controls (SIB and

CON groups) with and without ongoing GI disturbance. Thus, increased urinary IAG may be indicative of GI disturbance in children with ASD. More studies are required with larger sample size to repeat and confirm these results. Routine determination of urinary IAG in young children with ASD may enable timely identification of GI disturbance and direct appropriate interventions which could potentially improve the quality of life of affected individuals.

Similar levels of urinary CR and GAA with age adjustment were identified in children with and without ASD in this thesis. Although routinely measuring urinary CR and GAA in diagnostic protocols for ASD is unlikely to yield many positive cases of creatine deficiency syndrome, examining these compounds during the diagnostic work up process should be considered for all cases of suspected ASD. Early detection of creatine deficiency syndrome cases will guide implementation of dietary interventions which can dramatically improve outcomes for those individuals.

The next aim in this thesis was to analyse these large bowel fermentation products in children with and without ASD. Any differences in observed fermentation products between children with and without ASD could shed light on how GI function is affected in children with ASD and need to be interpreted in light of potential differences in the amount and type of dietary substrates. A modified FGIDs questionnaire was completed by caregiver(s) of each participant to assess the presence of FGID. Higher incidence of GI disturbance in ASD was confirmed in the current cohort. Large bowel microbacteria can metabolise undigested carbohydrate and protein. The end fermentation products include SCFA, phenols (i.e. phenol & *p*-cresol) and ammonia (Topping and Clifton 2001). It is understood that the measurement of these faecal fermentation product represents what remains following the generation of these products from the fermentation of dietary sources by the microbiota and their subsequent removal by absorption and/or utilisation by other gut bacteria.

Increased faecal levels of acetate, propionate, some minor SCFA and ammonia were found in children with ASD compared with controls. These changes could contribute to, or reflect, altered GI function. The alterations in large bowel fermentation products was not attributable to other conditions such as coeliac disease, Crohn's Disease and ulcerative colitis since children with and without ASD showing severe GI conditions were excluded

from participating. Hence, the alterations were indicative of a specific association between ASD and GI disturbance.

The findings of elevated levels of faecal propionate in this thesis are novel. Elevated levels of propionate in the systemic circulation could manifest in developmental delay, seizures and GI symptoms, resembling some aspects of ASD (Burlina *et al.* 1999*;* Wajner *et al.* 2004). MacFabe *et al* (2007, 2008) have developed a rat model where intraventricular injection of propionate into the CNS induces behaviours resembling ASD. The primary source of propionate in humans is the fermentation of undigested food by the colonic microbacteria. Most propionate absorbed by the GI tract is metabolised by the liver. Only a small amount of colonic propionate reaches the systemic circulation under normal conditions (Al-Lahham *et al*. 2010). Significantly increased faecal levels of propionate as reported in the thesis may reflect impaired utilisation of propionate in the colon of children with ASD.

Propionate in the gut can be converted to succinate, and then enter the tricarboxylic acid (Krebs) cycle. Methylmalonyl-CoA isomerase (mutase) is a vitamin B_{12}-requiring enzyme (5´-deoxyadenosylcobalamin) that catalyses the conversion of methylmalonyl-CoA to succinyl-CoA. It has been suggested that vitamin B_{12} deficiency is present in some children with ASD (Pineles *et al.* 2010). In the presence of vitamin B_{12} deficiency, the rate of propionate clearance from blood will be decreased with an accompanying accumulation of methylmalonyl-CoA and a corresponding increased urinary excretion of methylmalonic acid. Notably, increased urinary excretion of methylmalonic acid has been found in a small group of children with ASD (n= 8) (Wakefield *et al.* 1998).

It seems unlikely that the magnitude of the increase in faecal propionate levels observed in children with ASD will translate into or affect blood levels that will subsequently have neuropathological manifestations. It may be important to measure blood levels of propionate in future studies to examine this link. Having blood taken can be an unpleasant experience for children (particularly those with ASD) and caregivers are reluctant to agree. Thus, the current study did not collect blood samples and the link between faecal and blood concentrations of propionate could not be investigated.

Like propionate, one of protein fermentation products, ammonia, was found in higher levels in faeces of children with ASD than siblings in this study. Ammonia (present as the

ammonium ion) is mostly detoxified in the liver before entering the systemic circulation. Accumulation of ammonia in the brain can disturb metabolism, cause extracellular potassium accumulation and hence may increase neuronal depolarization (Felipo *et al*. 2002). Elevated plasma ammonia levels have been reported in children with ASD (Clark-Taylor and Clark-Taylor 2004; Filipek *et al.* 2004) and may be indicative of the urea cycle dysfunction in the liver (Felipo and Butterworth 2002; Gorker and Tuzun 2005). However, the observed increased faecal ammonia levels in children with ASD in this study may not necessarily cause large changes of ammonia in the blood and brain. Consequently, it is premature to conclude that the elevations in faecal ammonia levels lead to central neuropathological effects in ASD. Other faecal protein fermentation products, phenol and total *p*-cresol did not differ between children with and without ASD.

Measurement of both urinary and faecal levels of enteric bacterial fermentation products allow for the indirect elucidation of population and activity profiles of an individual's large bowel microbiota. It is also important to conduct metagenomic studies to characterise the role of the variations in GI microbial species in the observed differences in the GI disturbance between children with and without ASD. To achieve this aim, targeted GI bacteria, such as *A. muciniphila*, *Bifidobacterium spp.* and *Clostridium spp.,* were quantified. Previous studies found that certain GI bacterial species in faeces, particularly some *Clostridium spp.,* are present in higher abundance in children with ASD who are commonly experiencing GI disturbance (Finegold *et al.* 2002; Song *et al.* 2004; Parracho *et al.* 2005; Finegold *et al.* 2010). In contrast, this study showed no differences in *Clostridium spp.*. Several other targeted bacteria were currently found to be altered in children with ASD compared with controls such as lower numbers of *Bifidobacterium spp.* and *A. muciniphila*. A clear correlation between *B. fragilis* counts and GI disturbance was described in ASD participants. These findings contribute to the knowledge of changes in the GI tract that occur in children with ASD and may potentially guide dietary interventions or supplements, such as probiotics or prebiotics that optimise GI health in this population. Probiotics are live bacteria that, when administered, can provide health benefits to the host. Prebiotics are substances that selectively promote the growth and activity of specific bacterial species in the gut, particularly *Bifidobacterium spp.* and *Lactobacillu,* and they can also be incorporated in the diet.

The present study also recorded anti- and probiotic usage in the current cohort during the sample collection period. Anecdotal reports showed adverse effects of the antibiotics use in children with ASD at the early stage as antibiotics may disrupt the balance of indigenous GI microbiota and allow colonisation of opportunistic pathogens. Thus, antibiotics may be a trigger for ASD. Faecal analysis in this thesis was conducted before and after excluding those participants who had anti- and/or probiotics immediately prior to the study. The key findings remained unchanged which indicated the potential influence of anti- and/or probiotics did not affect the current results. Since a relatively small number of participants used anti- and/or probiotics, a definitive conclusion may not be appropriate at this stage.

Elimination diets are used in ASD to address various categories of food allergies. The most popular diet used in the management of ASD is the gluten-/casein-free diet. Although anecdotal evidence of its benefits is plentiful, available research data are conflicting (Elder *et al.* 2006; Milward 2008; Johnson *et al.* 2010; Whiteley *et al.* 2010). In the current study, participants implementing a gluten-/casein-free diet was identified, but there were no significant differences in urinary IAG, faecal fermentation products or faecal microbiota found between children with ASD on the diet or those not implementing gluten- and/or casein- free diets.

Recent studies provide direct evidence that microbiota can influence brain development and behaviour (Neufeld *et al.* 2010; Bailey *et al.* 2011; Cryan and O'Mahony 2011). Behavioural changes due to altered microbiota in neonatal life may still present in adulthood (Cryan and O'Mahony 2011). Thus, findings in this thesis revealed alterations in GI microbiota and may also increase the understanding of the interplay of the brain-gut axis in ASD. Insight into alterations in the GI microbiota in ASD will be important for developing strategies that might alleviate GI dysfunction in affected children. Research involving large sample sizes is required to investigate the effects of antibiotics, probiotics and nutritional interventions on the GI microbiota, GI function and behaviours in children with ASD.

The genetic contribution to the GI manifestations was not examined in this thesis. However, notably, the concentrations of urinary (e.g. IAG)/ faecal (e.g. propionate) metabolites and the frequency of GI disturbance in siblings were investigated and found to be intermediate between ASD and CON participants in this thesis. These interesting

findings suggested that siblings of children with ASD may have genetic pre-disposition to GI disturbance. Genetic studies are recommended to improve understanding of susceptibility genes that confer a risk of developing GI disturbance. There is compelling evidence that genetics play a key role in the aetiology of ASD (Szatmari *et al.* 2007; Morrow *et al.* 2008). Specifically, Campbell *et al* (2009) have provided genetic evidence supporting the link between GI dysfunction and ASD and reported an association between MET factor gene and ASD. Thus, the author concluded that prospective studies need to be conducted to determine the specific GI conditions that manifest in those individuals with ASD who have the MET gene variant.

In conclusion, highlights of the work presented in this thesis included findings regarding altered urinary and faecal metabolic profiles, as well as an imbalance in composition and diversity of GI microbiota in children with ASD compared with controls. A modified FGID questionnaire administered to caregivers revealed nearly half of children with ASD experienced ongoing GI problems. Findings in the current work have confirmed that GI disturbance is strongly associated with ASD.

8.2 Future Direction

ASD is a debilitating and often socially devastating neurodevelopmental disorder characterised by complex aetiology, variable presentation and widely divergent outcomes and carries a significant risk of various co-morbidities. Currently, diagnosis and subtyping of ASD are based on a cluster of behaviours and there are no validated laboratory tests to assist ASD diagnosis. Definitive diagnosis is based on the DSM-IV (APA 2000) or ICD-10 (WHO 1992). If an earlier diagnosis was possible or a diagnostic marker could confirm the specific pathology of an ASD subtype, targeted intervention may improve prognosis.

Diagnosis of ASD should ideally incorporate laboratory investigations such as biochemistry, haematology, assessment of the liver and kidney function, immune system assessment, as well as investigating bacteriology from stool. Assessment of inflammatory parameters in the GI tract may identify the underlying cause and guide implementation of early medical intervention. Biological alterations as identified in urine and faecal samples in children with ASD compared with controls and presented in this thesis have brought

new insights into the potential aetiology and pathophysiology of ASD that have the potential to assist early diagnosis and selection of interventions.

Some future directions have been highlighted throughout the thesis, such as using larger sample sizes to confirm and extend the findings. However, several additional issues should be considered when planning future research in this area. A broad array of micro-organisms inhabits the GI tract and produces fermentation products that can be regarded as beneficial or detrimental to host health. This thesis examined the presence and abundance of a limited range of GI bacteria that are emerging as markers of GI health. Evidence is also emerging that intestinal permeability (D'Eufemia *et al.* 1996; Horvath *et al.* 1999; de Magistris *et al.* 2010) in individuals with ASD is different compared with the general population. Although not evaluated in this thesis, a novel direction of high impact research in the future is investigation of mucosa-associated microbiota and the impact on intestinal hyperpermeability which may lead to increased absorption of neurotoxins from the gut.

Real-time quantification PCR is a powerful method of investigation, but is limited by normal primer biases as well as the incomplete nature of 16S rDNA databases which cannot cover all the bacteria. As more bacteria are sequenced and added to the database and studies are conducted with multiple primers the effects of these limitations will decrease. Future studies should examine a broader range of GI bacteria including mucosa-associated bacteria and encompass molecular-based profiling strategies (such as denaturing gradient gel electrophoresis; DGGE), to enable the diversity and dynamics of bacterial populations to be determined. Moreover, the possible neurotoxic effects of GI bacterial by-products have received little attention to date. The question regarding whether GI microbial by-products affect the manifestations of ASD and whether the application of synbiotics rather than probiotics will help the individuals with ASD improve their gut condition needs to be considered. Studies related with the gut-brain axis in individuals with ASD are also required.

Several theories that have been posed in this thesis are not fully substantiated by current data. In the first instance, simultaneous blood, urine and faecal sampling is required to provide confirmation, or otherwise, of these theories. Although, most targeted metabolites in this thesis are assumed to be derived from large bowel bacterial fermentation, the exact pathways or involvement of specific bacteria are not conclusive at this stage. Clear links

between blood, urinary and faecal levels of these compounds as well as identification of specific GI microbacteria involved in their production will reveal the underlying pathophysiology of specific GI subtypes in ASD. Furthermore, linking the concentrations of intermediate products of the pathways (e.g. succinic acid is an intermediate product in the propionate pathway) and bacterial activities (e.g. test the H_2S concentration to monitor the activity of sulpha reducing bacteria) with current results may assist to further understand the aetiology of ASD.

To date, large intestinal permeability has not been investigated in ASD, in part due to the limited availability of techniques to assess large intestine permeability. Such studies are warranted as inflammation has been found in both the upper and lower GI tract of children with ASD (Wakefield *et al.* 2000; Horvath and Perman 2002a). Altered microbiota profiles in stools of children with ASD have been shown to have potential to cause inflammation and increased intestinal permeability. De Magistris *et al.* (2010) measured faecal calprotectin (FC) concurrently with intestinal permeability and reported elevated levels of FC in 24% ASD. FC is a protein produced by intestinal granulocytes and has proved to be a useful tool in the study of GI inflammation. Almost a quarter of the participants with ASD had pathological levels of FC which could account for mild GI inflammation. Faecal lactoferrin also has value as a marker of GI mucosal inflammation (Lamb and Mansfield 2010). A range of theories have been proposed to explain the hyperpermeability observed in ASD, including bacterial overgrowth or an imbalance in GI microbiota that in turn cause GI inflammation. Therefore, studies measuring the association between hyperpermeability, bacterial overgrowth and GI inflammatory markers are required (Garvey 2002; McGinnis 2004).

Although GI problems occur commonly in individuals with ASD, symptoms may be atypical. Due to difficulties with communication, individuals with ASD may not indicate they are experiencing GI symptoms which may only be evident in aberrant behaviour. Consequently, these GI disturbances may be under-reported to medical practitioners and undiagnosed. The temporal relationship between the emergence of the GI symptoms and the onset of ASD, or at least its behavioural presentation, also remains unclear. Further insight into the timing of onset of GI disturbance in affected children with ASD is required. Specifically, it is important to determine if GI symptoms are related to the causes of ASD

or are a consequence of its progression. Therefore, subsequent studies should also include a prospective evaluation of the GI symptoms in ASD.

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Appendix- A Instructions of Urine Samples Collection

University of South Australia

Instructions for the Collection of Urine Samples for Autism and Gastrointestinal Disturbance Study

Autism Research Group, Sansom Institute for Health Research, University of South Australia

1. **The following items will be provided to you for storing and collecting two urine samples from your child. These items include:-**
    a) **Five urine collection bags for children without toilet training**
    b) **Two labelled sterile containers (with yellow lids)**
    c) **Two protective plastic bags**
2. **Please collect the sample from the FIRST MORNING URINE. If possible, collect the urine sample directly into the sterile container with the yellow lid (it doesn't have to be full). If there is difficulty collecting a sample directly into the container, use the urine collection bag provided to assist collection. Please refer to the instruction sheet provided in the urine collection bags for further instruction.**
3. **Ensure the yellow lid on the container is firmly closed. Put the covered container inside the protective plastic bag and IMMEDIATELY put inside the FREEZER (provided). Freezing the urine sample is very important to prevent sample deterioration.**
4. **To collect the second urine sample, repeat steps 2 and 3. Once again, the first urine of the morning is required. The two urine samples should be collected over a maximum of 7 days with at least 1 day between the two samples. This design allows an average morning urine profile of each child to be identified.**
5. **Please do not remove the two urine samples from the freezer until they are collected by the researcher.**

***** Thank you for your participation in the study. *****

Appendix- B Instructions of Faecal Samples Collection

University of South Australia

Instructions for the Collection of Faecal Samples for Autism and Gastrointestinal Disturbance Study

Autism Research Group, Sansom Institute for Health Research,

University of South Australia

The following items will be provided to you for collecting and storing poop samples from your child(ren). These items include:

d) **Six labeled blue sample bags and six cable ties for each bag to secure the contents/child**

e) **Six large clear plastic snap-lock bags**

f) **A portable WAECO freezer to freeze the samples**

If you need any more bags please contact Lynn on 0432 203 386

We will collect poop samples for 48 hours from your child(ren). All the above items will have been delivered to your home in advance. We suggest you collect the poop samples over the week-end weekends and begin collection from midnight Friday until midnight Sunday. For each sample collection, please follow the directions below.

1. **When you anticipate that your child will poop, please write the date and time clearly on the label on the blue bag**
2. **Carefully place the blue plastic bag in the toilet so it sags in the middle (Figure 1). Please keep the bag above the water in the pan**



Figure 1

3. **Keep the poop free from urine. Please ask your child(ren) to empty their bladder into the toilet before collecting samples (i.e. before placing the blue collection bag in the toilet). Warn your child not to flush and do not throw any toilet paper to the sample bag. **If it is unavoidable to prevent urine being included with the poop sample, please carefully pour the excess urine in to the toilet afterwards, mention it to Lynn and write on the label.***
4. **Samples from a child who is not toilet trained may be collected by placing a square of Gladwrap™ in the child's nappy or potty. Place the Gladwrap™ square containing the poop in to the blue bag.**
5. **Please do not cut the top off the bag as the laboratory weighs the sample and the bag.**
6. **Please check that the label is visible and will not be caught in the tie. Expel as much air as possible before twisting the bag and folding over. Securely tie the blue bag just above the sample with a cable tie. Please ensure that the cable tie is locked tight.**
7. **Please place the blue bag inside the clear plastic snap-lock bag and seal.**
8. **Immediately place your samples in the portable freezer provided.**
9. **Wash your hands.**
10. **Please give us a call when the samples (for all your children who are participating) are ready, we will collect them at a time that is convenient for you.**
11. **Please do not remove the poop sample from the freezer until they are collected by the researcher.**

***** Thank you for your participation in the study. *****

Appendix- C Participant Information Sheet

PARTICIPANT INFORMATION SHEET

Investigation of factors that is diagnostic of autism and predictive of outcomes

Investigators: Dr Manya Angley (08) 8302 1227
Dr Michael Sorich (08) 8302 1230
Dr Robyn Young (Flinders University) (08) 8201 5104

Co-Investigators: Prof Mary Barton, Dr Richard Couper, Dr Cobus Gerber, Prof Ross Butler, Ms Lynn Wang

Introduction

You and your child(ren) are invited to participate in a medical research study being conducted jointly by the School of Pharmacy and Medical Sciences at the University of South Australia and the School of Psychology at Flinders University. Please read the following information carefully so you can make an informed decision about whether or not you and your child(ren) wish to participate in the study. This Information Sheet will provide details of what is involved in the study and why it is being undertaken. To achieve the aims of the study, we need to recruit children with autism and also typically developing siblings of children with autism. If you agree to participate, you will be asked to sign the attached consent form (if you have more than one child participating, a separate consent form will need to be filled out for each child). You will be given a copy to keep as a record. This is a research project and you and your child(ren) do not have to be involved. If you do not wish to participate the attention your child(ren) with autism receive through the Autism SA will not be affected in any way.

Purpose of the study

It is believed that the make up of a child's genes (DNA), and the way that these genes interact with the environment, may influence both susceptibility to developing autism and how the condition presents over time. Environmental influences may include dietary factors and medications. In this study, we will compare the body fluids of children with autism with those of typically developing siblings of children with autism and controls with no family history of autism. By analysing your child(ren)'s body fluids (saliva, urine), it is hoped that we can discover which genes and environmental factors influence the occurrence of autism and predict the subsequent course of the condition. In addition, there is considerable evidence in the literature that gastrointestinal abnormalities may be contributing to the aetiology of autism. Sometimes abnormal urinary compounds are present due to certain bacteria in the gut. These bacteria may be associated with gut disturbance. We are interested in exploring the relationship between the urinary chemicals and gut bacteria in the faeces. As a result, we may be able to improve the diagnosis and management of this autism in the future. In particular, we hope to be able to identify the interventions that will result in the best possible outcomes for each individual child with autism.

Overall aim of study

This study aims to identify genetic and environmental factors that may influence a child' susceptibility to autism and predict the subsequent course of the condition.

Requirements of participation in the study

You are not required to change any of your child(ren)'s treatments or interventions, but your child(ren) will be required to have the following additional procedures if you agree that they may participate in this study:

1. Urine collection

We will require your child(ren) to provide two morning urine samples. These are to be obtained within a seven day period and must be separated by at least one day. These samples will be collected directly into specimen containers or using adhesive bags that are used routinely to collect urine samples in young children if your child is not toilet trained. You will be advised on the best way to collect urine with a set of instructions included in the sample collection kit. These urine samples will be used to detect whether minute quantities of chemicals, formed in your child's body in different quantities can be used as an early detection and prediction system for autism. This test will also enable the particular type of your child's autism to be classified. Urine samples will be collected at home will need to be stored in the freezer – we will provide you with small collection containers, and a storage bag to place in your freezer to keep samples away from other freezer contents. Samples need to be frozen to protect them from changes that can occur to their contents if they are stored at room temperature. The urine sample will be kept for up to three years for use in this specific study. If you are willing to allow your child(ren)'s urine to also be used in later studies related to autism after appropriate Human Research Ethics Committee approval, there is area for you to indicate this on the consent form. Your child(ren)'s urine will not be used for any other purpose other than autism research. You are under no obligation to agree to longer (indefinite) storage of your child(ren)'s urine for use in other studies.

2. Faecal sample collection

We will require your child(ren) to provide 48 hours of "poop" (also called a stool) samples. You will need to follow the **<<Instructions for the Collection of Faecal Samples for Autism and Gastrointestinal Disturbance Study >>.** These samples will be used to analyse gastrointestinal bacterial fermentation products and extract DNA to analyse the presence or absence of specific bacteria. Faecal samples will be collected at home and stored in the freezer (provided). Samples need to be frozen to protect them from changes that can occur to their contents if they are stored at room temperature. The faecal samples will be kept for up to three years for use in this specific study. If you are willing to allow your child(ren)'s faecal samples to also be used in later studies related to autism after appropriate Human Research Ethics Committee approval, there is area for you to indicate this on the consent form. Your child(ren)'s faecal samples will not be used for any other purpose other than autism research. You are under no obligation to agree to longer (indefinite) storage of your child(ren)'s faecal samples for use in other studies.

3. Access to clinical information

We will require your permission to access information that has been recorded during initial and subsequent psychological assessments by Autism SA for your child(ren) with autism.

We will require you to answer a questionnaire about your child's diet as well as complementary and conventional medicines including anti-, pro/prebiotics used.

We also require you to complete the questionnaire of Functional Gastrointestinal Disorders (FGIDs) to determine the level of your child(ren)'s gut functioning.

Duration of study

It is expected that the study will be conducted for up to 3 years.

Possible risks

This research study will not expose your child(ren) to any additional risks.

Possible benefits

Although taking part in this study may not directly benefit your child(ren) personally, the aim of this study is to investigate factors that are diagnostic of autism and predictive of outcomes. Potentially there are significant benefits to the wider autism community.

Compensation

Every care will be taken to ensure your child(ren) does not experience any discomfort or distress when DNA and urine samples are obtained. Indemnity insurance is available for any research-related injury. If you believe that you have a research-related injury, further information concerning the availability of compensation can be obtained from any of the investigators listed at the top of this sheet.

Right to refuse or withdraw

You are under no obligation to be consent to your child(ren) being involved in this study. If you do not wish to participate, the attention your child(ren) with autism receives through Autism SA will not be affected in any way. If you elect for your child(ren) to participate you may withdraw your child(ren) at any time without prejudice to the further attention your child with autism receives through Autism SA. You do not need to specify why you want to withdraw your child(ren) from the study.

Costs/Out of pocket expenses

You or your child(ren) will not be paid for your participation in this study. You or your child(ren) will not be charged for the faecal or urine samples required for this study. This is a non-sponsored study, i.e. the investigators do not receive money from any drug company or medical institution.

Confidentiality

Information collected during this study will be treated with the strictest confidence. The faecal/urine samples, and data arising from this study will be de-identified, associated with a code, date of birth and gender only. No data will be stored with your child(ren)'s full name or contact details. Only the investigators listed at the top of this Information Sheet will be able to identify your child(ren) from the code they have been assigned. The results of this research may be presented at meetings or in publications, however your child's identity will not be disclosed. Data from this study will be stored confidentially until your child(ren) are 25 years old in a locked cabinet in the Autism Research Laboratory within the Sansom Institute for Health Research, University of South Australia.

Who to contact about the study

Questions regarding this study can be answered by contacting any of the investigators named at the top of this information sheet. This study has been reviewed and approved by the University of South Australia Human Research Ethics Committee and the Flinders University of South Australia Ethics Committee. Should you wish to discuss the project with someone not directly involved, in particular in relation to matters concerning the approval process, information about the conduct of the study, or your rights as a participant, or should you have any concern or complaint, you may contact Vicki Allen, Human Research Ethics Committee Executive Officer (University of South Australia) on (08) 8302 3118.

Appendix- D Questionnaire for Medications Study

QUESTIONNAIRE FOR MEDICATIONS STUDY

Sansom Institute for Health Research, University of South Australia

ID:	**Sex: M / F**	**Entered: ASD/SIB/CON**
Date of Birth (day/month/year):	**Today's Date (day/month/year):**	**Age of child (months):**

To parents/ carers:

Please tick the relevant boxes to identify past and current treatments your child has tried.

If there is a treatment that the child has tried but is not listed, please specify the treatment and duration of time in the provided spaces (labelled other).

Background:

This study involves the collection and analysis of the urine and faecal samples of children who have entered our gastrointestinal disturbance and autism study. The purpose of this study is 1) to analyse the urine to determine the relative levels of molecules found in urine; 2) analyse gastrointestinal bacterial fermentation products in faeces; 3) to extract gastrointestinal bacteria DNA in faecal samples to analyse for the presence or absence of specific bacteria. The body contains a wide range of molecules. The types and levels of these molecules change as the body undergoes normal maintenance and behaviour. The pattern of types and levels of molecules can provide very important information regarding the way in which the body functions; or existing dysfunction (such as in disease).

The urine (molecule) profile of each individual is thought to be generally similar when compared to other individuals who are of the same age, sex, and health. However when comparing individuals who differ in terms of health status, different body functions in each individual will result in different urine profiles. Recent studies have suggested that it may

be possible to distinguish people in terms of health status (i.e. individuals with a certain condition versus those without the condition) using metabolomics.

Our earlier studies have shown that many urine samples from children with autism, contained unexpected molecules compared to urine samples from typically developing siblings and healthy unrelated controls. Various bacteria are normally present in everyone's gastrointestinal tract. We think the unexpected molecules may be produced by an unusual bacterial profile and could be associated with abnormal gastrointestinal function. This study will examine urine and faecal samples simultaneously and we will determine if there is a relationship between participants' urinary molecule profiles and their faecal bacterial profiles.

Changes in diet, environment and lifestyle (external factors) may also modify the urinary profile and the diversity of GI bacteria. In order to uncover the underlying characteristic profile in children with developmental problems versus typically developing children (siblings and unrelated); changes in diet, medication (prescription and complementray) need to be taken into account. Therefore in addition to collecting the urine and faecal samples we need to explore other factors that might be involved in the urine profile and diversity of GI bacteria of your child(ren).

MEDICATIONS

PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT: A: 0-4 weeks B: 1-3 months C: 3-6 months D: 6-12 months E: Greater than 12 months

TREATMENT	**CURRENTLY USING** PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT:	**HAD USED IN THE PAST** PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT:	**NA**
MEDICATIONS			
Anti-anxiety			
Diazepam (Antenex, Valium)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Other anxiolytic (please specify)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Antidepressants			
Citalopram (Cipramil)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Fluoxetine (Prozac, Lovan)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Paroxetine (Paxil)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Sertraline (Zoloft)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Fluvoxamine (Luvox)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Other antidepressant (please specify)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Anti-epileptics			
Carbamazepine (Tegretol, Teril)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Lamotrigine (Lamictal)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Phenytoin (Dilantin)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Sodium Valproate (Epilim, Valpro)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Other anti-epileptic (please specify)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Anti-psychotics			
Olanzapine (Zyprexa)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Risperidone (Risperidal)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Other antipsychotic (please specify)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Anti-biotics			

Amoxycillin	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Cephalexin	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Amoxycillin/clavulanic acid	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Other antibiotic (please specify)	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Anti-fungals											
Fluconazole (Diflucan)	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Nystatin (Nilstat)	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Other antifungal (please specify)	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	

NA, never had used.

MEDICATIONS

PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT: A: 0-4 weeks B: 1-3 months C: 3-6 months D: 6-12 months E: Greater than 12 months

TREATMENT	**CURRENTLY USING** PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT:	**HAD USED IN THE PAST** PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT:	**NA**
MEDICATIONS			
Anti-virals			
Aciclovir (Zovirax, Acihexal, Lovir)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Transfer factor (Antiviral)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Other anti-viral (please specify)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
GI medications			
Bethanechol (Urocarb)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Famotidine (Pepcid)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Omeprazole (Acimax, Losec)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Probiotics	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Prebiotics	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Ranitidine (Zantac)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Secretin (Secrenase)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Other GI medication (please specify)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Sleep Aid			
Antihistamine (Various)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Melatonin	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Other sleep aid (please specify)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Stimulants			
Dexamphetamine	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Short acting methylphenidate (Attenta, Ritalin)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Long acting methylphenidate (Ritalin	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	

LA, Concerta)											
Other stimulant(please specify)	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Others											
Clonidine (Catapres)	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Intravenous IG (Antiviral)	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Naltrexone (Revia)	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	
Any other medication (please specify- attach a separate list if necessary	□ A	□ B	□ C	□ D	□ E	□ A	□ B	□ C	□ D	□ E	

NA, never had used.

VITAMINS / MINERALS

PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT: A: 0-4 weeks B: 1-3 months C: 3-6 months D: 6-12 months E: Greater than 12 months

TREATMENT	CURRENTLY USING PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT:	HAD USED IN THE PAST PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT:	NA
VITAMIN/MINERAL SUPPLEMENTS			
Colloidal Minerals (eg Maximol)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Colostrum	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Essential fatty acids (please specify e.g. fish oil)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Evening primrose oil	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Iron	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
L-glutamine	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Magnesium	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Zinc	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Calcium	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
High dose-vitamin therapy	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Selenium	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Ultra Muscleze	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Vitamin A	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Vitamin C	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Vitamin E	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Pyridoxine (Vitamin B6)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Vitamin B12	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Niacin/Nicotinic acid	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Folate	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Multi-vitamin	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
DMG (Dimethylglycine)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	
Chelation (please specify e.g. DMSA)	☐A ☐B ☐C ☐D ☐E	☐A ☐B ☐C ☐D ☐E	

NA, never had used.

ALTERNATIVE DIETS / DETOXIFICATION / OTHER

PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT:
A: 0-4 weeks B: 1-3 months C: 3-6 months D: 6-12 months E: Greater than 12 months

TREATMENT	**CURRENTLY USING** PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT:	**HAD USED IN THE PAST** PLEASE SPECIFY DURATION OF TIME CHILD HAS BEEN ON TREATMENT:	**NA**
ALTERNATIVE DIETS			
Additive-free	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Casein-free	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Egg-free	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Gluten-free	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Nut-free	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Seafood-free	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Organic diet	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Preservative-free	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Sugar-free	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Yeast-free	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
DETOXIFICATION			
Chelation	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
Reduced L-glutathione	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
OTHERS			
OTHER:	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
OTHER:	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
OTHER:	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
OTHER:	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
OTHER:	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	
OTHER:	☐ A ☐ B ☐ C ☐ D ☐ E	☐ A ☐ B ☐ C ☐ D ☐ E	

NA, never had used.

*** Thank you for your participation in this study ***

Appendix- E Functional Gastrointestinal Disorder Questionnaire

Gastrointestinal Disorders Questionnaire for Autism and Gastrointestinal Disturbance Study

Autism Research Group, Sansom Institute Health Research,

University of South Australia

ID	**Sex: M / F**	**ASD/ SIB/ CON**	**Date of Birth**

This questionnaire is to determine further information to assist the progress of the autism and metabonomics study that you have entered your child(ren). This study is being conducted by the Sansom Institute, at the University of South Australia.

There is considerable evidence in the literature that gastrointestinal abnormalities may be contributing to the aetiology of autism. Some of the unexpected findings may be due to abnormal gastrointestinal bacteria in children with autism that are not present in control subjects. The next study we are planning will involve collecting faecal samples from children with autism, their siblings and unrelated controls to conduct faecal analyses.

We also need to determine by parent report if the children have a medical history of gastrointestinal abnormalities and their history of antibiotic / probiotic usage.

We would be very pleased if you could respond to the questions below.

1. Do you have any concerns about your child's bowels? **Y / N**
2. Does he / she have constipation (by this we mean pain, difficulty or delay in passing a motion)? **Y / N**

   Has he / she ever received treatment (laxatives, enemas) for it? **Y / N**

   Please give treatments

   _____
3. Does he / she have diarrhoea? **Y / N**
4. Does he / she have persistent diarrhoea (defined as a change in bowel habit for the individual resulting in significantly more

frequent or looser stools)? **Y / N**

5. Does he / she complain of recurrent abdominal (tummy) pain? **Y / N**
6. Does he /she have problems with recurrent vomiting? **Y / N**
7. Does he / she complain of abdominal distension or seem bloated? **Y / N**
8. Does he / she have problems with excessive flatulence (wind)? **Y / N**
9. Has there ever been any blood present on the stool? **Y / N**
10. Have you ever been concerned about your child's growth? **Y / N**
11. Is your child on a special diet? **Y / N**

    If so which? _____

12. Are you concerned about the range of foods that your child has in the diet? **Y / N**

13. Have you ever seen a dietician or other professional person for dietary advice for your child? **Y / N**
14. Have you ever seen a doctor specifically because of a concern that your child may have a bowel disorder (excluding gastroenteritis)? **Y / N**
15. Have you ever seen a doctor who specializes in bowel disorders (gastroenterologist)? **Y / N**
16. Has your child been specifically diagnosed as having a bowel disorders? **Y / N**

    If so what was diagnosed? _____

17. Have you given your child a treatment because of a bowel disorder? **Y / N**

    If so what? _____

Appendix- F The Calibration Data and Graphs for Creatinine and IAG.

Table 1. The concentrations, accuracy and coefficient of variations for calibration curves (n = 6) of creatinine.

	Creatinine (n = 6)		
STDs (nominal concentrations) (ng/ml)	**Observed concentrations (ng/ml)**	**Accuracy**	**Coefficient of variation**
34	36 ± 0.7	104	1.9
68	67 ± 0.2	98	0.3
170	175 ± 7.1	103	4.1
339	354 ± 2.1	104	0.6
679	697 ± 17.7	102	2.5
1700	1680 ± 42.4	99	2.5
2710	2645± 7.1	98	0.3
QC (ng/ml)			
102	105 ± 0.7	104	0.7
509	508 ± 9.9	100	1.9
2210	2245 ± 63.6	102	2.8

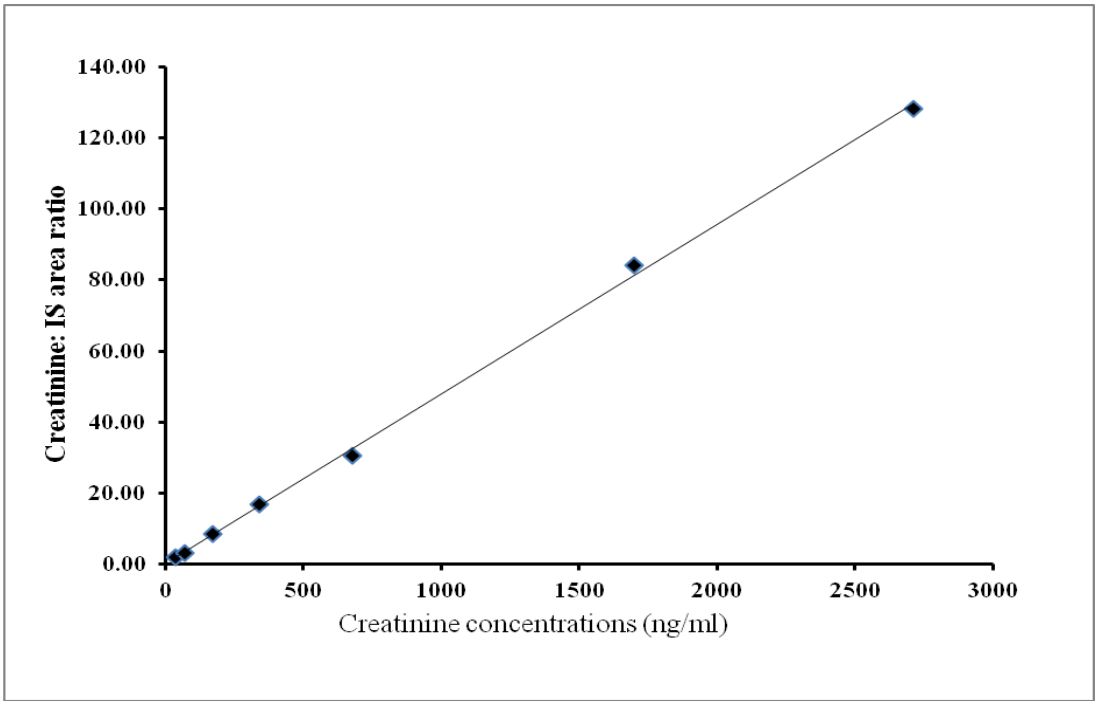


Figure 1 The example of creatinine calibration curve.

Table 2. The concentrations, accuracy and coefficient of variations for calibration curves (n = 6) of IAG.

	IAG (n =6)		
STDs (nominal concentrations) (ng/ml)	**Observed concentrations (ng/ml)**	**Accuracy**	**Coefficient of variation**
4	4 ± 0.1	101	2.5
8	7.9 ± 0.6	99	7.6
20	21 ± 1.8	103	8.6
40	38 ± 1.6	94	4.2
100	99 ± 5.2	99	5.2
200	192 ± 2.8	96	1.5
400	401± 9.9	100	2.5
QC (ng/ml)			
12	12.8 ± 0.4	106	3.1
60	63.4 ± 0.9	105	1.4
280	284 ± 11.3	101	4.0

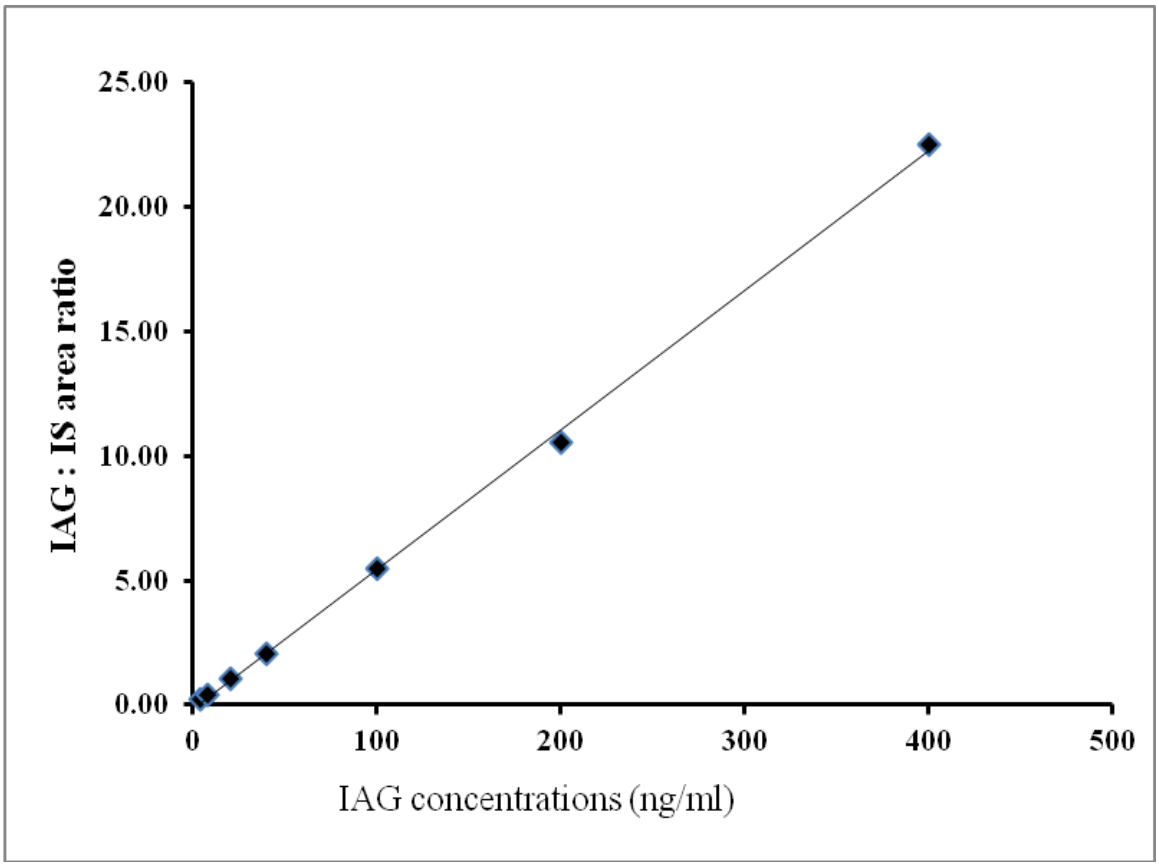


Figure 2 The example of IAG calibration curve.

Appendix- G The Calibration Data and Graphs for Creatine and Guanidinoacetate.

Table 1. The concentrations, accuracy and coefficient of variations for calibration curves (n = 6) of creatine.

	Creatine (n =6)		
STDs (nominal concentrations) (ng/ml)	**Observed concentrations (ng/ml)**	**Accuracy**	**Coefficient of variation**
200	207 ± 1.4	104	0.7
500	496 ± 2.1	99	0.4
1000	1015 ± 7.1	102	0.7
1500	1498 ± 3.5	100	0.2
2000	2030 ± 56.6	102	2.8
5000	4890 ± 28.3	98	0.6
9000	8740 ± 42.4	97	0.5
QC (ng/ml)			
100	100 ± 3.3	100	3.3
800	812 ± 1.4	102	0.2
7000	6860± 56.6	98	0.8

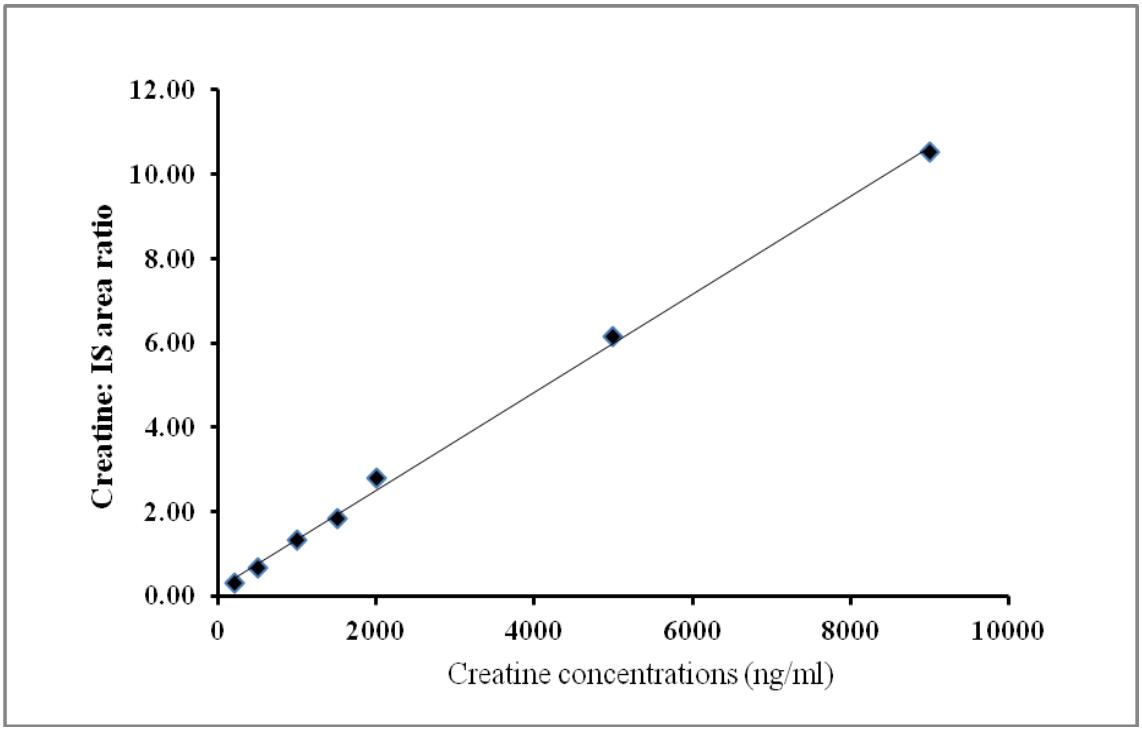


Figure 1 The example of creatine calibration curve.

Table 2. The concentrations, accuracy and coefficient of variations for calibration curves (n = 6) of guanidinoacetate.

Guanidinoacetate (n =6)			
STDs (nominal concentrations) (ng/ml)	**Observed concentrations (ng/ml)**	**Accuracy**	**Coefficient of variation**
50	49 ± 1.3	99	2.7
200	200 ± 8.5	100	4.3
500	497 ± 9.9	99	2.0
1000	1060 ± 28.3	106	2.7
1500	1525 ± 21.2	102	1.4
2000	1970 ± 14.1	99	0.7
5000	4861 ± 54.4	97	1.1
QC (ng/ml)			
100	98 ± 2.3	98	2.3
800	811 ± 10.6	101	1.3
3000	3045 ± 35.4	102	1.2

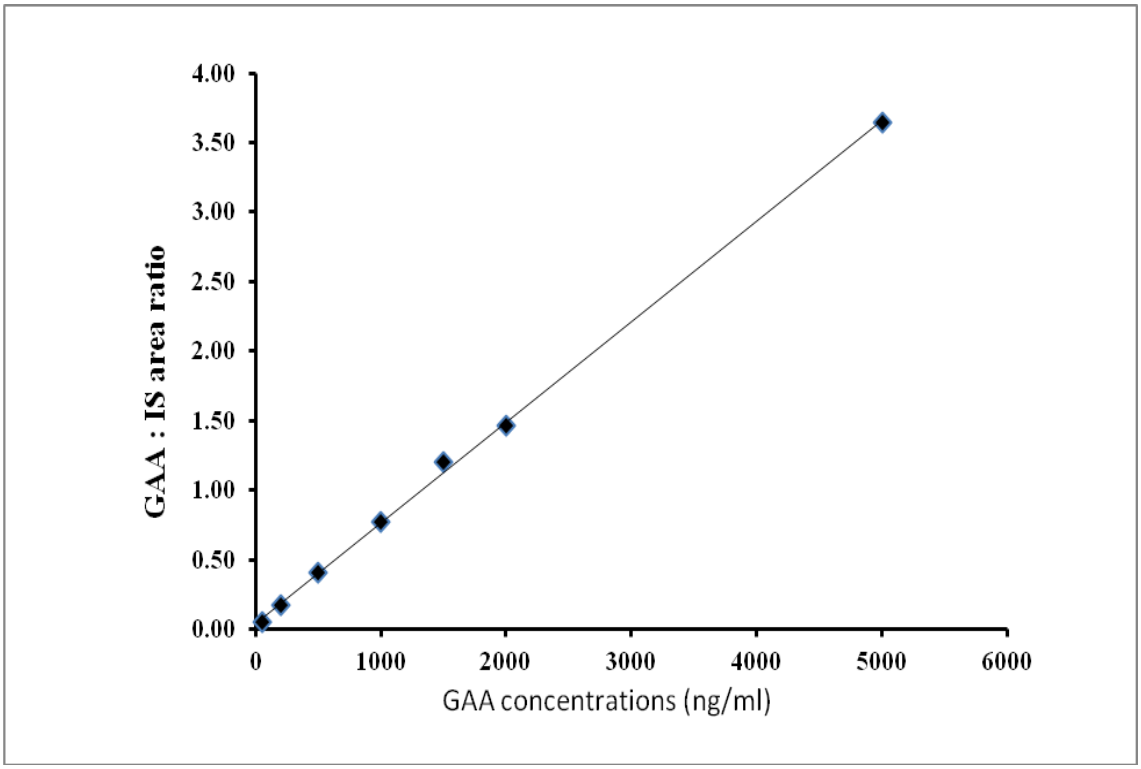


Figure 2 The example of guanidinoacetate calibration curve.