Environmental Factors Associated with Autism Spectrum Disorder:

A clinical study of microflora and micronutrient abnormalities

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Abbreviations

ACh	=	Acetylcholine
Ab	=	Antibody
ADOS	=	Autism Diagnostic Observation Schedule
AMMs	=	antimicrobial molecules
AP	=	Area Postrema
AP-1	=	Activator protein 1
APC	=	Antigen presenting cell
ANS	=	Autonomic nervous system
ASBA	=	Abnormal spontaneous brainstem activation
ASD	=	Autism Spectrum Disorder
ATEC	=	Autism Treatment Evaluation Checklist
BBB	=	Blood Brain Barrier
BP	=	Blood Pressure
CADDRE	=	Centres for Autism and Developmental Disabilities Research and Epidemiology Network
CDC	=	Centre for Disease Control and Prevention
CHARGE	=	Childhood Autism Risks from Genetics and the Environment Study
CNS	=	Central nervous system
CSF	=	Cerebrospinal fluid
CVO	=	Circumventricular organ
CVT	=	Central Vagal Tone
DC	=	Dendritic Cells
DIT	=	Developmental Immunotoxicty
DIN	=	Developmental Neurotoxicity
DNA	=	deoxyribonucleic acid
EAE	=	Experimental Autoimmune Encephalitis,
ECG	=	Echocardiogram
ELII	=	Early Life Immune Insult
ENS	=	Enteric nervous system
FODMAP	=	Fermentable, Oligo-, Di-, Mono-saccharides and Polyols
GI	=	Gastrointestinal
GABA	=	Gamma-aminobutyric acid
GALT	=	Gut associated lymphoid tissue
GF	=	Germ Free
GIT	=	Gastrointestinal tract
GP	=	General Practitioner
HPHPA	=	3-(3-hydroxyphenyl)-3-hydroxypropionic acid
IACC	=	The Interagency Autism Coordinating Committee
IECs	=	Intestinal Epithelial Cells
IR	=	Immunoreactivity
LC/MS-MS	=	Liquid chromatography-Mass Spectroscopy
LVT	=	Linear Vagal Tone
mAb	=	monoclonal antibody
MCP-1	=	macrophage chemoattractant -1
MIND	=	Medical Investigation of Neurodevelopment Disorders
MIP	=	macrophage inflammatory protein
MLN	=	mesenteric lymph node
MRI	=	magnetic resonance imaging
mRNA	=	messenger ribonucleic acid
MS	=	Multiple Sclerosis
NA	=	nucleus ambiguus
NE	=	norepinephrine
NF-KB	=	nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	=	Nuclear factor of activated T-cells
NIH	=	National Institute of Health
NK	=	natural killer

NMDA	=	N-Methyl-D-Aspartic acid
NSP	=	Non Soluble Polysaccharides
NTS	=	nucleus solitary tract
OVLT	=	organum vasculosum of the lamina terminus
PANDAS	=	Paediatric Autoimmune Neurological Disorder Associated with Streptococcus
PCR	=	polymerase chain reaction
PFC	=	prefrontal cortex
PRN	=	parabrachial nucleus
PRRs	=	pattern recognition receptors
Q-CHAT	=	Quantitative Checklist for Autism in Toddlers
RA	=	Rheumatoid Arthritis,
RANTES	=	‘regulated upon activation normal T-cell expressed and secreted‘
RCT	=	Randomised Control Trial
RS	=	Resistant Starch
SCFA	=	short-chain fatty acid
SEED	=	Study to Explore Early Development
SIBO	=	Small Intestinal Bacterial Overgrowth
SFO	=	subfornical organ
SLE	=	Systemic Lupus Erythematosus
SMR	=	Standardised Mortality Rate
T1D	=	Type 1 Diabetes
Th	=	T helper cell
TGF-B1	=	transforming growth factor beta 1
TLRs	=	toll-like receptors
Treg	=	T regulatory cell
USD	=	United States Dollar

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Abstract

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Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterised by impaired socialisation. The current project examines the hypothesis that ASD represents a broad range of distinct disease processes typified by environmental insult(s) during a period crucial for the development of any of the systems responsible for social integration skills, sharing simply the fundamental disruption to social functioning with various, definable systemic pathologies related to the initial insult conferring the heterogeneity of the condition. ASD will therefore have both modifiable environmental factors relating to the aetio-pathogenesis of the condition and likely, remediable disease processes.

Following an examination of the relevant literature this project presents the Variable Insult Model of Autism. As part of a wider research strategy, this project goes on to explore potential modifiable environmental factors in patients with ASD.

Zinc deficiency was explored as a potential environmental modifiable factor involved in the pathophysiology of autism and co-morbid disease. 72 patients with ASD were compared with 234 non-ASD controls. Mean serum zinc levels in the ASD group vs. the control group were 10.01 umol/l (SD 1.52 umol/l) vs. 11.61 umol/l (SD 2.14 umol/l, with a statistically significant difference - $p < 0.0001$, CI 1.2 – 2.1). The findings withstood correction for age and sex, and zinc did not correlate with diet or supplement use in the ASD group. Total lymphocyte count increased as zinc increased in the ASD group with zinc levels of 10.5 umol/l or above, suggesting zinc status is poor in patients with autism and this is affecting immune function.

Urinary metabolomics, quantitative PCR stool analysis and autonomic function were also explored in ASD, as biomarkers of systemic disease processes presenting potential modifiable factors. The urinary organic acids of 49 patients were analysed versus population norms. 90% of patients with ASD had at least one abnormality. A follow-up study of 122 patients revealed succinic acid and 2-hydroxyhippuric acid were significantly raised in the ASD group versus population means ($p = < 0.0001$ and <0.0001 respectively). Quantitative PCR analysis was conducted on 29 patients with autism versus 7 age-matched controls. Firmicutes to Bacteriodetes ratio was significantly elevated in the autism group versus the controls 69:41 (SD 8) vs. 54:46 (SD 8) ($p < 0.003$). A follow-up study of 143 patients and 12 controls showed consistent abnormalities in the composition of firmicutes and bacteriodetes ($p = 0.005$) and this withstood correction for age and sex ($p = 0.009$), suggesting an on-going abnormality in gut flora composition in the ASD-cohort. Autonomic profiles were available in 45 patients with ASD. There was marked variability in vagal tone, however in 11 patients with ASD who had both autonomic profile and qPCR stool analysis there was suggestion of a positive correlation between vagal tone and microflora composition (represented by firmicutes to bacteriodetes ratio) ($p < 0.003$).

In summary, evidence suggests there are modifiable environmental factors associated with the aetiology, pathophysiology and disease evolution in ASD, and this is worthy of further consideration and investigation. From the preliminary results presented here, zinc status is poor in ASD and may be affecting immune function; gut flora abnormalities appear common and may be affecting neurological function in ASD.

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Dedication

To my wife, Fatma: for your unwavering support and selfless sacrifice that made this work possible.

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CHAPTER 1
Introduction

1.1 Introduction to Autism

Autism Spectrum Disorder (ASD) is a developmental condition affecting a persons cognitive, emotional and social functioning. Signs of the illness are present before the age of three, and tend to be noticeable by 18 months of age. The initial presentation of the illness can be insidious, sudden, regressive or early. Over three times more males develop the condition than females. Currently there are no recommended treatments for the condition [1,2].

ASD is a heterogeneous condition affecting an individual's ability to communicate and socialise and often presents with repetitive movements or behaviours. It tends to be severe with less than 10% achieving independent living with a marked variation in the progression of the condition [3–5]. To date the literature supports a multifactorial model [2] with the largest, most detailed twin study demonstrating strong environmental contribution to the development of the condition [6].

The Autism Diagnostic Observation Schedule (ADOS) is an interview-based assessment exploring the DSM-V diagnostic criteria. It is generally accepted as the diagnostic test of choice. Whilst there is a great deal of research exploring biomedical markers, there are none widely used at present.

The aetiology, pathophysiology and disease evolution remains unknown. Several key constants though, are emerging from the growing body of research. Firstly, neuroinflammation and other immune abnormalities have been readily and repeatedly demonstrated[7–13]. Secondly, various environmental risk factors have been implicated [14–27] (recent reviews include, prenatal factors[28], maternal factors[29] and environmental pollutants[30]). Finally, abnormal microflora composition and/or species are often present[31–34].

1.1.1 Epidemiology

Autism Spectrum Disorder was first identified by Kanner in 1938[35]. A heightened fear response, introversion and difficulty with complex tasks, such as socialisation, predominated the first 11 cases. Other common features included repetitive/obsessional behaviours, chronic tonsillar problems and feeding difficulties. Over the subsequent 10 years Kanner discovered 50 further cases[36]. Kanner subsequently reviewed the first 11 patients at 30-year follow-up. Only one known patient achieved employment[37].

Recent evidence also suggests a high level of disability in affected individuals, with 60-75% achieving poor or very poor outcomes in adulthood[38]. However, case detection rates

are now substantially higher. Lotter et al., described a prevalence rate of 1 in 3000 in 1966 (this includes both autism and childhood psychosis)[39]. The Centre for Disease Control (CDC) reported a prevalence of 1 in 110 in 8 year olds in 2006[40], a rate of 1 in 88 in 2008[41] and a rate of 1 in 68 in 2010[42].

Previous twin studies suggested a predominant genetic component; however these studies were poorly designed and had weak power[43,44]. Indeed both studies reported a 0% dizygotic twin rate, highlighting the inadequate power of these studies. A recent twin study published in July 2011 (sponsored by the CDC and in collaboration with Stanford University, the University of California and Department of Health for the State of California) was well designed with a substantial statistical power. 134 twin groups were studied and clinically evaluated prior to statistical analysis. The study concluded: environmental factors have a strong causative role in autism, and indeed the environmental factors were of more significance than genetic factors[6].

1.1.2 Morbidity and Mortality

Shavelle et al investigated the mortality rate of ASD[45]. Over 13,000 patients with ASD were assessed between 1983 and 1997. Mortality rate amongst the ASD population was more than twice that of neurotypical peers. Standardised Mortality Ratio (SMR) was estimated as 2.4. Certain causes carried significantly higher SMR (see Table 1).

Table 1 **Causes of Death in ASD with moderate to severe retardation and {no or mild mental retardation}.** Adapted from Shavelle et al[45] SMR (Standardised Mortality Ratio). n/s - no significant increase in SMR found.

Cause of Death	Early Childhood SMR 5-10yrs	Late Childhood SMR 10-20yrs	Adulthood SMR >20yrs
Drowning	90.6 {14.1}	n/s	n/s
Digestive	n/s	40.8	5.9
Respiratory	n/s	24.5	9.4
Cancer	n/s	12.0 {3.8}	2.4 {1.6}
Nervous and Sense	n/s	6.4 {15.9}	4.1
Seizures	n/s	n/s	30.8 {33.1}
Circulatory	n/s	n/s	3.7 {2.2}

Similar mortality rates have been reported in other studies[46–48], and a follow up study by Shavelle et al., was confirmatory[49]. All studies reviewed here consistently report an increased mortality rate for ASD, and a substantially greater risk in female ASD patients.

1.1.3 Disease Progression

There have been several studies evaluating diagnostic stability over time. Turner et al reassessed two year olds diagnosed with ASD at four and half years of age[50]. Results showed diagnostic stability i.e. there was no change to the label of ASD. The same study also showed that 20% of children worsened between two years of age and four and a half years of age and 20% improved. Within the parameters addressed 60% remained relatively stable. No reason was identified for the variation.

Levy et al have recently reviewed the literature regarding long-term outcome in ASD. Based on the journals reviewed, Levy et al extrapolated a cognitive improvement in 20-55%, cognitive stability in 20-70% and cognitive loss in 10-15%[38]. Again no reason was identified for why some ASD patients suffer a progressive illness and others make some recovery.

There has been some progress in the last two decades. A socially 'good' outcome has improved from 10% to 20%[51]. Although much work still needs to be done: less than 4% were found to achieve independent living[52]; Eaves et al reported 10% achieved long-term, romantic relationships, although at the time of evaluation none were in a relationship[53]. Howlin et al reported 2 current marriages within a cohort of 68[51].

ASD as a group carry a poorer prognosis than other developmental disorders in almost all domains[38].

1.1.4 Public Health Issues

Autism Spectrum Disorder was recently identified as the most costly condition in the UK, estimated to cost the UK economy 32 Billion pounds per year [54]. Loss of earnings, high social, education and healthcare needs and high dependence on family members contribute to these costs. The wider impact on family life and individual suffering is difficult to define. Likely ASD as a whole carries a high impact generally.

Given such stark financial costs, the debate regarding rising incidence is tempered. Reducing the rates of ASD by even 10% would be one of the greatest economic contributions to the UK, reducing pressures on social, education and health services and allowing families to achieve their potential contribution. To achieve this, greater focus on modifiable environmental factors is required. Evidence exists that prenatal and maternal

care[29], environmental toxicants such as pesticides, air pollution and VOC's[30] and prenatal supplementation[25] all constitute modifiable environmental risk factors. As yet though, the evidence is not powerful enough to affect meaningful change.

Globally the cost of ASD is somewhat mitigated by the lack of interventions in low and middle-income countries[55] . Human suffering though is expected to be higher. Loss of income and contribution to the local economy is also substantial[56]. Due to the high costs of implementing supportive services, there is no real possibility of expanding service provision in most low and middle-income countries in the short or medium term[55]. Other solutions are required. Medical interventions managing co-morbid health, reducing pain and improving resilience may prove the most cost-effective intervention in low, middle and high-income countries.

Population studies exploring the environmental conditions prenatally (including paternal), maternally and during early childhood are urgently required. Without the guidance of such high power studies, research in autism will remain fragmented and divergent. Birth cohorts are underway, mainly in the high-income countries. Whilst few birth cohorts are targeted specifically at ASD, there remains usable data for identifying environmental factors in child development and a number of birth cohorts are exploring this[57–60]. Two cohort studies specifically relating to ASD are the CDC's Study for Exploration of Early Development (SEED Study) and the MIND Institute's Childhood Autism Risks from Genetics and the Environment Study (CHARGE Study). The SEED study is exploring child development in relation to natural progression of the disorder and service requirements, whilst the CHARGE study is exploring environmental factors retrospectively, and pathophysiology prospectively in patients with confirmed ASD. Both studies begin with patients already diagnosed with ASD. Prenatal, maternal and birth studies examining causal links remain few and far-between.

1.1.5 Environmental aspects of ASD

Evidence for modifiable environmental risk factors is significant. Gardner et al undertook a recent meta-analysis exploring prenatal and maternal risk factors in the development of autism. Whilst significance was found in several domains, the need for further more extensive studies was apparent. Possible risk factors identified included advanced maternal and paternal age at birth, maternal gestational bleeding, gestational diabetes, being first born vs. third or later, maternal prenatal medication use, and maternal birth abroad[28].

Xenobiotic exposure has recently been reviewed in detail[30] and a strong correlation was found for autism risk and air pollution, pesticides and volatile organic chemicals (VOCs). Air pollution is worthy of considerable note, given the increasing evidence for the affects of air pollution on Cognitive Impairment and Neurobehaviour [23,61–68]; Immunotoxicity [69];

Neurotoxicity [63,70]; Autonomic effects[71–73] and autism specifically [20,22,24,74–77].

There are several proposed mechanisms for how pollutants lead to neurodevelopmental disorders including and specific to ASD. Developmental Neurotoxicity (DIN) and Developmental Immunotoxicity appear to be the most promising. Hertz-Picciotto et al provides a detailed review of Developmental Immunotoxicity (DIT) in relation to neurodevelopment disorders, presenting a time-line of autism risk factors versus crucial developmental windows[78] (figure 1).

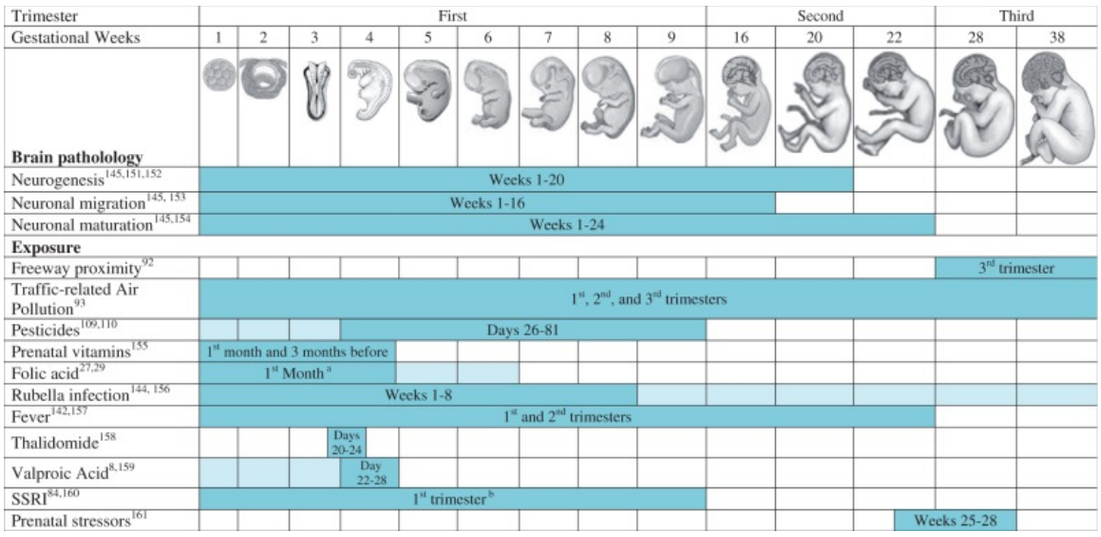

Figure 1 Summary of certain environmental factors associated with autism, the timing of the insult in relation to in-utero neurodevelopment[78]. Age of gestation is presented along the top with environmental factor presented down the left column. Note: Prenatal Vitamins and Folic Acid is noted here as a modifiable environmental factor with a protective effect over the development of autism.

Xenobiotic exposure in early life may lead to altered immune function throughout life. The type of immune dysfunction relates to the type of xenobiotic involved and the timing of xenobiotic exposure. Of note, dose-dependent effects are not as applicable in the developing immune system[78]. Dietert et al also describes several similarities between Early Life Immune Insults (ELII), including Developmental Immunotoxicity (DIT), and ASD. Dietert et al describes possible mechanisms and the similarities. Gender differences, time-windows for immune development and the corresponding variable presentation in both ELII and ASD make for a compelling argument[79].

Developmental Neurotoxicity (DIN) has been reviewed recently[80], implicating numerous xenobiotics in the pathophysiology of neurodevelopmental disorders including autism. Pesticides, VOCs, Metals and flame-retardants were implicated as causative for DIN, and suggested as possible aetiological factors involved in ASD.

Studying DIT and DIN is fraught with difficulties. In both cases the insult can be transient,

the organs affected not accessible to simple investigation and there remains the challenge of multiple-exposure effects. Population based prospective studies with frequent environmental and biological sampling are needed to investigate the association between a developmental immunotoxic or neurotoxic agent and neurodevelopment. Complicating matters further is the modest, but significant genomic variation in xenobiotic metabolism and hence resistance or vulnerability to environmental exposures[81]. One expects appreciation of metabolomics, transcriptomics and genomics together with quantitative measures of environmental pollutants in multi-modal analysis will be required to delineate environmental risk factors.

A series of papers from the CHARGE study attempted to account for these research challenges[14,16,82]. Blood mercury levels showed no correlation between the ASD group and controls[14]. There was however correlations between Lead and Mercury levels and gene expression in the ASD group versus controls. A higher level of lead conferred increased transcription factors for inflammation and other immune related mediators in the ASD group but not with the higher lead levels in the control group[82]. Higher mercury levels showed similar correlations with increases in cell morphology, amino acid metabolism and antigen presentation gene transcription with increasing mercury levels but no such changes in neurotypical controls as mercury levels increased[16]. These findings are conducive with a DIT type pathophysiology. In-utero or early post-natal exposure(s) causes an altered immunophenotype predisposing to abnormal responses to further immunological challenges.

From the same CHARGE study, a lack of positive correlation between total levels of Polybrominated diphenyl ethers in ASD cases versus controls has been reported[83]. Of note both controls and ASD cohort showed markedly elevated levels in comparison to levels 7 years previously[84]. A positive correlation between autism and proximity to freeway (<310m) was found[19]. Children born within 500m of agricultural land sprayed with pesticides carried a higher risk of developing autism, with a relative risk of 6.1 (95% confidence interval, 2.4–15.3)[21]. A longitudinal study measured dichlorodiphenyltrichloroethane (an organochlorine pesticide) levels and found a correlation with development indices for those exposed prenatally[85].

A review of pesticides and child development highlighted the likelihood of a negative association and recommended healthcare professionals to advise women attempting to conceive and those already pregnant to avoid exposure to pesticides[86].

There has been extensive literature published regarding immunotoxicity of pesticides including, organophosphates and immunotoxicity[87], pesticides and immunotoxicity with discussion of compensatory mechanism[88] and human immunotoxicity of pesticides[89].

Impaired NK cell activity, altered cytokine response and impaired phagocytosis are frequently found in both human and non-human subjects in both chronic and acute pesticide exposure. More studies are required relating to pesticides causative role in ASD. For example, it remains unknown whether exposure to pesticides through food, prenatally, maternally or during early childhood increases the risk of autism, and whether continued exposure to pesticides after the development of autism worsens the outcome. The same is true of air pollution and indoor pollutants such as VOCs and flame-retardants.

1.1.6 Immune Abnormalities in ASD

Neuroinflammation

Perhaps one of the most substantive studies in the last decade was conducted at the John Hopkins Institute, and involved an analysis of autopsy specimens and CSF samples from affected individuals and controls[8]. The results indicated a neuroinflammatory response, regardless of age (in patients between 5 - 46 yrs., of age), involving excess microglial activation and increased pro-inflammatory cytokine profiles in the ASD group. The study carries high statistical significance[90] and indicates an inflammatory state probably exists in the brains of these patients. Similar findings were found in a more recent autopsy study of microglia densities in fronto-insular and visual cortices of patients with ASD versus controls, and found a statistically significant ($p = 0.0002$) increase in microglial density in both regions[7]. Other immune abnormalities have also been found indicating an inflammatory state. Transforming growth factor beta 1 (TGF-B1) is reduced in ASD cohorts versus controls and individuals with other developmental disorders and was found to be inversely proportional to behaviour outcomes (irritability, lethargy, stereotypy and hyperactivity) as well as with levels of social adaptability[91].

Natural Killer cells (NK cells) are abnormal in sub-groups of ASD. NK cells respond to macrophage-derived cytokines and are essential in tumour prevention and host anti-viral activity. Enstrom et al found a significant reduction in NK cell cytotoxicity and a 2.5 fold increase in KSP-37, an NK gene normally induced during active viral infection. They concluded that ASD patients have activated but resting NK cells with increased levels of cytolytic proteins and an altered response to stimulation with changes in gene expression[92]. Supporting these findings, cancer mortality rates are higher in ASD[45], and the only identified risk factor for mortality associated with the recent H1N1 outbreak was developmental delay[93].

There have been studies making correlations between measures of immune functions and cytokine profiles with behavioural measures in ASD (Table 2). Significant correlations were shown between certain behavioural indices and the chemokine's, macrophage chemoattractant MCP-1, macrophage inflammatory protein (MIP)-1β, eotaxin and

‘regulated upon activation normal T-cell expressed and secreted’ factor (RANTES). RANTES was associated with lethargy, stereotypy and hyperactivity. Eotaxin was associated with hyperactivity, visual perception, fine motor control, expressive language, communication and daily living skills, and socialisation. MCP-1 was associated with visual perception. These associations, if proven to be functional, raise many questions pertaining to the immune systems connectivity to the nervous system and involvement in neurobehavioural illnesses. Of importance here is the probability of immune involvement in the core features of ASD. These findings also raise the possibility of assessing behavioural changes in ASD through a quantitative measure, and addressing ASD through targeting these immune abnormalities.

Table 2 A summary of previous studies identifying a relationship between immune function and specific behaviour symptoms in ASD patients.

Studies (chronological)	No.	Age	Assessment method	Immune measure	Behaviour Measure
(Ashwood et al., 2008)[91]	143	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, ABC	Plasma levels of active TGFβ1	Lower TGFβ1 levels were associated with lower adaptive behaviours and worse behavioural symptoms
(Iwata et al., 2008a)[94]	37	20–25	ADI-R	Plasma levels of P-selectin	Lower levels of P-selectin associated with poor social development
(Heuer et al., 2008)[95]	271	2–5	ADI-R, ADOS, ABC	IgG levels in plasma	Decreased IgG associated with increased aberrant behaviours
(Grigorenko et al., 2008)[96]	1059	n.s.	ADI-R and ADOS	Genotyping of the MIF gene, and plasma levels of MIF(n=20)	Plasma MIF levels were positively correlated with worse scores on ADOS for social impairment and imaginative skills
(Onore et al., 2009)[97]	60	2–5	ADOS, ADI-R, MSEL, VABS and ABC	Induced cytokine response to PHA	Negative correlation between PHA induced IL-23 production and sociability scores of the ADOS
(Enstrom et al., 2010)[98]	30	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, ABC	Monocyte TLR ligand stimulation	More impaired social behaviours and non-verbal communication are associated with increased production of IL-1β and IL-6 after TLR4 stimulation
(Ashwood et al., 2010)[99]	139	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, ABC	Induced cytokine response to PHA and LPS	Pro-inflammatory or TH1 cytokines were associated with greater impairments in core features of ASD as well as aberrant behaviours; GM-CSF and TH2 cytokines were associated with better cognitive and adaptive function
(Goines et al., 2010)[100]	466	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, ABC	Antibodies directed against a 45 or 62 kDa cerebellum protein	Children with antibodies directed against a 45 kDa cerebellum protein had increased, lethargy and stereotypy; children with antibodies against a 62 kDa cerebellum protein showed increased aberrant behaviours on the VABS composite standard score
(Kajizuka et al., 2010a)[101]	62	6–19	ADI-R	Serum levels of PDGF	Increased serum levels of PDGF-BB homodimers positively associated with increased restricted, repetitive and stereotyped patterns of behaviour and interests
(Ashwood et al., 2011c)[102]	175	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, ABC	Plasma Chemokines CCL2, CCL5 and eotaxin	Plasma chemokine levels associated with higher aberrant behaviour scores and more impaired developmental and adaptive function
(Ashwood et al., 2011b)[103]	223	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, ABC	Plasma levels of cytokines IL-1β, IL-6, IL-8 and IL-12p40	Elevated cytokine levels in plasma were associated with more impaired communication and aberrant behaviours
(Ross et al 2013)[104]	16	3-31	ADI-R,	GMcsf, INFγ, IL-12p70, IL-1β, IL-6, IL-8, TNFα and IL-10	Elevation of cytokines correlated with autistic symptoms in patients with 22q11.2 deletion syndrome

1.1.7 Neurological abnormalities in ASD

Neuroanatomical studies have been unable to generalize. A population based longitudinal neuroimaging study has though not been undertaken. Most neuroimaging studies have involved older children, and most are higher functioning and have been compared to typical controls. Insufficient confidence has been achieved that abnormalities identified on neuroimaging studies represent more than secondary effects of developmental delays with no specific regions identified as autism specific. It is noted that the Midbrain had the greatest variability in size, albeit the majority were within normal parameters (See table 3 for summary).

Table 3 Summary of a meta-analysis[105] of structural neuroimaging finding in ASD

	Area	Effect Size	Standard Deviation	Variation
Reduced size	Midbrain	-0.77	-1.52 to -0.02	90.2%
	Vermal Lobules VIII - X	0.43	-0.8 to 0.06	49.5%
	Corpus Callosum	-0.28	-0.52 to 0.03	0%
	Vermal loubules VI - VII	-0.27	-0.51 TO 0.03	52%
Increased size	Cerebellar	0.72	0.4 to 1.03	0.0%
	Cerebral hemisphere	0.62	0.39 to 0.86	0.0%
	Caudate	0.41	0.12 to 0.71	0%
	TBV	0.32	0.16 to 0.49	22%

Comparisons of structural abnormalities versus functional deficits have been made with some associations identified for these specific sub-groups (table 4), although results have not been consistent.

Table 4 **A summary of structural abnormalities versus functional deficits meta-analysis[106] in ASD**

Domain	Area	Finding	Study
Social Impairment	Amygdala	⇓ volume	Nacewicz et al[107]
Language Impairment	Nil association found		Nacewicz et al[107]
Repetitive and Ritualistic Behaviours	Pre-Frontal Cortex	⇑ volume	Langen et al[108]
	Anterior Cingulate	⇑ volume	Hollander et al[109]
	Inferior Parietal Cortex	⇑ volume	
	Caudate	⇑ volume	
Sensory	Brainstem	⇑ volume	Jou et al[110]
Anxiety	Amygdala	⇓ size	Nacewicz et al[107]

Functional neuroimaging has similar restrictions. A recent meta-analysis demonstrated cortical hypoconnectivity as a general feature of higher functioning autism in comparison to mainly typical controls[111]. The majority of controls were neurotypical, the cohorts were exclusively older children and there remained marked variation in the areas affected. Those within the identified pattern tended to show an increased activity of the sub-cortical structures with a decrease connectivity of the higher cortical structures, consistent with an interrupted or delayed development (see table 5).

Table 5 **A Summary of connectivity on functional neuroimaging**[111,112]

Connectivity	Structural Area
Hypoconnectivity	Globally
	Cortical structures
	Fusiform, Superior Temporal Gyrus, Paralimbic
	Between hemispheres
Hyperconnectivity	Sub-cortical
	Thalamus
	Parietal somato-sensory cortex
	Globus pallidas

With the exception of neuroinflammatory changes, most reported neurobiological abnormalities in ASD are inconsistent. This is in-keeping with the heterogeneity of the group and may suggest a pathophysiological process beneath the neurological dysfunction with variable expression in the neurological system.

1.1.8.1 Autonomic Dysfunction

The Autonomic Nervous System is the most abundant peripheral nervous tissue, communicating information centrally to cerebellum, hypothalamus, amygdala, insular cortex and other cortical areas. The ANS also communicates instructions from these areas peripherally to exert motor and stimulatory functions on cardiac, respiratory, gastrointestinal, immunological and endocrine systems. It is often divided into sympathetic and parasympathetic divisions. The dominance of each division has clear and measurable affects, albeit there is a complex pattern of interaction within such dominance[113,114].

Autonomic involvement in ASD has been widely researched for over 30 yrs. More recently a controlled trial was undertaken exploring in detail the nature and type of autonomic involvement[115]. Real-time R-R variability together with continuous monitoring of blood pressure and breathing rhythms were assessed in an ASD cohort versus controls. Over 80% of the ASD cohort (n = 15) were found to have a reduced vagal tone, highly suggestive of low central parasympathetic tone. Field et al reviewed the developmental significance of vagal tone in detail[114]. Vagal tone in the neonate was found to predict neurodevelopmental outcome more accurately than birth weight, socio-economic status or co-morbid medical conditions.

Given the ANS is involved in the majority of sensory information received by the central nervous system, any disruption to such a system is likely to have wide-ranging affects on higher cortical development. In a longitudinal follow-up Gogtay et al examined the order of cortical development using repeat MRI and concluded '*higher-order association cortices*

mature only after lower-order somatosensory and visual cortices, the functions of which they integrate, are developed'[116]. The development of a normal parasympathetic tone is crucial for adequate neurodevelopment.

Additionally, vagal tone has been found to exert anti-inflammatory effects (the cholinergic anti-inflammatory pathway)[117,118]. One must also consider the commonest neuroanatomic abnormalities identified in autism follow the autonomic pathway (brainstem, cerebellum, hypothalamus, amygdala and prefrontal cortex).

1.1.8 Neuroimmune considerations

Microglia are hematopoietic in origin. Astrocytes are neural in origin. Microglial cells are immune cells and have the ability to form synaptic projections[119–121]. Astrocytes are neuronal cells with significant immunological function [review[122]].

Microglia serve a protective role in the CNS. Well known to serve as CNS phagocytes, microglia also have roles in synaptogenesis[121] and reducing glutamate excitotoxicity[119]. Microglia can detect nerve death and then signal the surrounding neurons to delay attempts at compensatory synaptogenesis[121], presumably in an attempt to prevent abnormal cortical development within a sub-optimal environment. Tremblay et al showed that microglia may contribute to synaptogenesis under the stimulation of light, yet under adverse light conditions (simulating a stressful stimuli) microglia were shown to up-regulate phagocytic activity and may cause an inhibitory affect on synaptogenesis[123]. Further, it has been suggested normal microglial function is required for adequate synaptic pruning during normal development[120].

There are several studies suggesting the immune-derived Microglia cells communicate neurologically with nerve cells, serving as an essential channel between immune system and neurological system[119–121,123]. The complexities of such communication have not been delineated.

Astrocytes help to regulate the blood brain barrier (BBB). They are major producers of RANTES, MCP-1, Il-8 and IP-10. As well as regulating the BBB, astrocytes appear to impart steady-state anti-inflammatory forces presumably to insure the specific media of the CNS is maintained by preventing the increased vascular permeability typical of pro-inflammatory forces[122]. Barcia et al has shown astrocytes can also form synaptic projections with T-cells[124].

Summary of Introduction to Autism

Various risk factors have been identified, various pathologies demonstrated and autism itself is highly variable. The shared outcome is impaired socialization beginning in early childhood. Autonomic abnormalities are often reported, although the findings are varied. Neuroanatomical abnormalities are reported but findings rarely apply to more than 40% of the cohorts examined, and the lack of controls and prospective studies appears a barrier to further discovery that may be difficult to surmount. Immune findings are also variable, but appear to correlate with autism symptoms, are more easily examined and could be followed up prospectively. Environmental factors appear the most promising area for examination in relation to identifying modifiable risk factors for the development of autism, and can be measured non-invasively.

1.2 Introduction to Microflora

The large intestine is colonised by majority presence of 150 individual species of bacteria. Accumulatively the number of individually coding genes present in the collective microbiota has been estimated to be substantially greater than that of the human host. Over 90% of the species are thought to be from the bacterial phyla of Firmicutes and Bacteriodetes[125].

With over 100 trillion cells and relative consistency of key species over time, the human gut is one of the most successful habitats in our biosphere[126]. Needless to say such success has not come easy. The complexities underpinning the host-microbe, microbe-microbe interactions are only beginning to be understood. The selection process too is sensitive and complex. It probably takes place at delivery, in the neonatal period and throughout infancy[126], with possibly some form of selection/deselection process continuing throughout life[127]. Even beyond this evidence is emerging for variation in adulthood depending on environmental factors[126,128–130].

Gut microflora have a clear role in human health and in maintaining homeostasis. Microflora breakdown digestive resistant starches producing short-chain fatty acids that are utilised by the host. Acetate is produced by microflora fermenting available food; it is absorbed into the host circulation and utilised as fuel for heart, skeletal muscle and brain. Butyrate is also produced and is the preferred energy supply for the coloncytes in the bowel[131]. Microflora also produce essential amino acids, vitamins and other co-factors[132–134].

1.2.1 Microflora and immune, neurological and neuro-immunological function

The gastrointestinal tract comprises the greatest network of nerves outside the human brain[135], and the greatest immunological representation in the entire body[136]. The neurological and immunological systems of the gastrointestinal tract develop in-utero and throughout early childhood[137]. The gut remains a vulnerable developmental area.

Establishing a healthy microflora depends on both neurological and immunological systems. Motor and secretory functions of the enteric nervous system maintain the appropriate transition of food through the GI tract and protect the mucosa, maintaining digestion, absorption and GI integrity[138]. These functions are critical in preventing excess bacterial growth and invasion. Beyond this, it has been suggested that the sensory component of the GI tract, probably through vagal afferents, is involved in the selection processes of microflora[135]. These sensory nerves are typically unmylineated and again vulnerable to environmental toxicants. The exact mechanisms remain ill-defined.

Selection

Selection must begin with the exposure to the microbes that will eventually form the microflora, most likely through the vaginal cavity during birth; faecal-oral route, soil, dust and food through infancy and inanimate objects in the environment throughout life. Colonisation and composition of microbes present on these sources differ, and will differ further depending on environment. For example, the dust analysed from farms was compared to urban domiciles and found to contain different compositions of firmicutes and bacteriodetes, leading to the postulation that one of the allergy-protective factors associated with farm-living is the exposure to a different microbial composition in dust[139]. It remains unknown what affect detergents, antibiotics, pesticides and flame-retardants have on the composition of microbes present in dust, in the vaginal cavity, on inanimate objects and in food in early human life.

Exposure to these organisms then gives way to survival of the organisms during transition through the GI tract. Again the presence of detergents, pesticides, preservatives and antibiotics likely affects the survival profiles of such microbes. Digestive processes such as gastric acid production, digestive enzymes and timely transit through the stomach and small intestine all confer selective pressures on microbes. Numerous factors also exist at the gut lumen wall that can influence the success or not of various pathogens, through local and systemic immune mechanisms, which has been the subject of much of the scientific investigation available on the subject of host selection and maintenance of gut flora.

Immune

Separating the external environment of the gut lumen from the internal environment is the Intestinal Epithelial Cells (IECs). They express pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and C-type lectin receptors. These receptors together with other mechanisms attempt to regulate the number of microflora and the type. Triggering these receptors generates several local and system-wide responses, including the secretion of antimicrobial molecules (AMMs), stimulation of T-cells and innervation of vagal nerve afferents. They also provide a crucial role in the maintenance of the epithelial tight junctions, increasing the tight junctions when under greater microbial stimulation, presumably to prevent microbe invasion[140,141].

The type of microbe permitted to colonise the GI tract is also, at least partially regulated by the IECs PRRs. For example certain C-type lectins can inhibit the growth of gram-positive bacteria[136]. Other products of the IECs, such as the soluble lectin, galectin, support local and systemic immune responses. IECs also secrete IgA into the lumen on demand. IgA is a local antibody neutralising infiltrating pathogens and other foreign material. Interestingly the IECs will simultaneously produce a proliferation producing ligand (APRIL) that will stimulate B-cells to switch to IgA production[142].

Dendritic Cells (DC) are antigen-presenting cells present in the GI mucosa. In the GI tract they are dynamic, extending their dendrites further into the lumen when harmful bacteria are detected. The total number and representation of DCs are dependent on constant stimulation from microflora, as demonstrated by the significantly lower numbers of DCs present in germ-free (GF) mice[143]. By receiving stimulation from microflora the DCs are in a better position to fend off pathogenic microbes; another example of symbiotic host-microbe interaction.

Th17 and T-reg cells have been extensively studied and appear key players in the GI adaptive immune response. Th17 cells are differentiated from naive CD4 T-cells in the presence of certain cytokines. Th17 cells control infections and are required to prevent pathogenic bacteria invading. They are then more inflammatory and produce a 'controlled inflammation' in the GI tract. In GF mice Th17 levels are much lower, indicating another aspect of GI immunity that requires some stimulation from the commensal flora to maintain adequate defenses[144].

T-regulation cells emanate from the Thymus under certain conditions. They are pivotal in regulation and tolerance, thus preventing autoimmune disease and excess inflammatory response. Certain microbial flora can induce T-reg proliferation and hence exert an indirect anti-inflammatory effect[144]. Lee et al, provides a useful diagrammatic representation of the delicate balance of GI immune function in relation to dysbiotic gut flora, suggesting too low a level of specific gut flora and too high a level of certain gut flora can trigger the same over-representation of Th17 cells (*figure 3*).

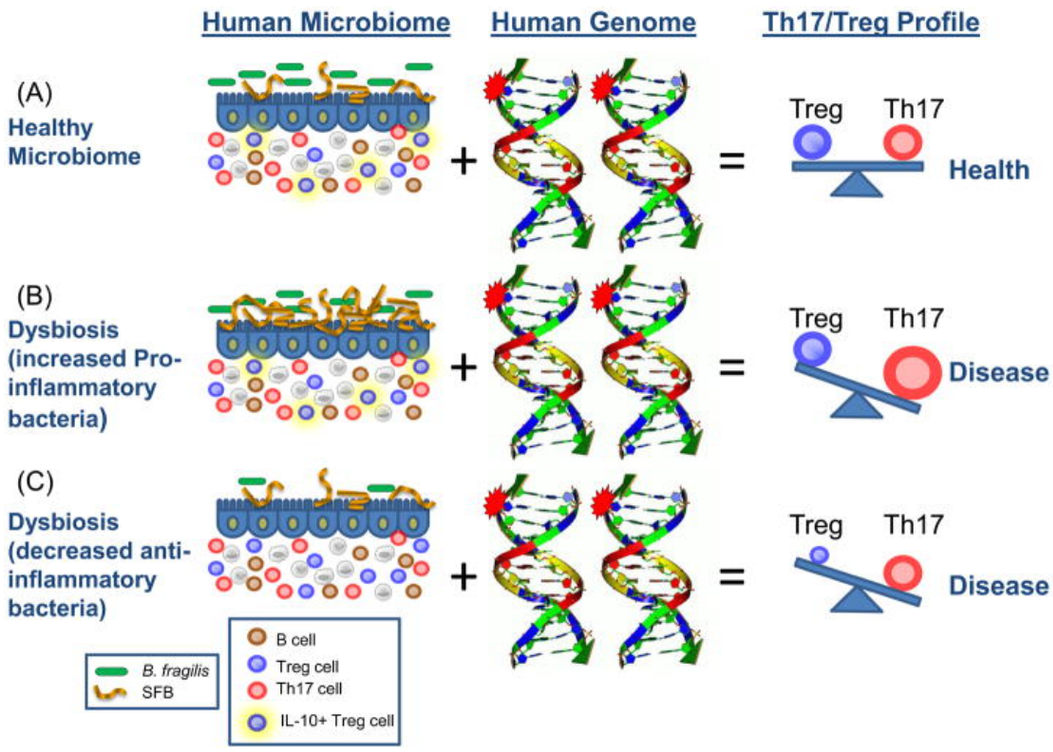

Figure 2 Proposed affects on T-regs and Th17 cells following alteration in microflora composition. A normal balance of microflora maintains enough stimulation of the immune system to produce Treg cells and Th17 cells providing a controlled response to potential invading pathogens (A). An excessive growth of certain microflora leads to a disproportionate production of Th17 leading to excessive pro-inflammatory forces (B). A deficiency in certain microflora leads to too little stimulation of the immune system leading to lower levels of T-reg cells, thus leading to a disproportionate representation of the pro-inflammatory Th17 cells (C)[144].

1.2.2 Microflora and Systemic Disease

An outstanding piece of research[145] examined the consistent neutropenia GF mice suffer[146]. Neutrophils are a vital immune cell. They are produced in the bone marrow and circulate the blood and tissues providing a rapid cellular response to microbial invaders. Neutrophils are normally present in the blood at a level of 2.0 - 6.5 x10^9/L. If they fall to less than 0.5 x10^9/L then the patient is highly susceptible to infection, and the severity of infection is considerably worse. If the count falls below 0.25 x10^9/L, then even a low virulent bacteria, despite the most effective antibiotics, can cause a fatal infection. Clarke et al demonstrated that a peptoglycan produced by gram-negative microflora, via a specific PRR, stimulate neutrophil function, and indeed can restore neutrophil function and protection against Streptococcus and Staphylococcus infections in GF mice[145]. This finding supports a growing body of research demonstrating the importance of microflora in priming, maintaining and regulating systemic immune function. Whether Round et al's suggestion that the microflora has greater regulation over host immune function than the host does[141], remains to be seen.

Researchers continue to examine the microflora affects on immune and non-immune diseases. There is already a considerable body of literature regarding the association between microflora and systemic allergy, including asthma and dermatitis[147]. In mouse

models of multiple sclerosis (MS), GF mice showed lower disease severity[148]. Abnormalities in microflora have been linked with the development and the progression of rheumatoid arthritis (RA)[149]. GF mice with known susceptibilities to RA achieved complete protection from disease (see table 5). Type 1 Diabetes (T1D) and microflora have been examined and found to have some associations[150]. Interestingly, there does not appears to be an optimum universal composition of microflora that protects against disease, instead a complex interaction between host gene vulnerability, environment and microbe seems to affect disease outcome.

Table 6 ***Effects of Germ Free (GF) status on spontaneous autoimmune disease models***[151]

Disease	Animal model	Genetic background	Outcome in GF animals
EAE	RR	SJL/J mouse	Complete protection
T1D	BB/W	BB/W rat	No change
T1D	NOD × *Myd88*$^{-/-}$	NOD mouse	Increased diabetes
T1D	NOD	NOD mouse	No change
RA	HLA-B27	HLA-B27 rat	Complete protection
RA	*Il1rn*$^{-/-}$	BALB/c mouse	Complete protection
RA	K/BxN	KRN TCR C57BL/6 × NOD mouse	Suppression
SLE	MRL-*lpr*	MRL-*lpr* mouse	No change
Autoimmune Gastritis	*Aid*$^{-/-}$	BALB/c mouse	No change
Ankylosing spondylitis	B10.BR	C57BL/10 mouse	Complete protection
Multi-organ inflammation	NOD × *Aire*$^{-/-}$	NOD mouse	No change

EAE - Experimental Autoimmune Encephalitis (transgenic model of multiple sclerosis (MS)),
T1D - Type 1 Diabetes,
RA - Rheumatoid Arthritis,
SLE - Systemic Lupus Erythematosus

Multiple Sclerosis (MS) has many similarities to ASD. Both involve neuroinflammation, with microglial activation, and the increasing rates of both conditions have been linked to environmental factors[6,152]. It is also interesting to note that MS very rarely presents before 3 years of age and ASD always presents before 3 yrs. of age. Perhaps plaque formation in MS requires a mature neuro-immune response?

Pro-inflammatory microbiota can probably exacerbate central nervous system inflammatory conditions, and has been reviewed recently[151,153]. Berer et al, summarises the alteration in the chance of developing autoimmune disease in GF mice (table 5), and the effects of altering microbiota composition on autoimmune diseases known to affect the brain (table 6). Interestingly, Berer et al, attributes a significant proportion of the rising rates of autoimmune disease to dietary factors[151].

Table 7 ***Effects of specific microbial colonization in autoimmune disease models***[151]

Disease	Animal model	Induction	Bacterial strain/products	Effect on disease
EAE	C57BL/6 mouse	MOG_{35-55}/CFA	*Lactobacillus paracasei* and *Lactobacillus plantarum*	Suppression
EAE	C57BL/6 mouse	MOG_{35-55}/CFA	PSA from *Bacteroides fragilis*	Suppression
EAE	C57BL/6 mouse	MOG_{35-55}/CFA	*Pediococcus acidilactici*	Suppression
EAE	SJL/J mouse	$PLP_{139-151}$/CFA	*Pediococcus acidilactici*	Suppression
EAE	Lewis rat	Spinal cord homogenate or MBP/CFA	*Lactobacillus casei* Shirota	Increased
EAE	Lewis rat	MBP/CFA	*Bifidobacterium animalis*	Slight delay
T1D	NOD mouse	Spontaneous	VSL#3 mix (Bifidobacteria, Lactobacilli and *Streptococcus salivarius subsp. thermophiles*)	Suppression
T1D	NOD mouse	Spontaneous	*Lactobacillus casei*	Suppression
RA	K/BxN mouse	Spontaneous	Segmented filamentous bacteria	Increased
RA	GF *Il1rn*$^{-/-}$ mouse	Spontaneous	*Lactobacillus bifidus*	Increased
Ankylosing spondylitis	GF B10.BR	Spontaneous	*Lactobacillus brevis*	No change
Ankylosing spondylitis	GF B10.BR	Spontaneous	*Staphylococcus* sp. and *Veillonella* sp.	Increased

EAE - Experimental Autoimmune Encephalitis,
T1D - Type 1 Diabetes,
RA - Rheumatoid Arthritis,
SLE - Systemic Lupus Erythematosus

1.2.3 Methods used for examining microflora.

Measuring gut flora has proved challenging. Initially culture based methods were the only option available. Culture has numerous drawbacks in relation to measuring gut flora composition. Primarily the culture conditions provide an absolute limitation for the identification of species, and a terminal limitation for the measurement of bacterial levels. Culture may be useful for identifying specific pathogens merely to determine if they are present or not. Even still, and throughout culture techniques, there is the risk of cross-contamination during collection, transportation and preparation of sample to incubation[154].

Polymerase Chain Reaction (PCR) technology has allowed greater chance that the composition of gut flora can be more accurately delineated. Specific PCR probes target previously charaterised DNA segments in the microbe being studied. Given that there are shared DNA sequences across phyla and there are specific DNA sequences down to a species level, there is the possibility of building a picture of the actual composition of gut flora. So long as the sample is collected relatively cleanly and DNA replication suspended in a suitable medium, contamination is less of a concern, particularly when concerned with compositions of groups or phyla. Contamination remains an issue when attempting to isolate down to a species level, particularly when such species are not dominant and hence unlikely to be at high enough numbers to prevent contamination concerns[155]. PCR probes can be costly, and as such the sample size used for analysis can be small, thus increasing the concern with contamination or a non-representative sample. There is also the unresolved question as to the relevance of bacteria passed in stool versus bacteria associated closely to the mucosa.

PCR analysis is likely a significant improvement over culture techniques in relation to determining compositions of gut flora. It does though depend on selecting PCR probes, and whilst broad and narrow probes are available, certain microbes may not be considered, and certain phyla may be missed. Overall, PCR remains the current choice for examining gut flora and the ease of repeat examination and the non-invasive technique of PCR stool allows for easier access to controls.

1.2.4 Metabolomics

Metabolomics is the study of human metabolism through analysis of biochemical end products. As such, metabolomics is not a new methodology, merely improved techniques and computing power enables this distinct field to expand, identifying disease patterns and novel findings leading to mechanistic exploration[156]. In the clinical setting the non-invasive urinary metabolomics is preferred, and is typically undertaken by mass spectroscopy.

Urinary analysis via mass spectroscopy has been investigated in ASD patients previously. Nicolson et al examined urinary proteomics in a small sample of ASD patients versus sibling and unrelated controls. Amongst the abnormalities identified, abnormal bacterial metabolites were found[157]. The study had low numbers and was potentially confounded by the large distance between specimen collection (Australia) and analysis (UK).

Several recent metabolomics studies have been undertaken in ASD. Low study numbers, differing techniques and different end-points prevent a generalization to ASD. Elevated succinic acid has though been previously demonstrated to be elevated[158].

1.2.5 Abnormalities in Microflora in Autism

Abnormal compositions of microflora have been found in ASD. Higher proportions of certain species (e.g. clostridium) and lower proportions of other species (e.g. bifidobacterium) have also been discovered. Methodological differences vary considerably among the studies reviewed, however a general trend towards higher firmicutes to bacteriodetes ratio seems to emerge following review of the studies.

Critical appraisal of the studies listed in table 8 can be found at appendix 2. Williams et al 2011 appears the most statistically powerful study, and as such is reviewed below together with a few other prominent pieces of research in the area[34].

Williams et al., is arguably the most intimate study of microflora in ASD patients with methodological and analytical vigour. Quantitative PCR was utilized to measure microflora compositions in biopsy specimens taken at the ileum and cecum in 12 ASD patients and 8 Controls. Clinical imperative for colonoscopy meant only those with active bowel symptoms were included. Stringent collection methods and consistent analytical technique made for a good comparison. Confounding factors (such as diet, probiotic use, co-morbidities, medications and supplementation) were recorded and the study results analysed against such variables. In addition to microflora composition, Williams et al., evaluated mRNA levels coding for disaccharidase enzymes and hexose transport (to assess carbohydrate digestion and absorption) and undertook a widespread qPCR bacterial probe looking for unique bacterial differences between ASD patients and controls.

Table 8 **A summary of microflora studies in ASD patients**

Study group	Methodology	Significantly higher in ASD	Significantly lower in ASD	Study
13 ASD, 8 CON	Bacterial culture, faeces	*Clostridium* and *Ruminococcus* spp.		Finegold et al.[31]
15 ASD, 8 CON	Quantitative PCR, faeces	*Clostridium* clusters I and XI, *Clostridium bolteae*		Song et al.[32]
58 ASD, 12 SIB,10 CON	Fluorescent in situ hybridization, faeces	*Clostridium histolyticum* group (*Clostridium* clusters I and II)		Parracho et al.[159]
33 ASD, 7 SIB, 8 CON	Pyrosequencing, faeces	Severe ASD (11 subjects) versus CON: Phylum level: bacteroidetes and proteobacteria Genus level: *Alkaliflexus,, Desulfovibrio, Acetanaerobacterium, Parabacteroides, Bacteroides*	Severe ASD (11 subjects) versus CON: Phylum level: firmicutes and actinobacteria Genus level: 14 genera, most significant and abundant: *Weissella, Turicibacter, Clostridium, Anaerofilum, Pseudoramibacter, Ruminococcus, Streptococcus*	Finegold et al.[160]
23 ASD, 22 SIB, 9 CON	Quantitative PCR, faeces	*Bacteroides fragilis* in ASD subjects with GI symptoms only (9 of 23)	*A. muciniphila* (ASD and SIB); *Bifidobacterium* spp. (ASD only)	Wang et al.[161]
58 ASD, 39 CON	Bacterial culture, faeces	*Lactobacillus* spp.; *Bacillus* spp.	*Bifidobacterium* spp., *Enterococcus* spp., *Klebsiella oxytoca*	Adams et al.[162]
15 ASD with GI symptoms, 7 CON with GI symptoms	Pyrosequencing and quantitative PCR, ileal and cecal biopsies	Cumulative level of firmicutes + proteo bacteria; *Sutterella* spp.	bacteriodetes	Williams et al.[34,163]
20 ASD vs. 20 Controls	16S rDNA-targeting quantitative real-time PCR		Diversity Prevotella, Coprococcus, and unclassified Veillonellaceae	Dae-Woo Kang et al[164]
10 ASD vs. 10 PDD-NOS vs. 10 siblings	Quantitative PCR, faeces	Clostridiaceae, Sutterellaceae, Enterobacteriaceae (e.g., *Proteus*, *Shigella*)	*Bifidobacterium spp.*,	De Angelis[165]
23 ASD, 22 SIB, 9 CON	Quantitative PCR, faeces	(Ruminococcus *gnavus and Ruminococcus torques*)		Wang et al. [166]

Firmicutes to Bacteriodetes ratio was elevated in ileal samples ($p = 0.003$) and cecal samples ($p = 0.022$) in ASD-GI patients versus control-GI patients. Whilst probiotic use confounded the ileal Firmicutes to Bacteriodetes increase, the lack of affect of probiotics on the cecal adiposity index suggests the abnormal ratio is representative of a true compositional change in the ASD-GI group versus controls. 100% of ASD-GI patients had bacteriodetes levels below the 25th percentile compared to 0% of the Control-GI patients in ileal samples and 86.7% vs. 0% in the cecal samples.

Alcaligenaceae sequences were also discovered in almost half the ASD-GI patients and none of the Control-GI patients. In a subsequent study, the research group found this to reflect the presence of suterella species[163].

Williams et al 2011., also discovered 93.3% of ASD-GI patients demonstrated at least one deficiency of mRNA's coding for disaccharidase enzymes, 80% at least two deficiencies and 73.3% had deficiencies in all three measured. 80% of ASD-GI patients were deficient in one and 66.7% deficient in both hexose transporters mRNA levels. These deficiencies in carbohydrate digestion and absorption were statistically significant relative to the control-GI group. There was some association with mRNA levels of the transcription factor caudal type homeobox 2 (CDX2) as a predictor of these deficiencies in both ASD-GI and control-GI groups, and CDX2 was significantly lower in the ASD-GI patients with all five deficiencies versus the other ASD-GI patients ($p = 0.037$). Therefore CDX2 level is a determining factor for these deficiencies, but there was no significant abnormality in CDX2 mRNA levels detected specific to ASD. Williams et al 2011, also attempted to determine whether any abnormality identified could be related to the aetio-pathogenesis of autism. Clostriadales group of bacteria were associated with onset of GI symptoms prior to or within one month of developing autistic behaviours (ileum $p = 0.015$, cecum $p = 0.019$).

Abnormal clostridia species have been found repeatedly in ASD[31–33], the theory of clostridia involvement first posited by Bolte E.R., Medical Hypotheses (1998). Bolte postulated clostridial toxin adversely affected neurotransmitter function that may result in neurobehavioural changes presenting as autism[167].

A clinical trial was carried out to assess the bowel and behavioural impact of anti-microbial therapy directed against the potential abnormal Clostridial species in ASD[168]. Oral vancomycin was used for 6 weeks. Behavioural measurements were carried out before and after, as well as clinical assessment of bowel symptoms. The numbers were low ($n = 11$); however the response to intervention was substantial and statistically relevant. All candidates improved in terms of behaviour and bowel symptoms – some scoring within the

neurotypical range. Discontinuation of Vancomycin after the 6-week trial period led to a gradual regression in almost all cases. As yet there has been no investigation of the combined approach of anti-microbial therapy and dietary modification. The trial is currently being repeated with larger numbers (personal communication, University of Arizona).

Summary of Microflora Abnormalities in ASD

There is reasonable evidence microflora compositions are abnormal in ASD. Greater attention is warranted as well, due to the increasing reports of improved ASD symptomology and developmental trajectories following interventions likely to alter gut flora compositions. From the evidence reviewed here, pursuing compositional changes of microflora in ASD patients presents a potential modifiable factor associated with disease.

1.3 Introduction to Zinc

Zinc is the second most abundant metal in the human body (second only to iron). It is essential for cellular life. Closely associated with DNA, zinc is a rate-limiting co-factor in hundreds of enzymatic processes including the polymerases underpinning protein synthesis generally [169]. At this basic level zinc is involved in gene expression and epigenetic mechanisms, with zinc deficiency manifesting across a diverse range of bodily processes and depending on individual genetic factors. Beyond the biochemical functions of zinc (not limited to co-factor metabolism), zinc has a biophysical role throughout the body. Zinc-finger motifs are proteins, often configuring cell receptors, where zinc has a crucial role in allowing functional folding to occur, thus permitting the receptor function [170]. Further, zinc has gained increasing attention given its role in cell-signaling [171,172]. Zinc has been demonstrated to provide immune and nervous system signaling, inducing T cell proliferation and activation, as well as mediating NMDA and GABA receptors centrally [173,174].

Zinc is predominately found in meat, fish, dairy, nuts and grains. Absorption is dependent on the digestion of proteins to release the mainly protein bound zinc, and the passage of zinc to the jejenum without microbial uptake or binding with inhibitors. The absorbed zinc transiently increases plasma zinc levels prior its incorporation into the extravascular space with the greatest quantity of zinc being present in bone and skeletal muscle with high concentrations being found in brain, testes, skin, kidney, liver and placenta [175,176].

Whilst zinc is considered a non-stored essential element, the extravascular pool serves as a form of zinc storage with the majority of zinc being bound to proteins such as albumin. During times of shortage a reduction in faecal loss occurs, followed by release of zinc from bone, skeletal muscle, and other organs through a shifting pool homeostatic mechanism. Bone appears to have greater resilience to zinc deficiency, and failure of bone growth is typically a late feature of marked zinc deficiency [175]. The most recent national nutritional survey calculated average zinc levels in 7 to 11 yrs. of age to be 15.5 ug/dl [177].

Prenatal zinc deficiency

There have been limited human trials with prenatal zinc supplementation, the majority of studies beginning zinc supplementation during gestation. Prenatal zinc supplementation has been shown to improve autonomic function in children versus controls [178,179], and this improvement was noticeable during gestation [180].

Maternal zinc deficiency has been shown to increase obstetric complications [181] and zinc deficiency may be involved in the increased obstetric risk associated with higher maternal age in a low socioeconomic group [182]. A recent meta-analysis of zinc supplementation

given during gestation demonstrated a statistically significant reduction in preterm labour [183]. The meta-analysis included only one study where supplementation began prenatally.

Congenital malformations have been reported in maternal zinc deficiency, albeit studies in this area are divided [184]. Prenatal and antenatal zinc supplementation has been demonstrated to ameliorate dysmorphology associated with ethanol in a transgenic model [185], and these results have been repeated [184]. Neonates with low serum zinc levels in comparison to controls had over nine times higher rate of Neural Tube Defects (NTDs) in a recent trial [186].

Studies of the affects of mild to moderate zinc deficiency on the fetus and early infant beyond congenital abnormalities are limited. Study design is generally hampered by the reliance on maternal plasma zinc levels, confounding additional nutritional deficiency or other socioeconomic factors and the lack of additional biomarkers for zinc deficiency. Perhaps even more confounding is the lack of prenatal supplementation in the majority of studies. Zinc is an essential co-factor in cell division, may affect key functions of the placenta such as barrier integrity, blood flow, protection against xenobiotics and autoreactivity. It may therefore be the period of prenatal zinc loading that has the greater impact on developmental disorders, and as yet there has been limited research into this area.

A recent meta-analysis of motor and cognitive outcomes in infants following maternal zinc supplementation found only 5 RCTs met the inclusion criteria and these studies considered together suffered substantial study heterogeneity [187]. A recent Cochrane review of zinc supplementation on mental and motor function in children also found a low number of high power studies and substantial study differences [188]. Of the 12 studies included, none supplemented zinc prenatally or maternally, and only two studies followed up beyond 13 months of age (Katz and Sazawal). Of the two studies examining motor and cognition after the age of 13 months Sazawal et al., examined only motor activity and reported significant improvements in motor activity ($p < 0.01$) and Katz et al., in a population based study examined motor milestones in a Nepalese rural community with a focus on age to first walking, and reported in infants commenced on supplementation (prior to 12 months of age and not severely malnourished) 12.2% of the control group took longer than 18 months to walk versus 9.3% of the zinc supplemented group (total participant number 748), but this was considered not statistically significant due to wide confidence intervals[189,190].

Since these two meta-analyses, further studies have been completed. One study supplemented 5 mgs/day elemental zinc to 36 children aged 5 to 7 years, and demonstrated improved serum zinc levels ($p < 0.0001$) and markedly improved intelligence ratings ($p < 0.001$) after 3 months of supplementation. The study was limited in power due to total number of participants and lack of controls [191], although assessments were well validated and substantive. Another study examined zinc with iron and folate versus iron and folate in

272 infants with final cognitive and sensorimotor assessments at 18 months of age. Significant improvement was reported in the zinc group, and this was only noticeable after 12 months of age following 6 months of supplementation [192]. Lima et al., compared neurodevelopmental outcomes in 167 over 5-year olds following a 12-month supplementation protocol. There were significant improvements in the zinc, vitamin A and glutamine supplementation group versus placebo, and these differences were sex-dependent. Zinc versus placebo also showed improvement in verbal fluency 8.3 (Mean SD 0.8) versus 6.9 (Mean SD 0.8)[193]. Christian et al 2011., evaluated cognitive and motor function in 7 to 9 year old Nepalese children who had received maternal supplementation with folate and iron versus folate, iron and zinc, and again received supplementation between 12 to 36 months of age. No benefit was identified. Whilst less than 30% of the original cohort were followed-up and the neurodevelopmental assessments were somewhat limited, the authors suggest the lack of supplementation before 12 months of age may have been a significant factor in the failure to maintain a positive effect of supplementation seen in the maternally supplemented children[194]. Siegel et al., examined Nepalese infants at 39 weeks and 52 weeks following variable durations of supplementation, and found some parameters improved and some worsened in the zinc, folate and iron group[195].

1.3.1 Zinc and Immune Function

The role of zinc and the affect of zinc deficiency on the immune system has underwent several reviews [196–201]. It has been suggested that under chronic zinc deficiency conditions the adaptive immune system is less efficient and dependence on the innate immune system occurs, despite the innate immune system also suffering impairment under zinc deficiency conditions [199,200]. With the general shift to a Th2 response, impaired phagocytosis, reduced antibody production and the reduced cell-mediated messaging that occurs with zinc deficiency (see table 9), it is of little surprise that zinc supplementation is utilised as a first line augmentation in the management of diarrhoeal illnesses and respiratory infections worldwide.

Zinc has been reported to be anti-inflammatory, and zinc deficiency to increase pro-inflammatory cytokines[201] and to increase CNS inflammasome activity[202].

Table 9 Summary of previous studies exploring a relationship between zinc and immune function

Immune Cell	Effect	Study
Neutrophil Granulocytes	Impaired recruitment	203
Neutrophil Granulocytes	Impaired chemotaxis	204
Neutrophil Granulocytes	Reduced total number	198,205
Natural Killer Cell	Reduced Activity	206–209
Macrophage	Impaired Phagocytosis	206
Neutrophil	Impaired Phagocytosis	206
Natural Killer Cell	Reduced total count	207,210
B-lymphocytes	Reduced total count	211
B-lymphocytes	Impaired antibody production	212,213
T-cell	Impaired development (in thymus)	214
T-cell	Peripheral function	215
CD-8	Proliferation	215
T Cell	Cytokine signalling	174,210,216
T-Cell	LPS activation pathway	172
Monocytes	Cytokine production (TNF-alpha & IL-6)	217

1.3.2 Nervous system and zinc

Zinc and the nervous system has been reviewed recently[218,219]. It is useful, given zinc's many functions, to consider the three broad categories: structure, cell-signalling and enzymatic co-factors.

Zinc is required for DNA and RNA polymerases, histone catalyses and DNA ligase. As such zinc is involved in most aspects of protein synthesis within the CNS, and is an independent factor involved in gene expression[220].

More recently zinc has been identified as a key component in structural proteins such as zinc-finger motifs[219]. These ubiquitous proteins often form the structure of receptors such as the oestrogen, thyroid hormone and glucocorticoid receptors in the brain [221]. The presence of zinc within these proteins allows folding and the formation of the functional structure of such receptors [222]. The affects of zinc deficiency on zinc-finger motifs, and whether zinc is liberated from such proteins under chronic zinc deficiency, remain unknown.

10-20% of CNS zinc is considered free, and is largely present pre-synaptically, and more often in the glutamenergic neurons. The release of zinc has been shown to modulate post-synaptic receptors including NMDA, GABA and voltage-gated calcium channel receptors [171].

Zinc is essential to normal brain development. Zinc has been implicated in olfactory, cerebellum and hippocampal development [223–226], and even mild zinc deficiency has been shown to affect memory and learning [227]. It has also been demonstrated that transient gestational zinc deficiency can affect memory and learning that persists into adult [228].

1.3.3 Zinc and Autism

Table 10 A summary of previous studies exploring a relationship between zinc and autism

Sample	Number	Result	Comments	Study
Hair and nail	95 (50 controls)	Low Zinc correlated with Low Functioning Autism (p<0.01) Zinc levels in hair and nails correlated with CARS scores (p<0.001).	Authors noted higher variability of zinc levels in ASD vs. controls.	229
Serum	37 (patients with Phelan-McDermit Syndrome)	Zinc deficiency correlated with attention deficit and hyperactivity (p<0.01) and seizures (p<0.001)	Authors also undertook transgenic work demonstrating alteration of ProSAP/Shank levels, and alterations in cerebellum and hippocampal volumes. Induced acute zinc deficiency in pups led to hyperactivity and over-responsiveness to acoustic stimuli.	230
Plasma	79 ASD & 18 controls	No correlation identified	Limited details on controls, and samples were not fasting. Study power relating to zinc was low.	231
Hair and Urine	25 ASD & 25 controls	Zinc deficiency in hair (p<0.003)	Low power study. Sample transit time high.	232
DNA-samples	761 low verbal autism ASD	*ZNF804A* SNP was associated with Low Verbal Autism (p<0.008)	Analysis of zinc associated transporter. No zinc analysis was conducted	233
Serum	230 ASD	20.4% deficient 50.8% in lower ten percent of mean normal values	Study lacked control group.	234
Hair	1967 ASD	50% under 3yrs had zinc levels below 2SD versus 30% of all ASD children analysed	High numbers, and age correlation significant. Lack of controls.	235
Plasma	102 ASD & 18 controls	Zinc deficiency correlated with hyperactivity (p<0.02) and fine motor skills impairment (p<0.005)	Limited details on controls, and samples were not fasting. Study power relating to zinc was low.	236
Hair	44 ASD	50% were deficient for zinc. Zinc negatively correlated with fear and nervousness (p<0.022) and verbal communication (p<0.017)	Low numbers and lack of controls hampers the power.	237
Serum	60 ASD vs. 60 Controls	Zinc deficiency was significant (p<0.001)	Zinc:Copper ratio was associated with symptom severity on CARS (p<0.001)	238

Zinc has been examined in autism (table 10). The lack of population based longitudinal studies hampers the generalisations to the aetio-pathogenesis of ASD. Taken together the studies conducted do though, suggest zinc deficiency is common in ASD. Correlation has been demonstrated between zinc levels and ASD symptoms and severity. Zinc deficiency generally has been found to increase sensory issues in ASD and impair cognitive function.

CHAPTER 2
Hypothesis, Aims and Objectives

2.0 Hypothesis

2.1 Variable Insult Model of Autism

It is the wide heterogeneity of ASD that poses the greatest challenge. Identifying a common pathophysiology is hampered by such diversity, as is the identification of management strategies, both behavioural and medical. Equally those faced with patient care often struggle to discern the range of presentations and the impact this has on management. ASD shares simply, a marked impairment with any of the faculties required for social integration. This can present with a lethargic, disinterested child or an agitated, distracted child or any number of features leading ultimately to impaired social integration.

It may be that in order to determine treatment response we must first delineate/categorise treatable groups. Indeed if some form of environmental insult occurs early on in development (be it infection, toxicant or other environmental stressor), then it may not only cause variable manifestations based on timing, nature and genomic individuality, it may also leave no discernible, or at least easily discernible trace. It may, as Hertz-Piciotta et al[15] and Dietert et al[79] suggest, merely be an event that primes or disrupts a critical window in development. Given the wide ranging heterogeneity of the disorder and the many faculties required for social integration it may be neither the insulting agent, the timing, the genomic vulnerability nor the system(s) affected that remain static or, when ASD is taken as a single consequential expression, statistically identifiable.

The Variable Insult Hypothesis of ASD:
Within the developing neurological, immunological and neuroimmunological systems there is vulnerability to environmental insult. Depending on the nature, timing and duration of insult neurological, immunological or neuroimmunological abnormalities may predominate, and the relative proportion each system is affected will vary accordingly, leading to the variation in pathologies identified in ASD and the heterogeneity in phenotypic presentation typifying the condition (see Figure 3).

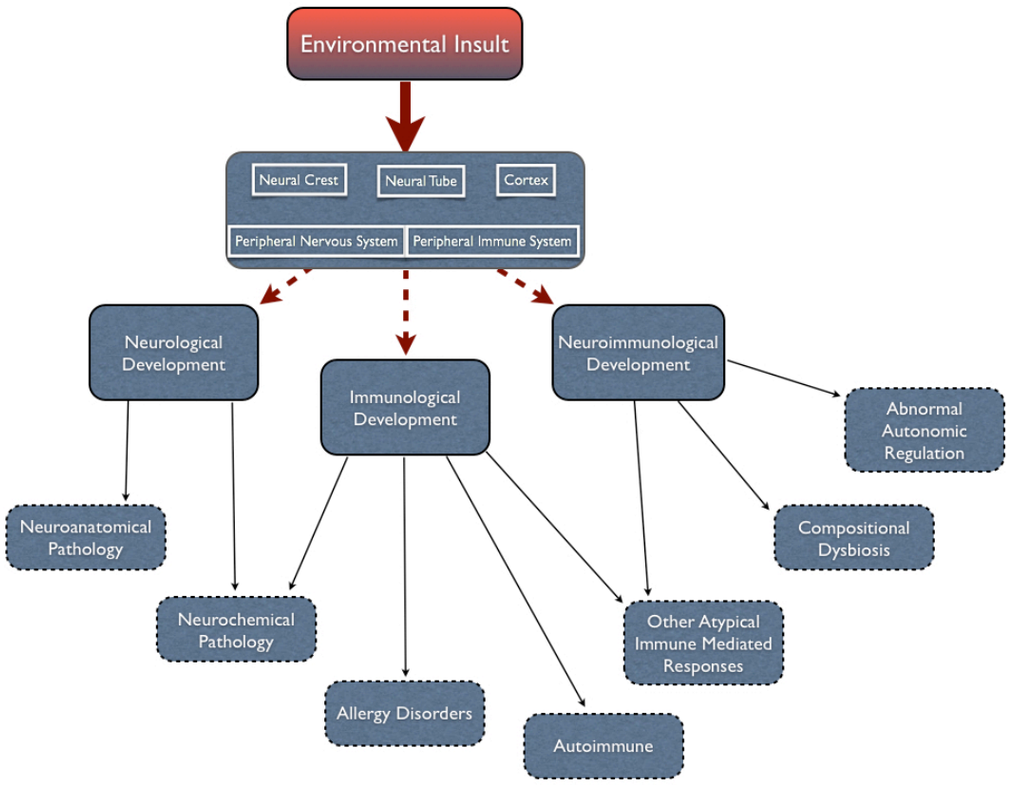

Figure 3 Variable Insult Model of ASD. Depending on timing, extent and type of environmental insult, dysfunction of neurological structure, immunological priming and autonomic set points can all lead to the common feature of autism – impaired social functioning.

Adequate social function depends on the successful integration of these systems, from peripheral neurological and immunological processing to central response to such information, and as such an environmental insult to any of these systems during early development can present with the consistent phenotypic presentation of poor social function as is typified in ASD with the variation in type, duration and extent of insult conferring the heterogeneity within the spectrum. As such the pathology of ASD relates to identifiable neurological, immunological and/or neuroimmunological abnormalities, and often this is likely to present as systemic pathophysiology.

2.2 Abnormal Microflora in ASD

The systems dictating the acquisition and regulation of the microbiota also depend on adequate development of neurological and immunological functions and are likely vulnerable in the same way social functioning is to a variety of environmental insults during early development. In this regard, abnormal microflora may be a secondary effect of the developmental insult (figure 4).

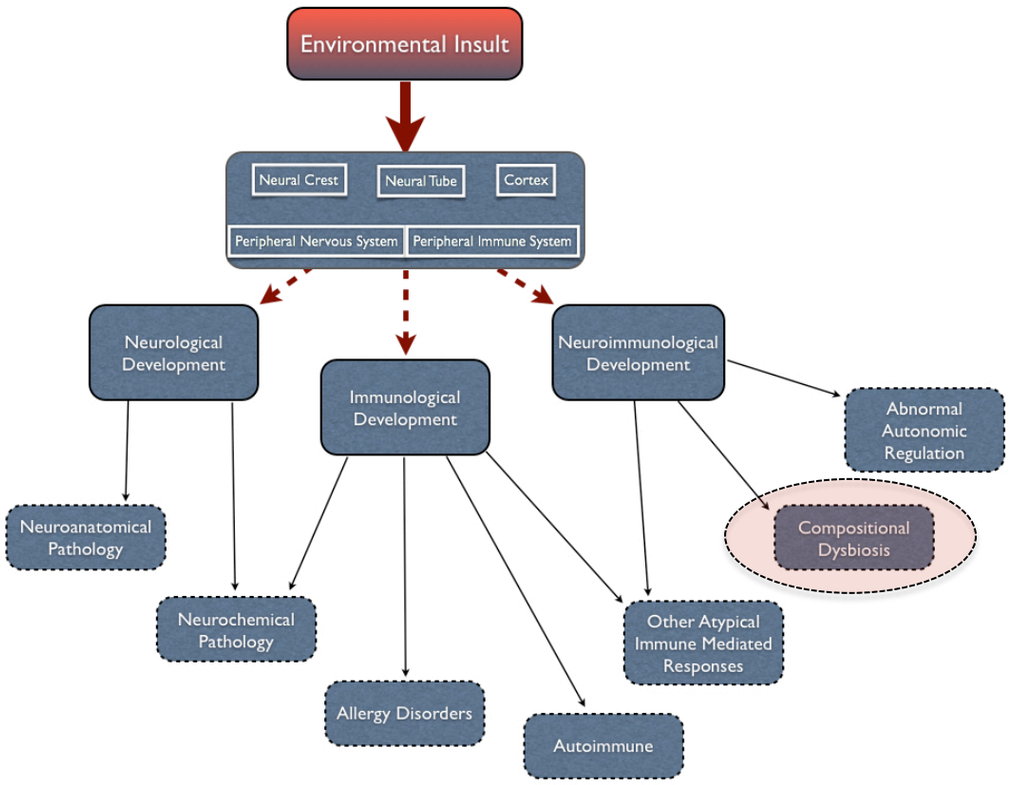

Figure 4 Variable Insult Model showing microflora abnormalities as a secondary effect of environmental insult. Abnormal composition of microflora (compositional dysbiosis) may be a secondary effect of disruption to neurological and immunological development following an environmental insult. It may still perpetuate the systemic pathology of autism, perhaps worsening neuroinflammation or nutritional deficiency, but here is considered as a secondary effect.

The possibility remains that it is the abnormal acquisition of microflora that constitutes an independent environmental factor capable of disrupting the systems necessary for adequate social functioning. In such a consideration the abnormal microflora constitutes the environmental insult (see figure 5). The colonization of the GI tract with an abnormal microbe could disrupt neurodevelopment through a variety of mechanisms, including generating endotoxins, promoting a pro-inflammatory state, excess stimulation of vagal afferents disrupting autonomic function and hence impairing sensory integration or through a direct immunogenic effect via autoantibody production.

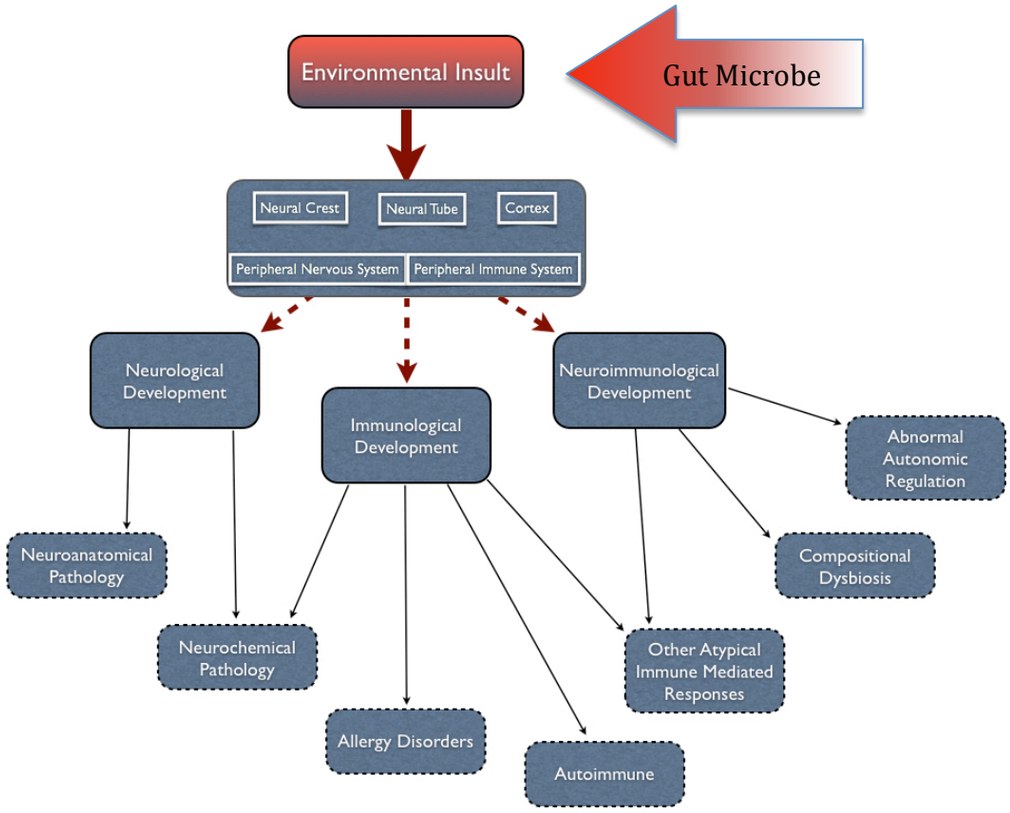

Figure 5 **Variable Insult Model with Gut Microbe(s) as the Environmental Insult.**

2.3 Zinc deficiency in ASD

Zinc deficiency can be considered in a similar way. Prenatal, maternal or early childhood zinc deficiency could be an independent risk factor for the development of autism. Zinc has a wide-range of activities, including being involved in epigenetics and protection against environmental exposures. Perhaps then, within the consideration zinc deficiency is an independent aetiological factor, it is the extent and timing of zinc deficiency combined with the genomic vulnerabilities and/or environmental exposures that lead to the heterogeneity of the condition.

Equally, zinc deficiency may simply be an outcome of chronic ill-health, compositional dysbiosis or chronic immune stimulation, and as such may still be an important determining factor for disease severity, progression and the development of co-morbid disease.

2.4 Study Questions

Both microflora abnormalities and zinc status are possible modifiable environmental factors in the aetio-pathogenesis and evolution of autism. Whilst there is evidence both microflora and zinc status are abnormal in autism, high-powered studies are required to determine more definitively whether such abnormalities are consistent in autism and the specific nature of such abnormalities. As such this project asks the following questions:

1. Are there microflora abnormalities?
2. Are there micronutrient abnormalities?
3. Do microflora abnormalities have direct neurological effects?
4. Do micronutrient abnormalities have any immunological effects?

In relation to the wider Variable Insult Model of Autism, it is hypothesized that there will be modifiable environmental risk factors in a significant proportion of individuals with ASD. In relation to the questions asked here, zinc deficiency is expected to be more prevalent in ASD versus controls; microflora metabolites excreted in the host urine are expected to reveal elevated levels in ASD versus population means and qPCR stool analysis is expected to reveal abnormal compositions of microflora in ASD patients versus controls with a shift towards the firmicutes phyla. It is hypothesized that autonomic function will correlate with microflora composition.

2.5 Aims and Objectives

Aim:

To identify modifiable environmental factors associated with the aetio-pathogenesis of Autism Spectrum Disorder in the context of disease prevention, harm reduction and successful management of the condition.

Objectives:

1. Investigate microflora abnormalities in ASD.
2. Investigate micronutrient status in ASD.
3. Investigate autonomic function in ASD.
4. Investigate immune function in relation to micronutrient status in ASD.
5. Develop trial design for measuring 'the affects of altering microflora composition in ASD on behavioural, cognitive, autonomic, immunological and biochemical measures: an RCT.'

CHAPTER 3
Methods and Materials

3.0 Methodology

To explore the Variable Insult Model of Autism, a literature review was conducted exploring potential generalities applicable to autism. Significant findings were examined for penetrance, power and suggested aetiology in relation to autism. A further literature review was undertaken exploring environmental factors associated with autism. Studies were analysed for statistical power and literature was examined for feasible mechanisms explaining any significant association. Finally, the literature review focused on promising modifiable environmental factors associated with autism aetio-pathogenesis, pathophysiology, evolution and co-morbid illness, towards identifying useful harm-reduction, disease prevention and treatment directions. Part of the results of this literature review was presented through publication in March 2014 "Neuro-immune Abnormalities in Autism and their Association with the Environment: A Variable Insult Model." [239].

Modification of microflora and correction of nutritional deficiencies were identified as potentially modifiable factors associated with autism.

A clinical database of initially 289 patients with ASD attending outpatient clinics were examined for markers of abnormal microflora and nutrient levels. Initially a random selection of 100 patients from this database were selected. The case files were examined for the presence of urinary metabolomics (urinary organic acids) results. Patients were excluded where they had been on antibiotics within three months or where the urinary analysis was not undertaken at initial consult. 61 cases were identified that met the inclusion and exclusion criteria. The case files were examined further for evidence of ASD diagnosis, and only those who had a Multi-Disciplinary Team (MDT) diagnosis of ASD were included. Out of the randomly selected 100 patients, 49 patients were eventually selected for further examination.

The medical records (n=49) were each scrutinsied individually to ascertain severity of ASD, presence of gut symptoms, supplementation use, medication use and whether a dietary modification had been in place. The results were analysed together, potential associations were sought, including total number of abnormalities, mean levels of each metabolite and any associations with age, sex, severity, supplement, medication or diet use. An examination of gut symptoms in relation to the abnormalities identified was also undertaken to help delineate whether the abnormalities were directly associated with gut pathology or whether such abnormalities may be related to the underlying ASD. Both parametric and non-parametric analysis were undertaken as normal distribution could not be inferred from these numbers or the general knowledge pertaining to urinary metabolomics in ASD.

Urinary metabolomics was undertaken utilizing the standardized LC/MS-MS Tandem Mass Spectrometry methodology via the Great Plains Laboratory. The author was not involved in the analytical process.

Next the same database of 289 patients with ASD attending outpatient clinics was explored in relation to qPCR stool analysis results. The case files were selected in a random manner until 60 cases with an initial qPCR stool analysis meeting the inclusion and exclusion criteria were found. Each case file was examined for validated ASD diagnosis, and only those with confirmed diagnosis through MDT assessment were included. The case files were then each examined for evidence of gut symptoms, supplement use, medication use, dietary modification and severity. Initially 7 non-ASD controls were identified who had underwent qPCR stool analysis at initial consult and had not taken antibiotics within three months. The results were collated and analysed. Both parametric and non-parametric analysis were undertaken as normal distribution could not be inferred from these numbers or the general knowledge pertaining to qPCR stool analysis in ASD. QPCR stool analysis is undertaken in the standardized method via Metametrix laboratories. The author was not involved in the analytical process. Such analysis includes a total composition score where bacteriodetes and firmicutes are calculated as a ratio percentage.

Autonomic profile tests were available in 45 patients with ASD from the same database of 289 patients. All patients had a confirmed diagnosis of ASD through MDT assessment. There were no exclusion criteria applied to the total 45 ASD-patients. The clinical case files of these 45 patients were analysed, the autonomic profile results extracted and analysed. Mean values were obtained for Vagal Tone, Heart Rate (HR), Mean Arterial Pressure (MAP) and age using the Neuroscope. The case files of the 45 patients were further examined for immune and microflora markers, and the autonomic variables were analysed against such markers in SPSS. Lymphocyte count, erythrocyte sedimentation rate (ESR) and platelet count were extracted from the case files where available. Lymphocyte count was extracted as a measure of general immune status (i.e. immunocompetence), and ESR and platelet count were included as inflammatory markers. Both parametric and non-parametric analysis were undertaken as normal distribution could not be inferred from these numbers or the general knowledge pertaining to autonomic function in ASD. Autonomic profiling was undertaken in the standardized method via a routine neurophysiology department. The author was not involved in the autonomic assessments.

The micronutrient investigations are stored on a separate database in a standarised format. Serum zinc levels were identified in 128 unique individual patients attending neurodevelopmental outpatient clinics. The patients were matched to the clinical database of 289 patients, and the notes of all 128 patients were individually scrutinized to insure only those with an MDT confirmed diagnosis of ASD were included in the study. The files were

also analysed to ascertain whether a previous diagnosis of zinc deficiency had been made and therefore the patient was or had been on zinc replacement therapy – such individuals were excluded from analysis. Only cases under 16yrs of age were included. Case files were analysed for immune related parameters e.g. lymphocytes, ESR and platelets, to ascertain if zinc status influenced immune function in ASD patients. Plasma manganese and chromium levels were also recorded as a method of recording general nutrient status. A control group was extracted from the nutritional database. Following exclusion of those over 16yrs of age, the control group consisted of 231 individual patients analysed in the same time period as the ASD-cohort. These results were amalgamated and analysed in SPSS. Parametric analysis (favouring Pearson's correlation) is the preferred method used here for analyzing any associations between the ASD population and the control group, as there is sufficient patient numbers and it is reasonable to expect equal distributions would be met. Lymphocytes, platelets and ESR were not recorded in the control population, and as such a one sided T-test was undertaken for the ASD-cohort against population means for these variables. Correlations were sought between zinc status, platelets, ESR and lymphocyte counts, and analysis was also undertaken where possible for supplementation use and dietary modification in the ASD-cohort. Both parametric and non-parametric analysis were completed due to the relatively small numbers present in each sub-group and the relationships between zinc and these immune markers are not well established and cannot reasonably be assumed to be of normal distribution. There was sufficient numbers in the full zinc ASD-cohort to undertake a multivariate analysis correcting for age and sex. It was not feasible due to size constraints to undertake multivariant analysis beyond age and sex. Serum zinc levels were analysed via the standardized Gas Chromatography Mass Spectrometry methodology via Biolab Medical Unit. The author was not involved in the laboratory measurement of zinc.

Following the completion of the above examination and analysis, a further data collection was undertaken. The outpatient clinic database, now comprising of 539 individual patients with ASD, was examined. A decision was made to extract a number of variables from 178 patients who had underwent initial investigations likely to include urinary and stool analysis. The primary aim of this extraction of data was to collate more data pertaining to mean succinic and 2-hydroxyhippuric acid levels (metabolomics) and firmicutes percentage via qPCR stool results. The same inclusion and exclusion criteria was applied as detailed above. With the increased numbers parametric analysis was favoured, but non-paramteric analysis was also undertaken.

CHAPTER 4
Urinary metabolomics in ASD

4.0 Results – Urinary Metabolomics

Urinary Metabolomics were analysed in patients with ASD, specifically five organic acids thought related to microflora, and then compared to population norms.

Table 11 Total Number of Urinary Metabolomics Abnormalities, Demographics and Variables in 37 patients with ASD

ASD Cohort (n = 37)	Results
Age (yrs.)	6.2 (Range, 2-19)
Sex Ratio (M:F)	3.63
Severity (Physician Rated)	5.3 (Range, 2-8)
Supplements	57%
Diet	53%
Medication	11%
Average number of abnormalities	1.86

49 cases were identified as meeting the inclusion criteria detailed above. Of these 49 cases, 37 had reliable recording (positively or negatively) of diet and supplement use. The metabolomics analysis included 75 individual markers, of which five were selected for inclusion within the statisticial analysis (2-hydroxyhippuric acid, succinic acid, 4-hydroxypheylacetate, 3-oxygultaric acid, hippuric acid and proprionic acid).

Table 11 presents the demographics, severity rating, supplementation, medication use, dietary modification and the average total number of urinary metabolomics abnormalities identified in the five metabolites analysed. The average age was 6.2 yrs with a range of 2 to 19 yrs of age, representing a young ASD population. The male to female sex ratio was 3.63 (M:F), consistent with the epidemiology of ASD, and suggests a good sample population. Severity rating (physician) was 5.3 (out of 10) with a range of 2 to 8, indicating mild, moderate and severe ASD were present in the cohort. Supplementation and diet use was 57% and 51% respectively. These rates of dietary modification and supplementation use are likely skewed higher due to the inclusion of

records where positive or negative recording of diet or supplementation use was made, as it is more likely no recording of diet or supplementation use equates to no supplementation or diet use and case files were no recording was made were excluded. 11% of patients were taking medication at the time of urinary analysis (table 11).

Overall there was an average of 1.86 abnormalities detected out of a possible 5 metabolites analysed. Number of participants with reliable recording of supplement and diet use in this cohort (n = 37) was insufficient for multi-variant analysis on medication, supplement and diet use. There was though similar total number of abnormalities regardless of supplement and diet use (no supplements vs supplements - 1.75 vs. 2.0 -, no diet versus diet – 1.81 vs 2.25 – see appendix 4).

Mean levels of the five metabolites were determined in the two dominant groups (table 12) – males 2 to 12yrs of age with a primary diagnosis of ASD (n = 26) and females 2 to 12 yrs of age with a primary diagnosis of ASD (n = 9). There was insufficient data available to analyse the two other groups – males > 12yrs of age (n = 2) and females over 12 yrs of age (n = 0).

Table 12 **Mean Levels of Urinary Metabolomics in ASD patients aged 2 to 12yrs (n= 35).** Figures in bold indicate a mean level above the laboratory reference range i.e. abnormally raised. (nr. = normal reference)

Urinary Metabolite	Male 2-12yrs of age with ASD N = 26	Female 2-12yrs of age with ASD N = 9
Propionic **(mmol/mol creatinine)**	190 (SD 167) (nr < 221)	34.6 (SD 13.9) (nr < 228)
Hippuric **(mmol/mol creatinine)**	377 (SD 383) (nr < 681)	414 (SD 368) (nr < 718)
4-Hydroxyphenylacetate **(mmol/mol creatinine)**	26.7 (SD 19.1) (nr < 33)	13.5 (SD (5.1) (nr < 31)
2-Hydroxyhippuric **(mmol/mol creatinine)**	**1.77** (SD 1.30) (nr < 1.3)	**1.61** (SD 2.17) (nr < 1.3)
Succinic **(mmol/mol creatinine)**	**30.2** (SD 28.4) (nr < 23)	**26.9** (SD 17.2) (nr < 16)

2-Oxyglutaric **(mmol/mol creatinine)**	0.32 (SD 0.34) (nr < 0.47)	0.24 (SD 0.32) (nr < 0.52)

The Mean levels of the five metabolites are presented in table 12. Both groups had a mean level of succinic acid above the population norm. In the male 2 to 12yr ASD-group succinic acid was 30.2 mmol/mol creatinine against a population mean of 13 mmol/mol creatinine, and the female 2 to 12 yrs ASD-group had a mean succinic acid level of 26.9 mmol/mol creatinine against a population mean of 9 mmol/mol creatinine. Mean 2-hydroxyhippuric acid was also above the population norm in both groups – male ASD-group 1.77 mmol/mol creatinine versus population mean of 0.8 mmol/mol creatinine and the female ASD-group 1.61mmol/mol creatinine versus a population mean of 0.8 mmol/mol creatinine. Standard deviations were high across the metabolites analysed. Overall, of the 35 cases analysed, 86% had at least one abnormality and 58% had at least 2 abnormalities identified on urinary metabolomics (appendix 4).

Out of the 37 cases there were only 9 female ASD patients, and only two Male patients above 12yrs of age. The male 2 to 12 yrs of age group consisted of 26 cases, 13 of which had a positive record of abdominal symptoms (including diarrhea, constipation or confirmed abdominal pain, and excluding suggested abdominal pain on examination). 13 cases out of the Male 2 to 12yrs of age cohort had a record of no abdominal symptoms. Further scrutiny of each individual file confirmed no evidence of abdominal symptoms including non-specific abdominal symptoms such as discomfort on defecation, variable appetite, night waking, difficulty gaining weight, abdominal bloating, excessive flatulence or behavioural disturbance following a meal.

Total number of urinary abnormalities were calculated and age, severity, supplement use, medication use and the presence of current dietary restrictions were recorded in relation to gut symptoms in the Male ASD group aged 2 to 12yrs (n = 26) (Table 13).

Table 13 Total Number of Abnormalities and Demographics in ASD Patients with and without GI symptoms (full data appendix 4)

	Without Abdominal Symptoms N = 13	With Abdominal Symptoms N = 13
Age	6.5 (Range 2 to 12)	3.8 (Range 2 to 8)
Severity	4.7 (Range 2 to 8)	6.5 (Range 4 to 8)
Supplements	75%	38%
Diet	58%	54%
Medication	9%	14%
Number of abnormalities	2.25	2.08

Comparing the male ASD patients aged 2 to 12yrs with and without GI symptoms, the two groups differed in age, severity and supplementation use. Those with abdominal symptoms were younger, more severe and had less supplement use. Diet and medication use were similar between the groups. Total number of abnormalities were similar between those with and those without abdominal symptoms (2.08 vs. 2.25 respectively). There were insufficient numbers to undertake multi-variant analysis.

Mean levels of the five urinary metabolites were calculated in Male ASD patients 2 to 12yrs of age with and without abdominal symptoms (Table 14).

Table 14 **Mean levels of urinary metabolites in Male ASD patients aged 2 to 12yrs without abdominal symptoms and Male ASD patients aged 2 to 12yrs with abdominal symptoms**

Organic Acids	Male 2-12yrs of age without abdominal symptoms N = 13	Male 2-12yrs of age with abdominal symptoms N = 13
Propionic (mmol/mol creatinine)	162.5 (SD 167.9) (nr < 221)	216 (SD 169) (nr < 221)
Hippuric (mmol/mol creatinine)	470 (SD 456) (nr < 681)	290 (SD 292) (nr < 681)
4-HPAA (mmol/mol creatinine)	23.9 (SD 14.9) (nr < 33)	29.2 (SD 22.7) (nr < 33)
2-HHA (mmol/mol creatinine)	**2.01** (SD 1.44) (nr < 1.3)	**1.55** (SD 1.17) (nr < 1.3)
Succinic (mmol/mol creatinine)	**24** (SD 16.1) (nr < 23)	**36.3** (SD 36.7) (nr < 23)
2-Oxyglutaric (mmol/mol creatinine)	0.30 (SD 0.39) (nr < 0.47)	0.35 (SD 0.3) (nr < 0.47)

Mean 2-Hydroxyhippuric acid and Succinic acid were elevated in both the abdominal and non-abdominal symptom male 2 yrs to 12 yrs ASD group. At this number (n = 26) there was no significance found on statistical analysis. An attempt to correct for age was made, and following correction for age the results suggested 4-Hydroxyphenylacetate levels were higher in the GI-symptom group (p =0.013), and raised proprionic acid trended towards significance (p = 0.184).

ASD urinary metabolomics (n=49)

The 36 cases where the diet and supplement variables were recorded reliably were analysed and compared to the full cohort of 49 cases. There were no significant differences in demographics between the two groups (see table 15), and hence the larger group was further examined.

Table 15 **Total number of urinary metabolomics abnormalities and demographics in ASD cohort (n = 49)**

ASD Cohort (n = 49)	Results
Age (yrs.)	6.2 (Range, 2-19)
Sex Ratio (M:F)	4.4
Average number of abnormalities	1.8

In the larger cohort (n = 49) the average age was 6.2yrs of age with a male sex ratio of 4.4, suggesting a good sample consistent with the ASD population. The larger cohort was analysed further for total count of abnormalities and mean levels of metabolites.

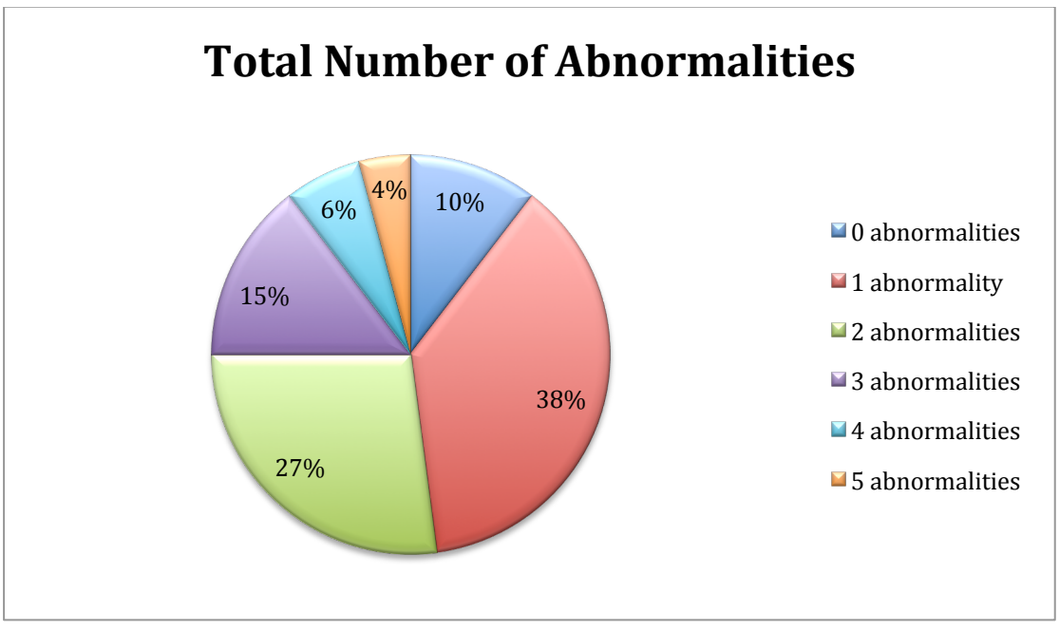

Figure 6 **Total number of raised Urinary Metabolomics as % of total ASD cohort (n = 49)**

90% of patients showed at least one abnormality on limited urinary metabolomics screening, and 55% of patients showed at least 2 abnormalities (see figure 6).

Total number of abnormal urinary metabolites (out of the five analysed) were examined

in relation to age and severity scores.

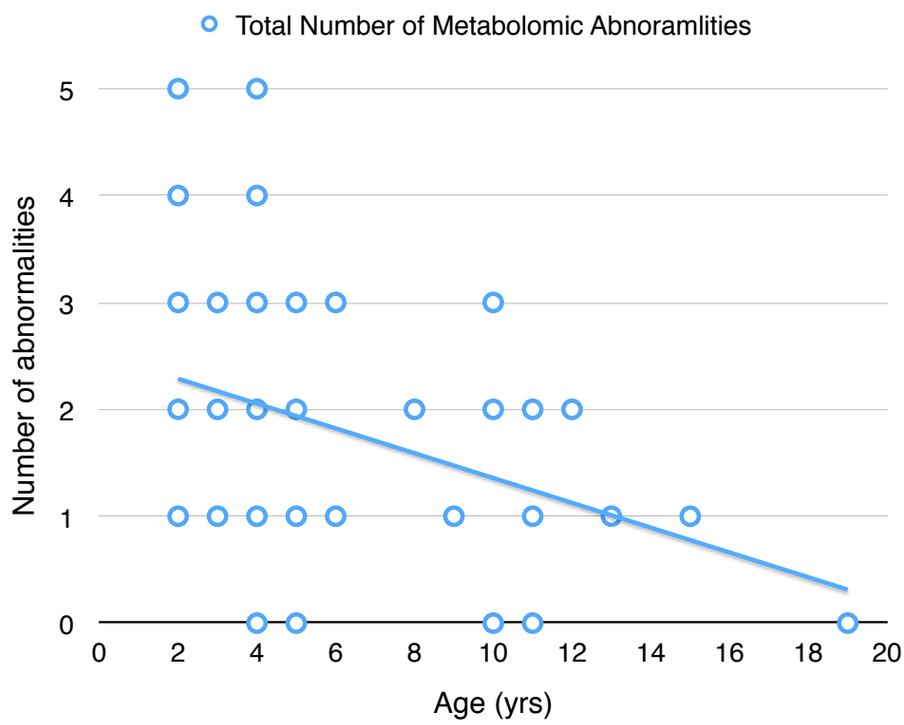

Figure 7 Scatter graph with best-fit line for Number of Abnormalities of urinary metabolomics versus Age in Patients with ASD (n = 49). The graph suggests a possible trend with reducing number of abnormalities with increasing age.

Numbers were limited across each specific age group, however a scatter graph demonstrated a trend towards a greater number of total abnormalities in younger patients versus older patients. On parametric analysis, number of abnormalities negatively correlated with age (Pearson's correlation = -0.38, p = 0.007), suggesting there were more abnormalities in younger patients with ASD and this reduced over time.

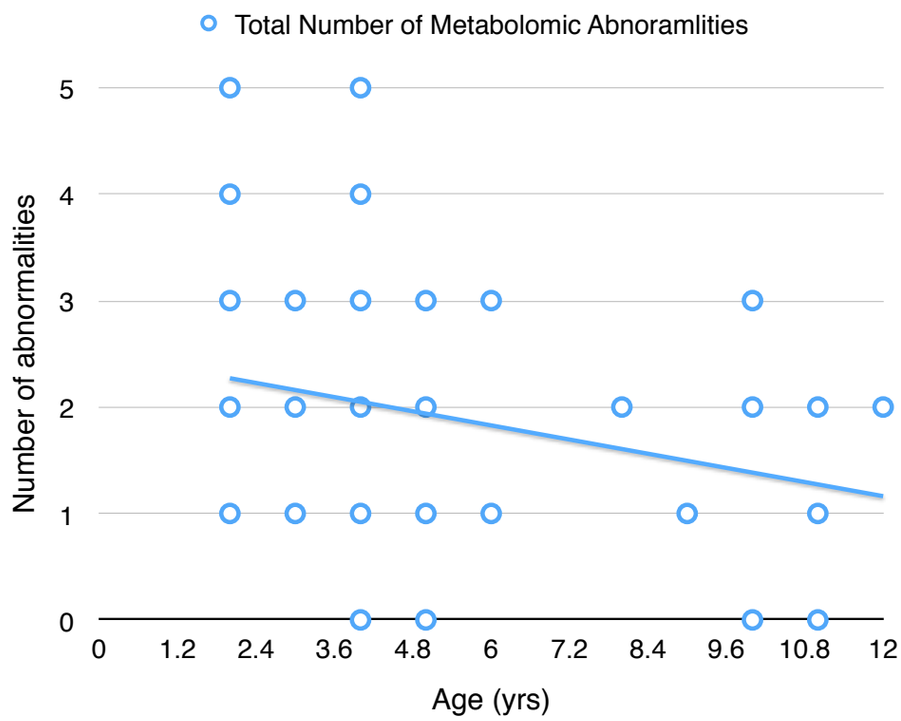

Figure 8 Scatter graph with best-fit line for Number of Abnormalities of urinary metabolomics versus Age in patients with ASD aged 2 to 12yrs of age. Trend line is maintained despite removing the older patients, suggesting total number of abnormalities on urinary metabolomics testing reduces with age.

The trend remained after removing patients over 12yrs of age. Parametric analysis demonstrated a trend towards significance (Pearson's correlation = -0.28, p = 0.064).

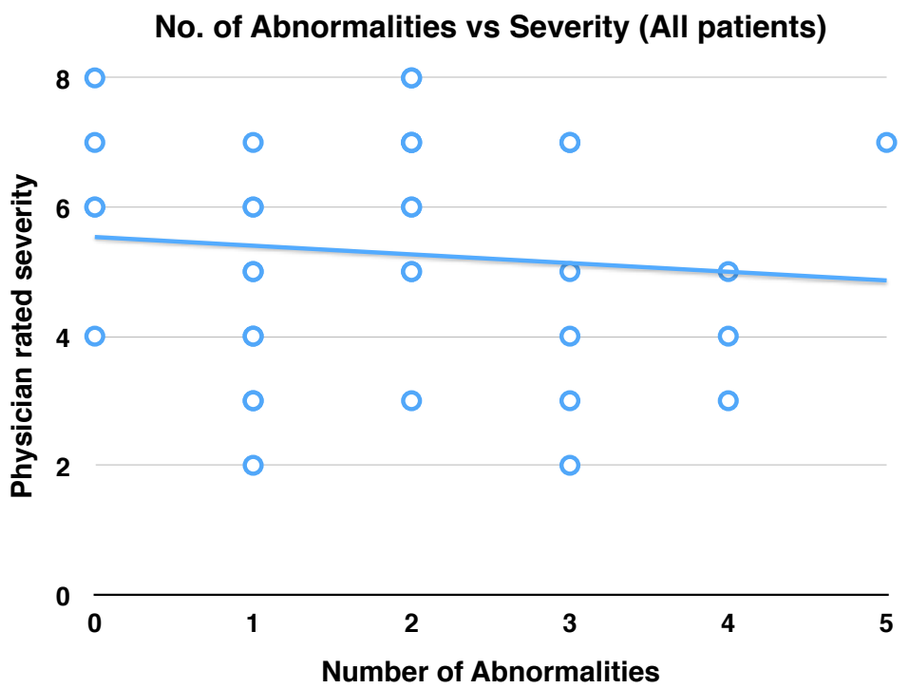

Figure 9 Scatter graph with best-fit line for Physician Rated Severity versus Number of Abnormalities on urinary metabolomics testing in patients with ASD (n=36). Scatter graph did not show significant trend, suggesting autism severity may not relate to number of abnormalities

Physician rated severity plotted against number of abnormalities suggested a small negative correlation. Parameteric analysis demonstrated no correlation between number of abnormalities and severity rating (Pearson's correlation = -0.01, p = 0.57). Removing one outlier (severity rating 7, number of abnormalities 5), increased the slope of the trend line towards a negative association between severity and number of abnormalities. No significance was identified between number of abnormalities and severity rating on parametric (or non-parametric) testing (Pearson's correlation = -0.19, p = 0.28).

Normal population ranges for the urinary metabolites analysed have been determined on an initial control group (n = 50, data not available) and subsequently readjusted based on samples received, as is standard laboratory practice for clinical specimens. Table 16 is the current normal values for the five metabolites analysed.

Table 16 **Normal laboratory reference ranges for urinary metabolites measured**

	Males 2-12 yrs. of age	Females 2-12 yrs. of age	Males 13+yrs	Females 13+yrs
Propionic (mmol/mol creatinine)	< 221 Mean 120 SD +/- 101	< 228 Mean 124 SD +/- 102	< 102 Mean 55 SD 47	< 209 Mean 109 SD 100
Hippuric (mmol/mol creatinine)	< 681 Mean 350 SD 331	< 718 Mean 362 SD 356	< 221 Mean 115 SD 116	< 614 Mean 314 SD 300
4-HPAA (mmol/mol creatinine)	< 33 Mean 18 SD 15	< 31 Mean 17 SD 14	< 18 Mean 10 SD 8	< 20 Mean 11 SD 9
2-Hydroxyhippuric (mmol/mol creatinine)	< 1.3 Mean 0.8 SD 0.7	< 1.3 Mean 0.8 SD 0.7	< 0.86 Mean 0.55 SD 0.31	< 1.4 Mean 0.8 SD 0.6
Succinic (mmol/mol creatinine)	< 23 Mean 13 SD 10	< 16 Mean 9 SD 7	< 4.4 Mean 2.4 SD 2	< 9.4 Mean 5.1 SD 4.3
3-Oxyglutaric (mmol/mol creatinine)	< 0.47 Mean 0.27 SD 0.2	< 0.52 Mean 0.29 SD 0.21	< 0.12 Mean 0.75 SD 4.5	< 0.34 Mean 0.19 SD 0.15

It is noted that gender differences and age differences exist in the normal population. Females maintain a generally higher level of metabolites with age versus males.

Mean levels of urinary metabolites were calculated in four specific groups from the full cohort, separated on gender and age group (Table 17).

Table 17 **Mean levels of five urinary metabolites in an ASD-cohort (n = 49).** Bold indicates mean values above the normal reference range.

	Male 2-4yrs of age N = 20	**Male 5-11yrs N = 16**	**Male 12yrs and over N = 5**	**Female 2-10 yrs. N = 9**
Propionic (mmol/mol creatinine)	257 (SD 224) nr < 221	115 (SD 106) nr < 221	51.8 (SD 19.6) nr < 102	55 (SD 59) nr < 209
Hippuric (mmol/mol creatinine)	402 (SD 318) nr < 681	271 (SD 240) nr < 681	**585** (SD 664) nr < 221	406 (SD 341) nr < 614
4-HPAA (mmol/mol creatinine)	28.4 (SD 19.5) nr < 33	19.7(SD 11.9) nr < 33	9.68 (SD 1.9) nr < 18	16.2 (SD (8.9) nr < 20
2-HHA (mmol/mol creatinine)	**1.73** (SD 1.25) nr < 1.3	**1.56** (SD (1.23) nr < 1.3	**2.14** (SD 1.84) nr < 0.86	0.88 (SD 0.51) nr < 1.4
Succinic (mmol/mol creatinine)	**28.9** (SD 29.3) nr < 23	**31.3** (SD 21.2) nr < 23	**12.4** (SD 12.5) nr < 4.4	**27.4** (SD 14.2) nr < 9.4
2-Oxyglutaric (mmol/mol creatinine)	0.47 (SD 0.54) nr < 0.47	0.34 (SD 0.5) nr < 0.47	0.026 (SD 0.06) nr < 0.12	0.36 (SD 0.60) nr < 0.34

At 12yrs and over male patients showed an increase in hippuric acid and 2-hydroxyhippuric acid in comparison to the younger age groups, against the expected normal trend, but the numbers were small (n=5). Mean 2-Hydroxyhippuric acid was above the reference range in male patients of all age groups, but not in female patients. Succinic acid showed the most marked and consistent abnormalities, whilst decreasing with age as expected, the mean level of succinic acid was consistently elevated throughout all patient groups (Raw data is presented in appendix 4).

The medical records database was analysed further. 178 individual cases were reviewed, and analysed specifically for the urinary metabolomics, succinic acid and 2-hydroxyhippuric acid, and the demographics recorded (appendix 5).

Table 18 Mean Succinic Acid and 2-Hydroxyhippuric acid levels in patients with ASD aged 2 to 12 yrs separated by gender (n = 105)

	Males 2-12 yrs. of age with diagnosis of ASD	Females 2-12 yrs. of age with diagnosis of ASD
2-Hydroxyhippuric (mmol/mol creatinine)	Mean **1.85** SD 2.46 N = 86	Mean **2.1** SD 0.7 N = 19
Succinic (mmol/mol creatinine)	Mean **28.5** SD 24.5 N = 73	Mean **19.1** SD 12.5 N = 19

Out of 178 cases, 116 had urinary metabolomics analysis at initial consultation (65.2%). 10 cases were over the age of 12yrs and hence were excluded from initial analysis. Mean levels of 2-hydroxyhippuric acid and succinic acid were calculated. One outlier was removed from the female cohort as the 2-hydroxyhippuric acid level was ten times the expected normal in that single case. The results are presented in table 18.

Comparison was subsequently made with the initial cohort (table 19).

Table 19 Comparison of Mean levels of succinic acid and 2-hydroxyhippuric acid in ASD patients aged 2 to 12 years of age separated by gender in the initial cohort (n = 43) versus the additional cohort (n = 106)

	Males 2-12 yrs. of age Follow-up cohort (n=86)	Females 2-12 yrs. of age Follow-up cohort (n=20)	Male 2-12 yrs of age Initial Cohort (n = 37)	Females 2-12 yrs. of age Initial Cohort (n = 8)
2-Hydroxyhippuric (mmol/mol creatinine)	Mean **1.85** **(nr < 1.3)** SD 2.46 N = 86	Mean **2.10** **(nr < 1.3)** SD 1.9 N = 19	Mean **1.61** **(nr < 1.3)** SD 1.21 N = 37	Mean **1.60** **(nr < 1.3)** SD 0.7 N = 8
Succinic (mmol/mol creatinine)	Mean **28.5** **(nr < 23)** SD 24.5 N = 73	Mean **19.1** **(nr < 16)** SD 12.5 N = 19	Mean **28.4** **(nr < 23)** SD 25.6 N = 37	Mean **27.4** **(nr < 16)** SD 14.2 N = 8

The mean level of 2-hydroxyhippuric acid in the male 2 to 12yrs of age follow-up ASD-cohort was 1.85 mmol/mol creatinine, succinic acid was 28.5 mmol/mol creatinine. Succinic acid was similar to the initial cohort, and 2-hydroxyhippuric acid was slightly greater in comparison to the initial cohort. The mean level of 2-hydroxyhippuric acid in the female 2 to 12 yrs of age ASD cohort was 2.1 mmol/mol creatinine, and succinic acid was 19.1 mmol/mol creatinine – both above mean population levels. 2-hydroxyhippuric acid was more elevated and succinic acid was reduced in comparison to the initial cohort in the female ASD-group. Both mean succinic acid and 2-hydroxyhippuric acid levels were elevated in relation to population means in males and females in both the initial cohort (n =45) and the follow-up cohort (n = 106).

The initial cohort and follow-up cohort were combined, scrutinized for duplication and then analysed (Table 20).

Table 20 Mean 2-Hydroxyhippuric acid and Succinic acid levels in the combined cohorts (n = 151). A separate column in the female 2-12yrs of age is presented for succinic acid with removal of outlier.

	Males 2-12 yrs. of age	Females 2-12 yrs. of age	
2-Hydroxyhippuric (mmol/mol creatinine)	Mean **1.8** **(nr < 0.8)** SD 2.2 N = 122	Mean 2.7 SD 4.2 N = 28	Mean **1.95** **(nr < 0.8)** SD 1.93 N = 27 Outlier removed
Succinic (mmol/mol creatinine)	Mean **27.5** **(nr < 23)** SD 22.6 N = 107	Mean **21.6** **(nr < 16)** SD 13.3 N = 26	

The combined cohort of 151 patients showed similar levels with raised 2-Hydroxyhippuric and succinic acid levels in both males and females 2 to 12 yrs of age with a diagnosis of ASD as the initial cohorts. A direct comparison to population means was undertaken (Table 21 & 22).

Table 21 One-sided T-test of mean levels of succinic and 2-hydroxyhippuric acid in Male ASD patients 2-12yrs of age versus population means

	Case vs. Population Means (Male ASD patients 2-12yrs of age)						
	Population mean (mmol/mol creatinine)	No. of ASD Cases	Case Mean (mmol/mol creatinine)	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
						Lower	Upper
Succinic Acid (mmol/mol creatinine)	13	106	27.5	0.000	14.46	10.12	18.79
2-Hydroxy-hippuric Acid (mmol/mol creatinine)	0.8	121	1.8	0.000	0.97	0.58	1.36

Mean levels of succinic acid and 2-hydroxyhippuric acid were significantly elevated in comparison to population means ($p < 0.0001$ and $p < 0.0001$, respectively).

Table 22 One-sided T-test of mean levels of succinic and 2-hydroxyhippuric acid in Female ASD patients 2-12yrs of age versus population means

	Case vs. Population Means (Female ASD patients 2 to 12yrs of age)						
	Population mean (mmol/mol creatinine)	No. of ASD Cases	Case Mean (mmol/mol creatinine)	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
						Lower	Upper
Succinic Acid (mmol/mol creatinine)	9	26	21.2	0.000	12.62	7.24	17.99
2-Hydroxy-hippuric Acid (mmol/mol creatinine)	0.8	28	1.95	0.027	1.87	.23	3.51

Mean levels of succinic acid and 2-hydroxyhippuric acid were significantly elevated in female ASD patients aged 2 to 12yrs of age in comparison to population means ($p < 0.0001$ and $p < 0.05$ respectively).

Succinic acid and 2-hydroxyhippuric acid were each analysed for age-related differences.

Figure 10 Scatter graph with best-fit line of mean succinic acid levels in Male ASD aged 2 to 12 yrs cohort versus patient age. Scatter graph suggested a lack of relationship between male patients with ASD and succinic acid levels.

Scatter graph (Figure 10) showed a widely distributed pattern of succinic acid levels

versus age in the male ASD patient 2 to 12 yrs group. Parametric testing of this larger cohort showed no correlation between succinic acid levels and age in the male ASD patient 2 to 12 yrs group (n = 107) – Pearsons correlation = -0.003, p = 0.98.

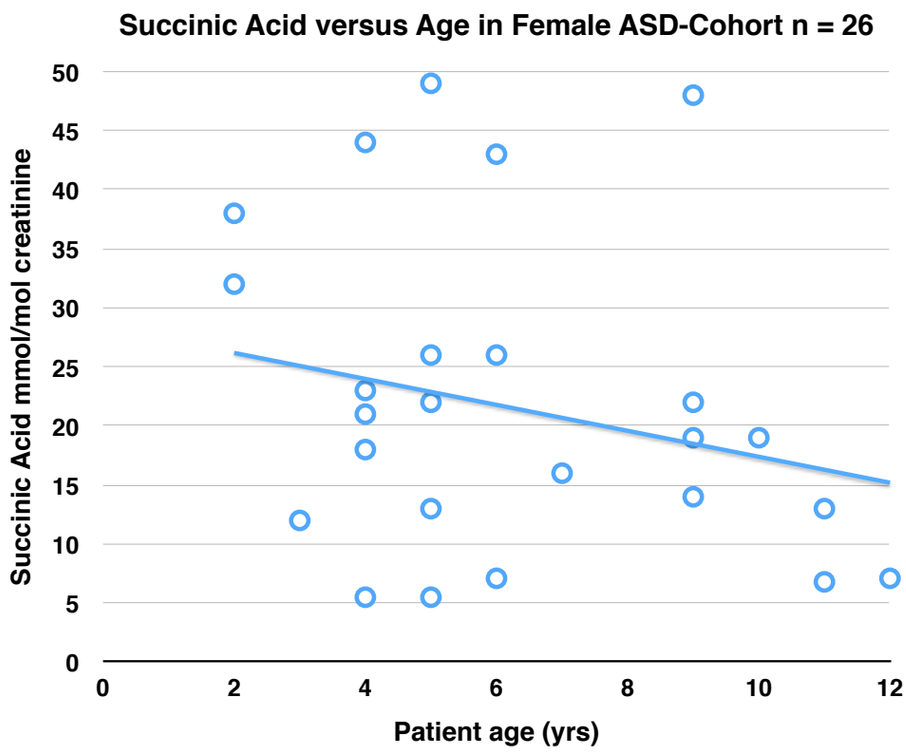

Figure 11 Scatter graph with best-fit line for mean succinic acid levels in female ASD patients 2 to 12yrs of age versus age. Scatter graph suggests succinic acid levels reduce with age in females with ASD.

Scatter graph (Figure 11) with best-fit line suggested a trend in reducing succinic acid with age in the female ASD 2 to 12yrs cohort. Parametric analysis of this larger cohort did not reveal any significance at these numbers (n = 26) – Pearsons correlation = -0.24, p = 0.236.

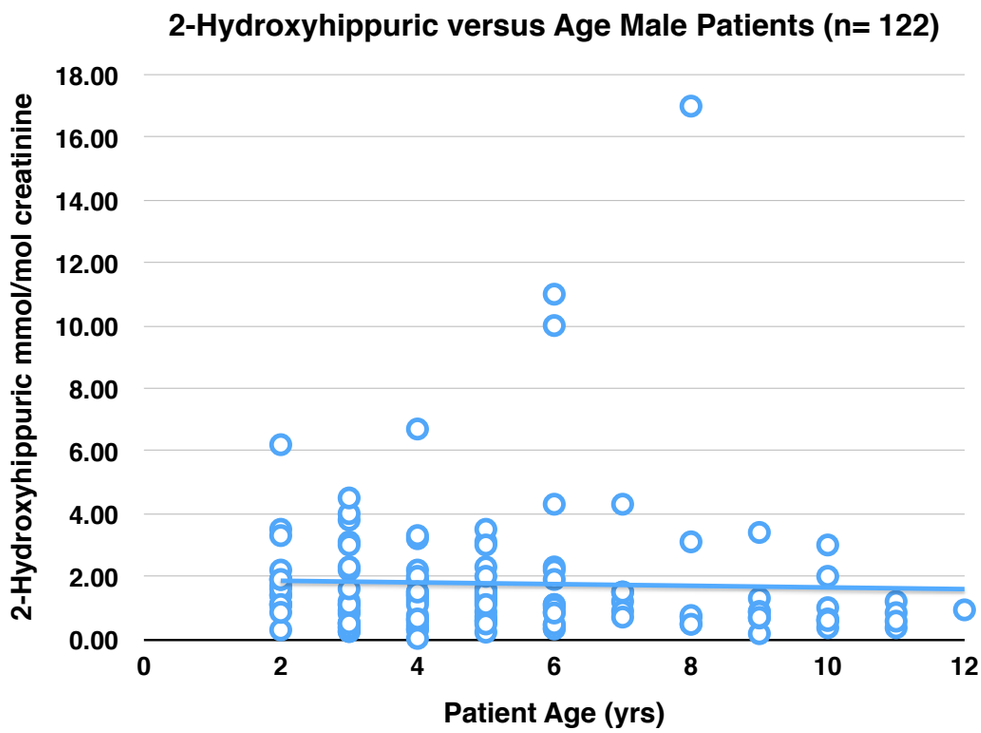

Figure 12 Scatter graph with best-fit line for mean levels of 2-hydroxyhippuric acid levels in Male ASD patients aged 2 to 12yrs versus age. Scatter graph suggests minimal relationship between 2-hydroxyhippuric acid levels and patient age, although several outliers are visible.

Scatter graph with best-fit line suggested minimal association with age and succinic acid in the male cohort. Parametric analysis of this larger cohort revealed no correlation between age and 2-hydroxyhippuric acid in the male ASD-cohort – Pearson's correlation = -0.032, p = 0.73.

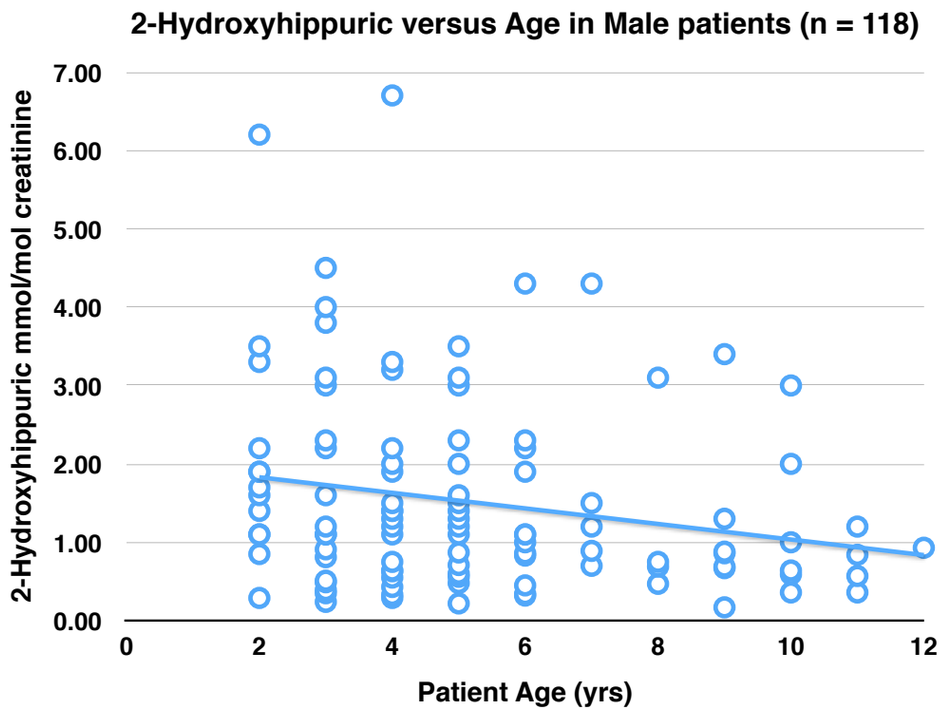

Figure 13 Scatter graph with best-fit line for mean levels of 2-hydroxyhippuric acid levels in Male ASD patients aged 2 to 12yrs versus age with 3 outliers removed. Scatter graph suggests some relationship between 2-hydroxyhippuric acid levels and patient age – as age increases 2-hydroxyhippuric acid decreases.

Removing the four outliers altered the best-fit line on scatter graph (figure 13). There remained no correlation – Pearson's correlation -0.032, p = 0.73.

The female group (2-12yrs) also showed no correlation with age in 2-hydroxyhippuric acid – Pearson's correlation = 0, p = 0.99. A post-hoc analysis of female patients under the age of 7yrs was undertaken.

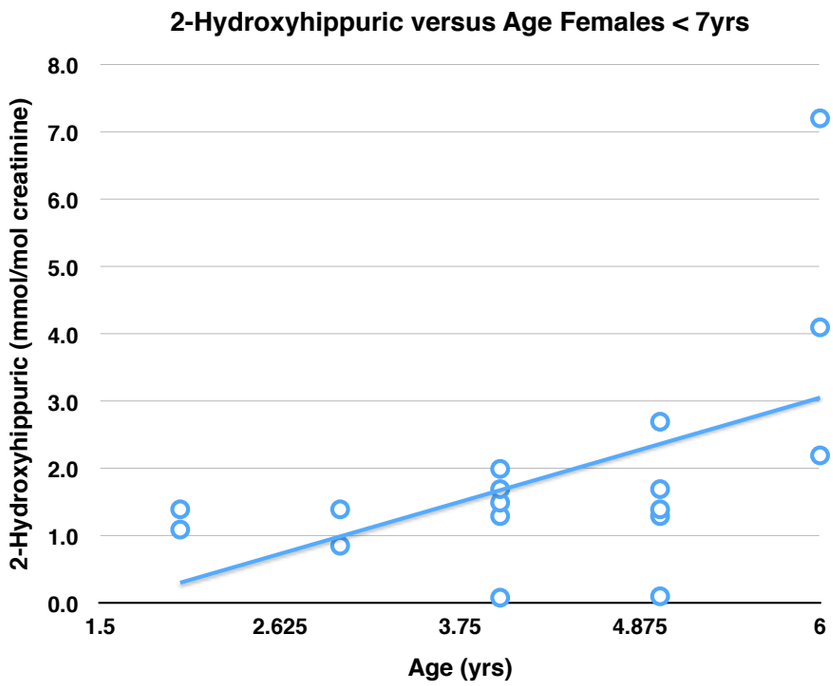

Figure 14 Scatter graph with best-fit line for mean levels of 2-hydroxyhippuric acid levels in Female ASD patients aged 2 to 7yrs versus age (n = 15). Scatter graph suggests a positive correlation between 2-hydroxyhippuric acid levels and age in female ASD patients below 7 years of age.

Below 7yrs of age scatter graph and best-fit line suggested a positive association between age and 2-hydroxyhippuric acid in female patients (n = 15) (Figure 14). Parametric analysis on the under 7yrs female ASD group trended towards a significant positive correlation – Pearson's correlation = 0.44, p = 0.06.

Table 23 **Summary of Metabolomics findings in ASD in the current study**

Variable	Group	Number	Finding	Correlation	Comment
Total number of abnormalities on metabolomics (x5)	Total ASD cohort	49	1.8 abnormalities	n/a	Only 10% had none of the 5 metabolites raised.
Succinic Acid	ASD-Male 2 – 12yrs	116	Mean Diff + 14.5 mmol/mol creatinine (vs. population norms)	P < 0.001	Against population norms
	ASD-Females 2-12yrs	26	Mean Diff + 12.1 mmol/mol creatinine (vs population mean)	P < 0.001	Against population norms
Age vs Succinic Acid	Males (2-12yrs)	107	No association	P = 1.00	Negative correlation expected. Reasonable power
	Females (2-12yrs)	26	No association	Pearson's Correlation. = -0.24, p = 0.24.	A slight trend towards positive correlation. Low numbers
2-Hydroxyhippuric acid	Male ASD patients 2 to 12yrs	121	Mean Diff + 0.97 mmol/mol creatinine	P < 0.001	Against population norms
	Female ASD patients 2 to 12yrs	28	Mean Diff + 1.87 mmol/mol creatinine	P = 0.027	Against population norms
Age vs. 2-Hydroxyhippuric Acid	Male ASD patients 2 to 12yrs	118	No correlation	Pearson's correlation = -0.03, p = 0.73	Against expected association
	Female ASD patients 2 to 12yrs	28	No correlation	Pearson's correlation = 0, p = 0.998	Limited numbers
	Female ASD patients 2 to 7yrs	15	Trend towards a positive correlation	Pearson's correlation = 0.44, p = 0.06	Limited numbers
Age vs Total Number of metabolites	Total ASD cohort	49	Negative correlation	Pearson's correlation = -0.38, p = 0.007	Whilst limited numbers, reducing total number of abnormalities with age

These results suggest both succinic and 2-hydroxyhippuric acid are elevated in patients with autism spectrum disorder versus expected population means. The results also suggest there is a loss of the expected decrease in these urinary metabolites with age.

CHAPTER 5
Microflora Composition via QPCR stool analysis

5.0 Results - QPCR stool analysis in patients with ASD

The composition of microflora was analysed in patients with ASD versus a control group via qPCR stool analysis.

Table 24 Percentage Firmicutes and Bacteriodetes levels on PCR stool analysis and demographics in ASD patients and in unhealthy control patients.

	ASD-Cohort N = 29	Controls N = 7
Age (yrs.)	6.14	7.2
Sex Ratio (M:F)	1.9	2.5
Autism Severity	5.8	N/A
Supplements	45%	43%
Diet	48%	43%
Medication	28%	14%
FIRMICUTES	65.8 (SD 7.8)	54.1 (SD 10.9)
BACTERIODETES	34.2 (SD 8)	45.9 (SD 10.9)
Abdominal Symptoms	67%	6/7 (86%)

Out of the 60 patients selected, 54 had undergone PCR stool analysis at initial assessment and were considered relatively naïve to treatment. 29 of the 54 had reliable record of age, sex, diet, supplements and medications. Seven controls were identified with reliable demographics. Age and sex ratios were reasonably well matched between controls and ASD-patients. In the larger cohort of 54 ASD-patients the sex ratio was 2.8:1 male predominance, less than the expected population ASD demographics, suggesting a modest female over-selection. Of the smaller cohort of 29 ASD-patients the sex ratio was more skewed to a female over-selection (table 24). The age range was good in both cohorts, and representative of a young ASD population. The control group consisted of non-ASD clinic attenders. As such the control group was considered as an unhealthy control group. In the controlled analysis, diet and supplement use were consistent between the control group and ASD-cohort, with greater medication use in the ASD cohort versus controls (28% vs. 14% respectively). Controls were more likely to suffer GI symptoms than the ASD-cohort (86% vs. 67%).

Mean levels of firmicutes and bacteriodetes were calculated for both the ASD-cohort and the control group.

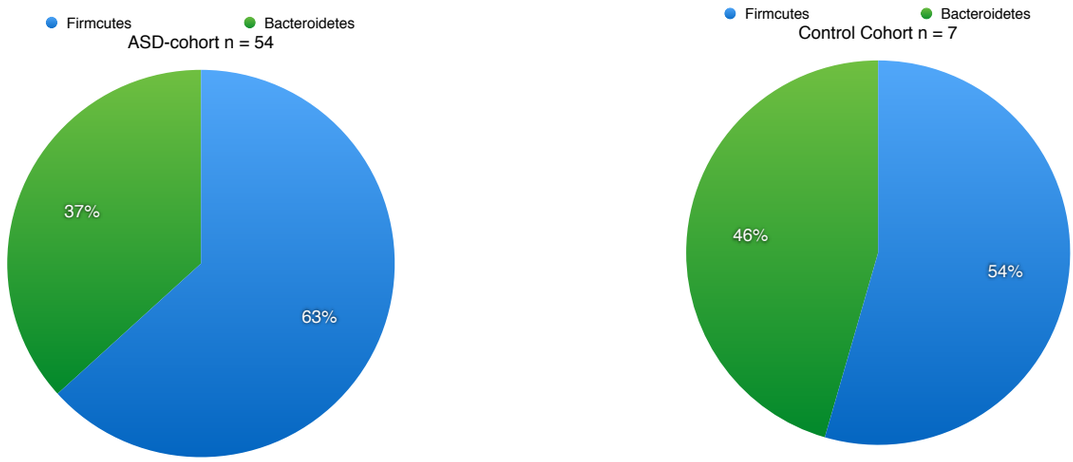

Figure 15 Proportion of firmicutes to bacteriodetes in the (A) ASD cohort (n=54) and (B) the control non-ASD cohort (n = 7). Firmicutes and bacteriodetes percentages calculated from qPCR stool analysis. Firmicutes appears significantly more dominant in comparison to bacteriodetes in the ASD-cohort than in the control cohort.

As indicated in table 24 and represented in Figure 15, firmicutes was higher in the patient group with a mean difference of 8.5 ($p = 0.007$, CI 2.427 to 14.5) and bacteriodetes was lower in the same fashion (raw data is presented in appendix 7).

A further 178 medical records were analysed for PCR stool analysis and, after checking for duplication, combined with the previous cohort (Table 25).

Table 25 Firmicutes and Bacteriodetes as percentage ratio, age and sex in ASD-cohort (n = 147) versus controls (n = 12)

	ASD-Cohort N = 147	Controls N = 12
Age (yrs.)	6.03 (Range 2 to 21)	6.4 (Range 1 to 16)
Sex Ratio (M:F)	4.0	1.42
FIRMICUTES	63.15 (SD 7.8)	54.75 (SD 10.4)
BACTERIODETES	36.85 (SD 8)	45.25 (SD 10.4)

There remained a significant difference in firmicutes to bacteriodetes percentage ratio between the ASD-cohort and the non-ASD control group of 8.6 (SEM 0.707, p = 0.005).

Age was analysed versus firmicutes in both the ASD and control cohorts.

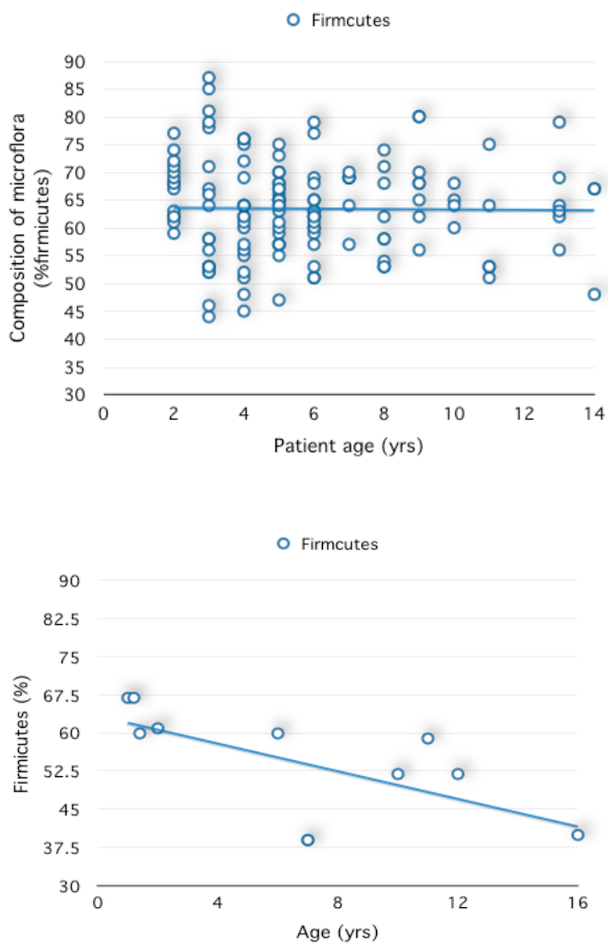

Figure 16 Scatter graphs with best-fit line for firmicutes versus age in the (top) ASD cohort 2 to 16 yrs and (bottom) control patients 2 to 16 years of age. Firmicutes percentage is calculated through qPCR stool analysis and is compared to age at time of sample analysis. Age appears to have no relation to firmicutes percentage in the ASD cohort (Pearsons correlation = -0.014, p = 0.872), whilst the control cohort demonstrates the expected decrease of firmicutes percentage with age (Pearson's correlation = -0.818, p = 0.007).

Figure 17 demonstrates the relationship of firmicutes with age in the control and ASD-cohort. There was no relationship found on parametric or non-parametric analysis between age and firmicutes in the ASD-cohort. The control cohort demonstrated a statistically significant negative correlation between firmicutes and age (Pearson's

correlation = -0.818, p = 0.007).

Both cohorts were analysed for the association between firmicutes and sex. There were no significant differences between male and female patients in relation to firmicutes in the ASD-cohort (pearson's correlation = 0.055, p = 0.511), or in the control cohort (Pearson's correlation = 0.012, p = 0.976).

Age and sex were then analysed within a multi-variant analysis to assess for confounding effects of either on the mean difference between levels of firmicutes in the ASD-cohort and the control cohort. The unadjusted correlation for the mean difference of firmicutes between groups was statistically significant (p = 0.005). The sex and age adjusted correlation remained significant (p = 0.009).

Firmicutes percentage was then analysed versus the presence or absence of abdominal symptoms.

Table 26 Firmicutes, bacteriodetes and other variables in patients with ASD with and without abdominal symptoms

	Without Abdominal Symptoms (n = 9)	With Abdominal Symptoms (n = 20)
Age (yrs.)	5.8	6.2
Sex Ratio (M:F)	2.25	3.5
Severity	5.0	6.2
Supplements	78%	60%
Diet	67%	60%
Medication	11%	40%
Firmicutes	65.5	68.6
Bacteriodetes	35.4	31.4

Of the 29 patients with reliable recordings of other variables, 20 ASD-patients had evidence of GI symptoms and 9 did not. Comparing the groups, the age and diet use was similar in both. There was a higher proportion of male patients in the GI symptom group. The non-GI symptom group had more supplementation use than the GI symptom

group (78% versus 60%). There was greater severity of ASD symptoms in the GI symptom group (table 26). Medication use was significantly higher in the GI symptom group. In this small cohort there were no statistically significant differences between the composition of firmicutes to bacteriodetes in the GI-symptoms ASD group and the non-GI symptom ASD group (see table 27).

Table 27 Parametric analysis of firmicutes and other recorded variables.

		Age	Sex	Severity	Abdominal Symptoms	Supplements	Diet	Meds
Firmicutes	Pearson Correlation	-0.01	0.06	-0.08	-0.21	-0.06	-0.04	-0.09
	Sig. (2-tailed)	0.87	0.51	0.69	0.30	0.77	0.83	0.65
	N	144	144	28	28	27	27	28

These results taken together suggest a significant compositional difference in the proportion of the two main bacterial phyla in patients with autism spectrum disorder, and these differences were not explained by diet, supplement or medication use.

Chapter 6
Autonomic Function in patients with ASD

6.0 Results - Autonomic Function in ASD

Autonomic function was examined in relation to microflora and inflammatory measures in patients with ASD.

Table 28 Autonomic Parameters (Vagal Tone, Mean Arterial Pressure and Heart Rate), immunological variables (ESR & Platelet Count) and microflora composition marker (microflora) in patients with ASD .

	N	Mean Value
Age *(Years)*	45	7.4 (Range 3-19)
Vagal Tone *(Linear Vagal Scale)*	45	10.46 (Range 1.25 – 38)
Mean Arterial Pressure *(mmHg)*	38	68.2 (Range 48 – 108)
HR *(Beats Per Minute)*	34	95 (Range 71 – 174)
ESR *(mm/hr)*	31	8.4 (4 – 30)
Platelet Count *(x10^9/L)*	31	323 (Range 233 – 472)
Firmicutes *(% versus bacteriodetes)*	13	66.5 (Range 54 – 80)

45 autonomic profiles were extracted from the case files of patients with ASD attending outpatient clinics. Of the 45 cases identified, 38 had Mean Arterial Pressure (MAP) recorded, 34 had Heart Rate (HR) recorded, 31 had ESR results, 31 had platelet counts and 13 had qPCR stool analysis. The average age was 7.4 yrs with a range of 3 yrs to 19 yrs indicating a predominantly young ASD-population. Average vagal tone was 10.48 with a broad range of 1.25 to 38. The average ESR was 8.4 mm/hr. Mean Adiposity Index (as % firmicutes) was 66.5 in-keeping with the mean levels reported in chapter 5.

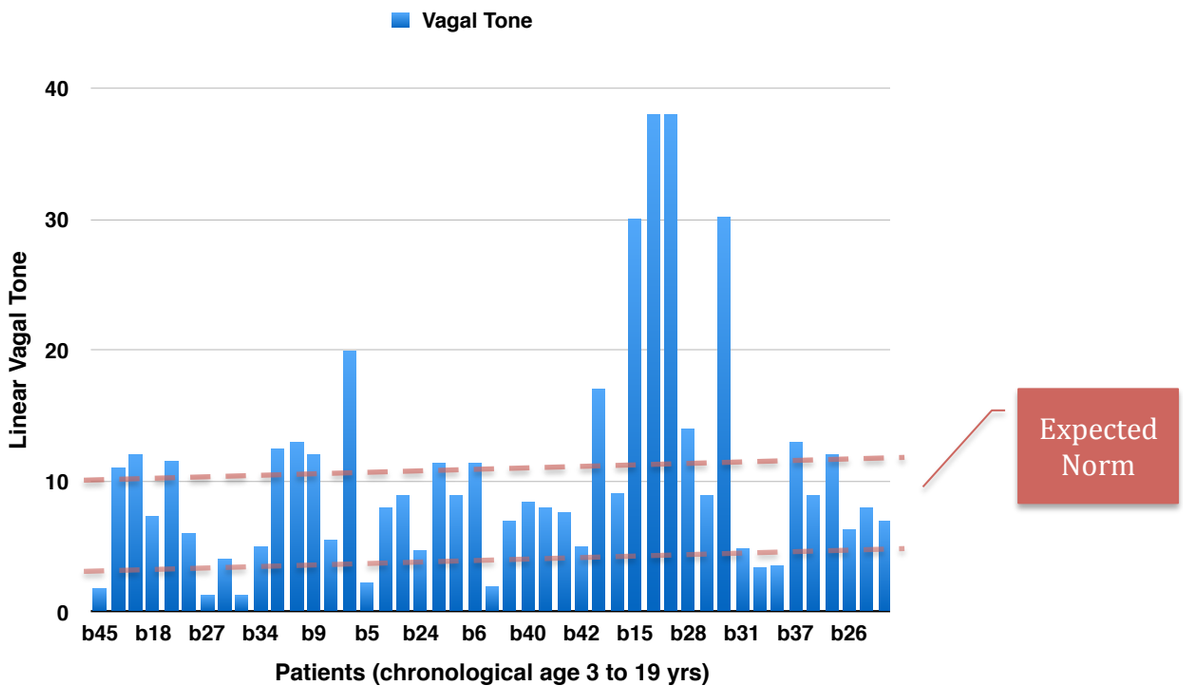

Figure 17 Vagal Tone in 45 patients with ASD presented as a bar chart with increasing age from left to right. The expected normal range is presented by the red dotted lines. Vagal tone is calculated through monitoring heart rate and blood pressure variability in real time.

Vagal tone (as measured on the Linear Vagal Tone scale) was markedly varied. 20 out of 45 were out-with the expected norms with no discernable pattern. Age was then plotted against vagal tone.

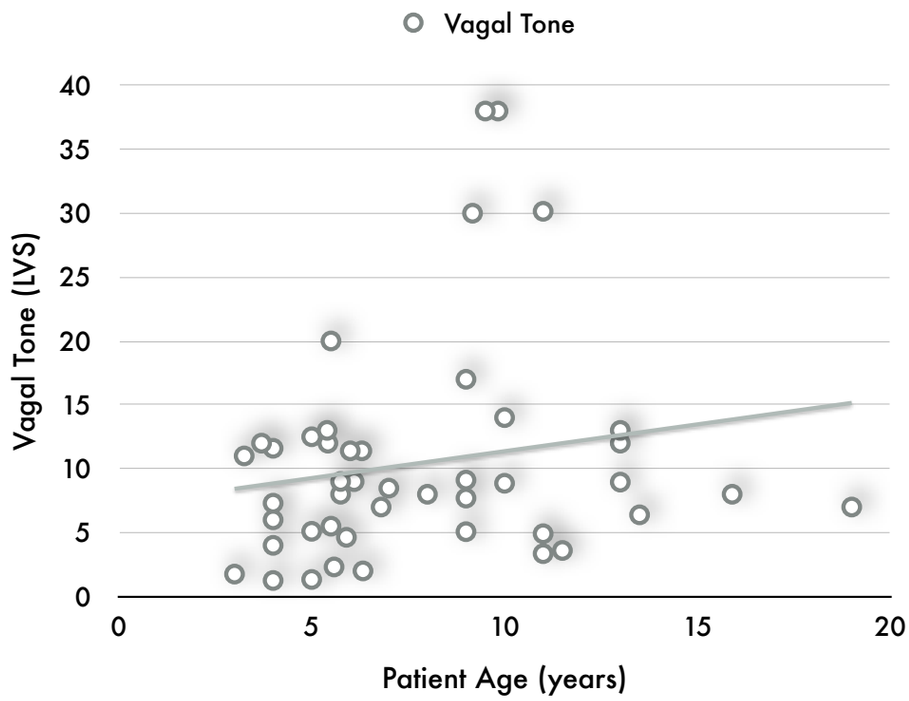

Figure 18 Vagal tone against Patient Age in ASD patients. Vagal tone is measured through continuous monitoring of heart rate variability and blood pressure monitoring.

The trend line indicated the expected normal increase of vagal tone with age, but this did not reach significance on statistical analysis (Pearson's correlation = 0.178, p = 0.242).

There were only 3 female patients indicating an over-representation of male patients. It was not feasible to examine for a relationship between vagal tone and gender.

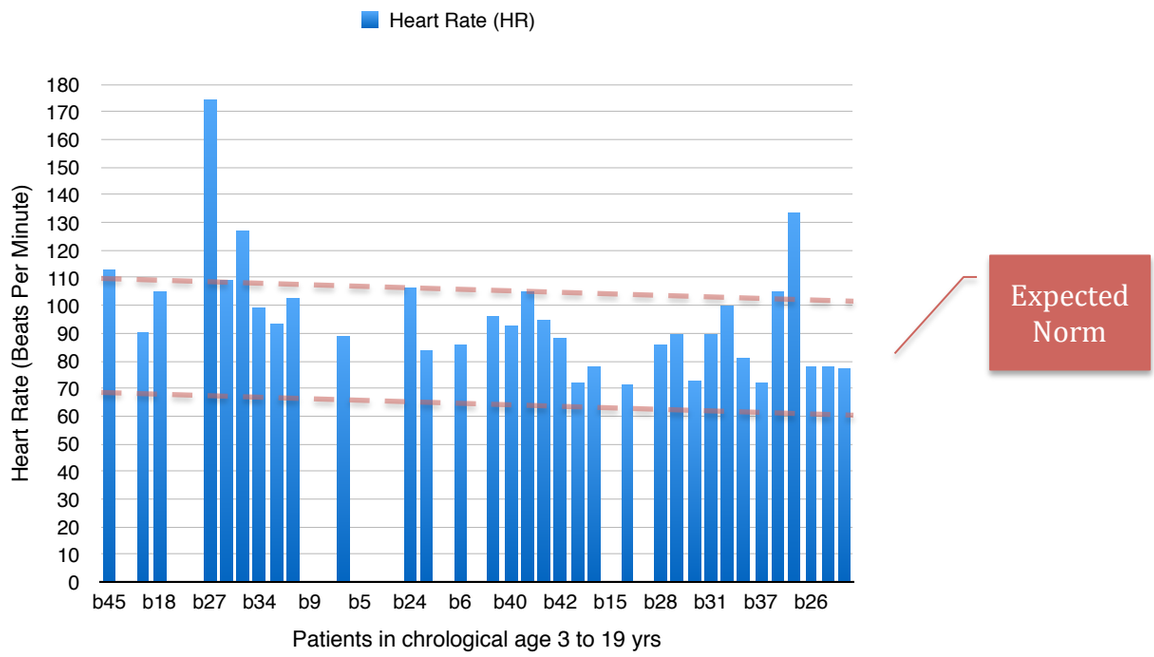

Figure 19 Heart Rate in patients with ASD presented as a bar chart with increasing age from left to right. The red dotted lines indicate the expected normal range of heart rate.

Average Heart Rate was also variable, but less so than vagal tone (Figure 20). Only three patients were out-with the expected norm for age.

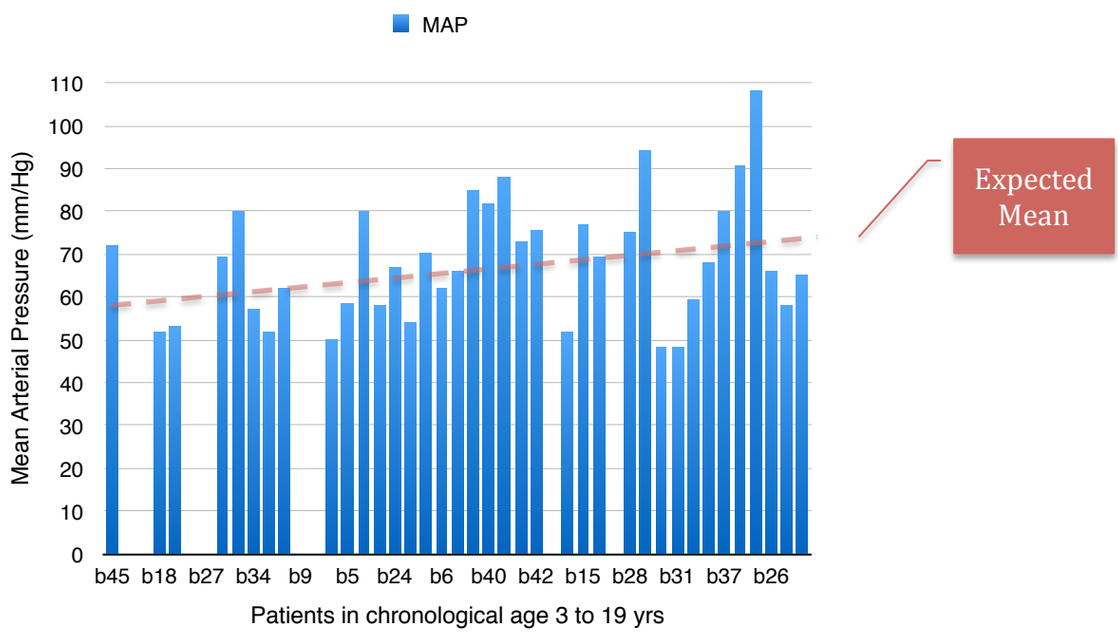

Figure 20 – Mean Arterial Pressure (MAP) in patients with ASD presented as a bar chart with increasing age from left to right. The red dotted line indicates the expected normal range in MAP.

Mean Arterial Pressure (MAP) is difficult to determine in children. Consensus of opinion is that MAP relates to height and age. Assuming an average height at the 50th percentile, the MAP shows modest variation against expected norms (figure 21).

Vagal Tone was further analysed against ESR, platelets and Adiposity Index.

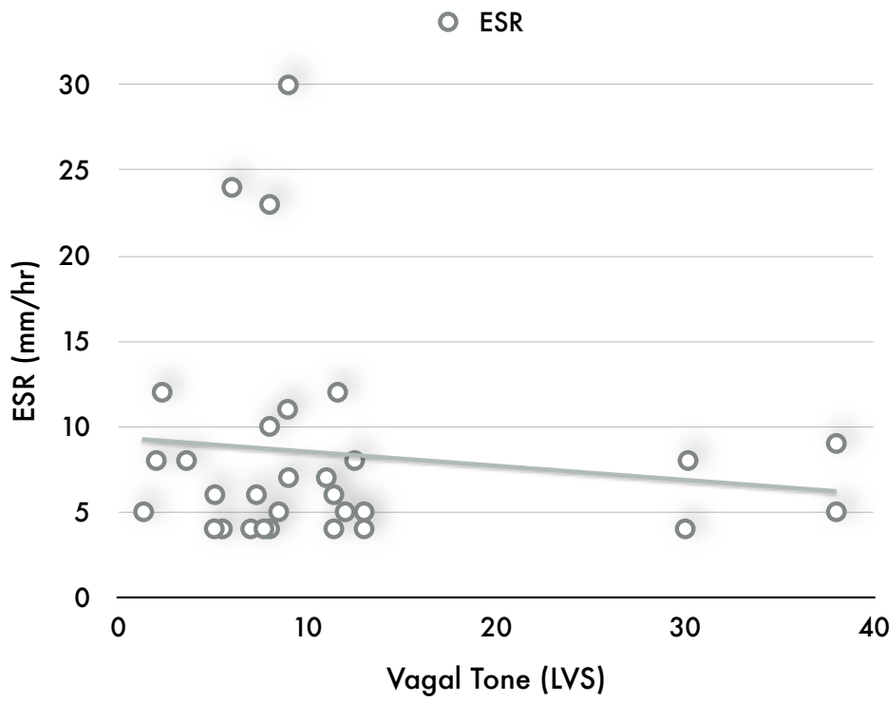

Figure 21 ESR plotted against Vagal Tone in patients with ASD (n = 31). ESR is calculated by measuring the rate of separation of whole blood over the period of an hour, and is represented as millimetres per hour – higher levels indicating higher levels of inflammation. Vagal tone is calculated through measuring of heart rate variability. ESR = Erythrocyte Sedimentation Rate

There initially appeared to be a trend towards increasing vagal tone correlating with decreasing ESR (Figure 22).

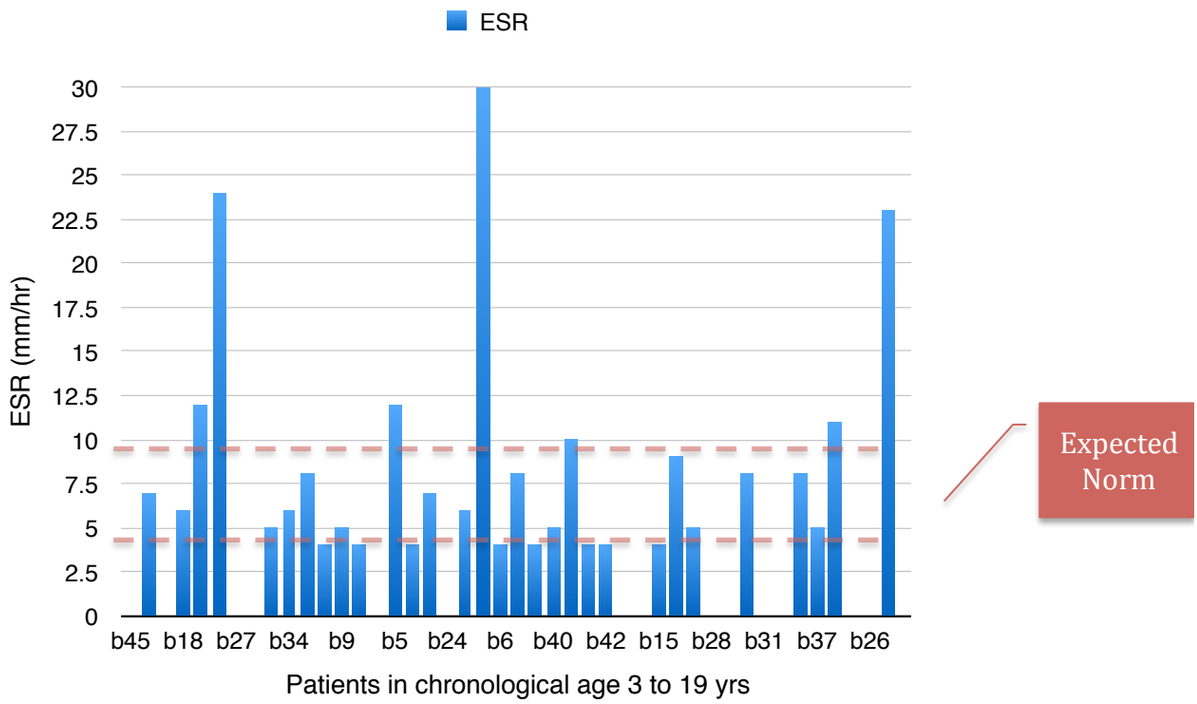

Figure 22 ESR levels in ASD patients with increasing age from left to right. ESR is calculated by measuring the rate of separation of whole blood over the period of an hour, and is represented as millimetres per hour – higher levels indicating higher levels of inflammation. The red dotted lines indicate the expected normal range of ESR.

ESR was generally within the expected norm (figure 23), and hence was analysed without the greatest three outliers.

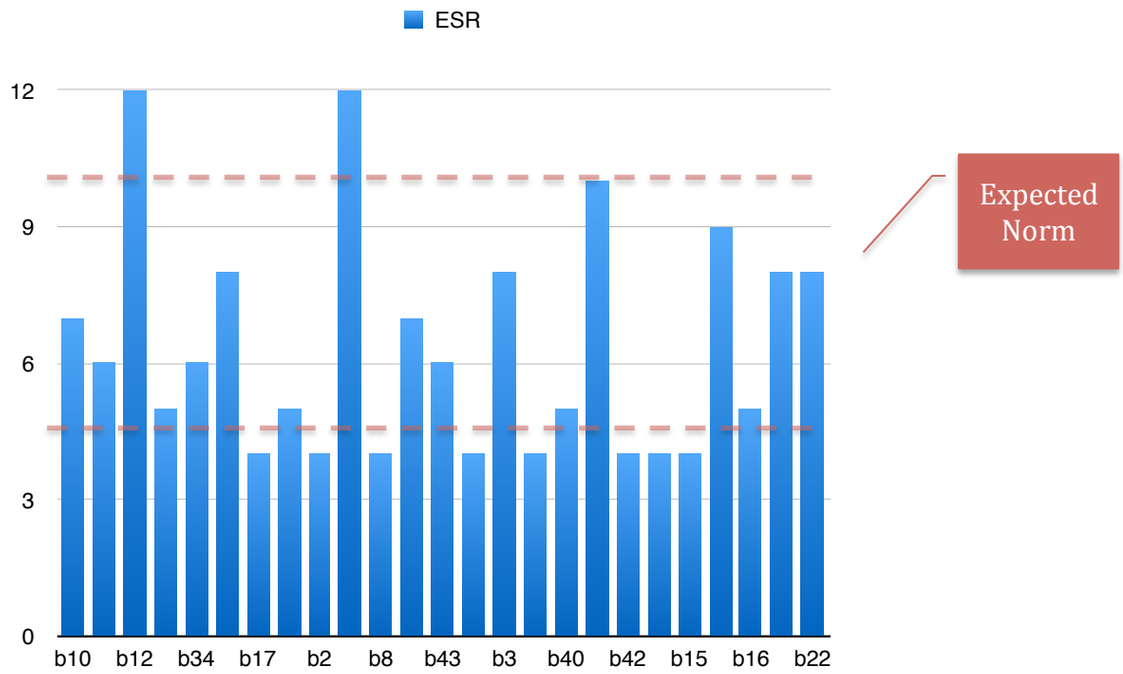

Figure 23 ESR levels in ASD patients with increasing age from left to right without three outliers. ESR is calculated by measuring the rate of separation of whole blood over the period of an hour, and is represented as millimetres per hour – higher levels indicating higher levels of inflammation. The red dotted lines indicate the expected normal range of ESR.

Removing the three outliers led to a relatively normal ESR distribution across the ASD-group (Figure 24).

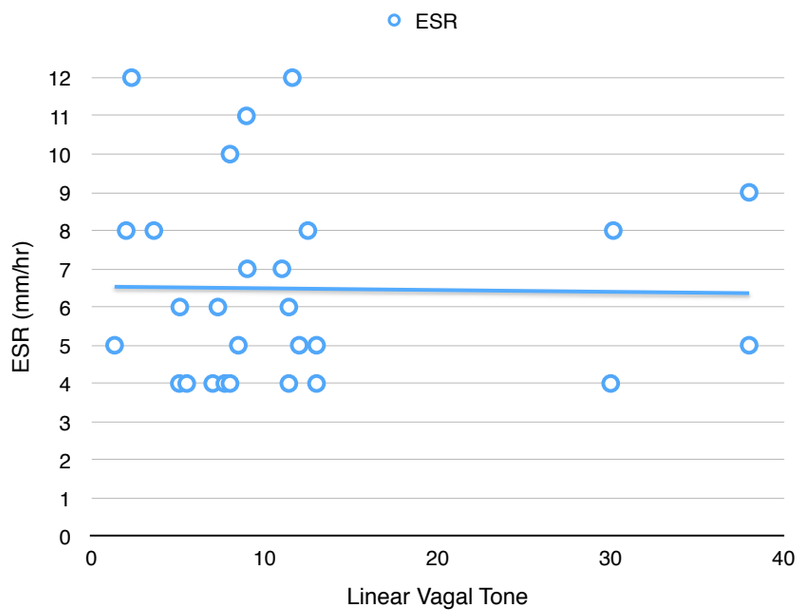

Figure 24 - ESR plotted against Vagal Tone in patients with ASD (n = 28) with 3 outliers removed. ESR is calculated by measuring the rate of separation of whole blood over the period of an hour, and is represented as millimetres per hour – higher levels indicating higher levels of inflammation. Vagal tone is calculated through measuring of heart rate variability. ESR = Erythrocyte Sedimentation Rate.

With the three outliers removed the suggested association between vagal tone and ESR on scatter graph was reduced further (Figure 25).

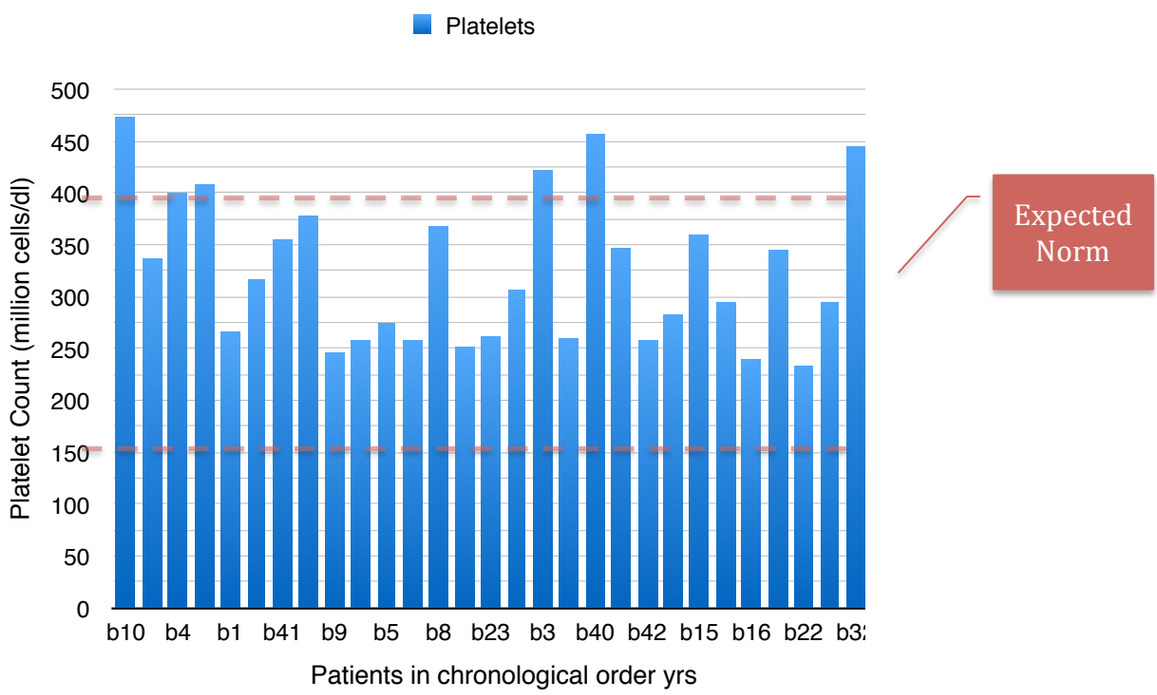

Figure 25 Platelet Count in ASD patients represented on bar chart with increasing age from left to right. Platelet Count is calculated by counting the number of platelets per field of vision under high-powered magnification and is automated.

Platelet count was within normal range (Figure 26).

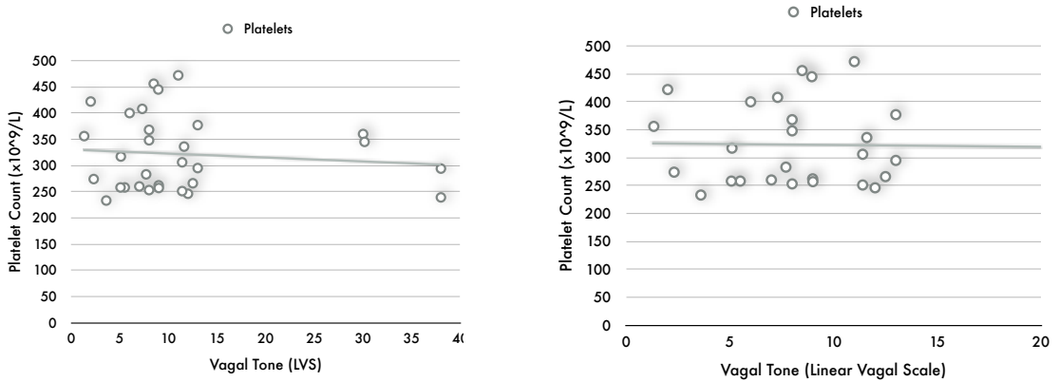

Figure 26 Platelet Count versus Vagal Tone in ASD patients (left) in full cohort (n=31) and (right) without four outliers (n = 27). Platelet Count is calculated by counting the number of platelets per field of vision under high-powered magnification and is automated. Vagal tone is calculated by continuous monitoring of heart rate variability.

As indicated in figure 27 there was no significant relationship identifiable between platelet count and vagal tone in patients with ASD.

Vagal Tone was analysed against adiposity index (% firmicutes) in 13 patients. Two patients were excluded at the extremes of the adiposity range (see table 29 – excluded patients highlighted in grey).

Table 29 Variables of 13 patients with ASD who had underwent both autonomic profile and adiposity index. MAP = Mean Arterila Pressure, HR = Heart Rate, ESR = Erythrocyte Sedimentation Rate.

Patient ID	Age	Sex	Vagal Tone	MAP	HR	ESR	Platelets	Adiposity Index
b1	5	0	12.5	52	93	8	266	54
b2	5.5	1	5.5			4	258	60
b3	6.33	1	2	66		8	422	63
b4	4	0	6			24	400	65
b5	5.58	1	2.31	58.4		12	274	65
b6	6.3	1	11.4	62	86	4	306	66
b7	19	1	7	65	77			66
b8	5.75	1	8	80		4	368	67
b10	3.25	1	11			7	472	68
b9	5.42	1	12			5	246	68
b11	6.8	1	7	85	96	4	260	70
b12	4	1	11.6	53		12	336	73
b13	9	1	7.7	72.6	95	4	283	80

Adiposity index (as firmicutes %) was then plotted against Vagal Tone (figure 28).

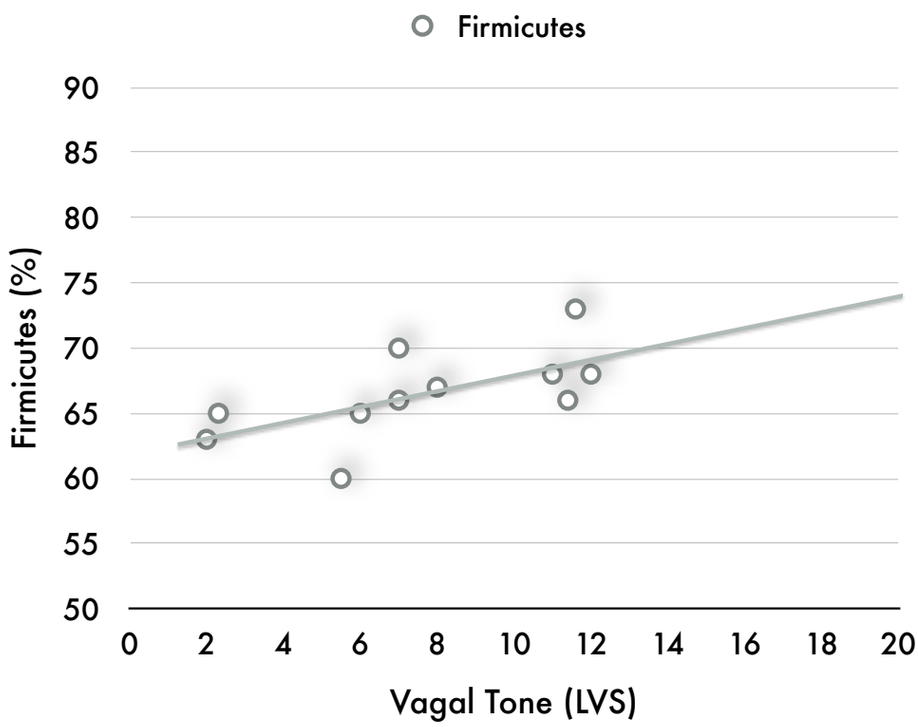

Figure 27 Firmicutes versus Vagal Tone in ASD patients plotted on a scatter graph with best-fit line (n = 11). Firmicutes is calculated through qPCR stool analysis. Vagal Tone is calculated through measurement of heart rate variability. Two outliers have been removed.

Plotted on scatter graph there appeared to be a trend towards a correlation between vagal tone and firmicutes percentage, but there was no correlation identified (Pearson's correlation = 0.067, p = 0.827, n = 13). Removing one outlier (patient b1 in table 28) brought the non-parametric analysis into significance (Spearman's Ro = 0.695, p = 0.012, n = 12). Removing one further outlier (patient b13 in table 28) brought both parametric and non-parametric analysis into significance (Pearson's correlation = 0.630, p = 0.038, n = 11; Spearman's Ro = 0.782, p = 0.004, n = 11).

Taken together these results suggest vagal tone may correlate with the composition of the two main phyla of gut bacteria, specifically a higher proportion of firmicutes leading to a higher vagal tone.

CHAPTER 7
Zinc Deficiency in Autism

7.0 Results - Zinc Deficiency in Autism

Zinc was examined as a potential environmental modifiable factor associated with ASD. After applying exclusion criteria there were 72 ASD cases and 234 controls. In the ASD group mean age was 7.0 yrs. of age (range 2-16) and in the control group was 10.1 yrs. of age (range 2-16). Male to female ratio in the ASD group was 3.8 and in the controls was 1.3 (Appendix 9 & 10).

Table 30 Means and standard deviation of plasma elements and white cells in ASD-Cohort

Cases Means and Standard Deviation					
	N	Minimum	Maximum	Mean	Std. Deviation
WCC (x10^9/L)	72	2.65	20.25	8.26	3.17
Lymph (x10^9/L)	72	0.15	9.58	3.69	1.91
Mono (x10^9/L)	72	0.24	1.17	0.55	0.19
Neurto (x10^9/L)	72	0.70	12.98	3.65	1.75
Eosino (x10^9/L)	72	0.00	1.24	0.30	0.28
Baso (x10^9/L)	72	0.01	0.25	0.07	0.05
Plasma Cr (x10^9/L)	72	8.8	40.4	15.258	4.7426
Plasma Mn (x10^9/L)	71	6.9	37.8	14.689	5.7126
Plasma Zn (x10^9/L)	71	6.80	13.50	10.0114	1.51796

In the ASD-cohort, mean values of the other micronutrient levels measured here (chromium and manganese) were all within normal range. Mean White Cell Count and differential were within the normal range, but lymphocytes were at the lower end of the normal range. Mean zinc levels were low at 10.01 umol/l (SD 1.52 umol/l) with a maximum level of 13.5 umol/l (Table 30).

Table 31 **Means and standard deviation of plasma elements in control group.**

Controls Means and Standard Deviation					
	N	Minimum	Maximum	Mean	Std. Deviation
Plasma Cr (umol/l)	234	5.1	95.1	16.415	7.0787
Plasma Mn (umol/l)	234	6.50	40.90	14.4822	4.92936
Plasma Zn (umol/l)	234	7.70	21.20	11.7632	2.14467

The mean level of all micronutrients analysed in the control group were within normal range, albeit zinc was at the low end of normal at 11.76 umol/l (SD 2.14 umol/l) with normal reference range 11.5 umol/l to 20 umol/l (Table 31).

Table 32 . **Comparative analysis between controls and ASD-cohort for zinc, manganese and chromium mean difference**

		t-test for Equality of Means				
		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Plasma Zinc (umol/l)	Equal variances assumed	.000	1.75175	.27332	1.21390	2.28960
	Equal variances not assumed	.000	1.75175	.22828	1.30098	2.20252
Plasma Manganese (umol/l)	Equal variances assumed	.766	-.20651	.69385	-1.57188	1.15886
	Equal variances not assumed	.784	-.20651	.75065	-1.69513	1.28211
Plasma Chromium (umol/l)	Equal variances assumed	.195	1.1571	.8905	-.5952	2.9093
	Equal variances not assumed	.113	1.1571	.7256	-.2750	2.5891

The ASD-cohort had a mean plasma zinc level of 10.01 umol/L (SD 1.52 umol/L) and the case controls had a mean plasma zinc level of 11.76 umol/L (SD 2.14 umol/L). The ASD-cohort had a significantly reduced plasma zinc level than controls (Mean Difference = 1.75 umol/L, $p < 0.0001$ CI 1.2 to 2.3). The results withstood correction

for age and sex. There were no significant differences between ASD and controls in relation to Manganese or Chromium (Table 32).

Table 33 Parametric analysis of serum zinc versus differential white cell count in ASD-cohort. Lymp = Lymphocyte, Mono = Monocyte, Neutro = Neutrophil, Eosino = Eosinophil, Baso = Basophil, Zn = Zinc

Correlations Zinc vs. Differential White Cell Count

		Lymph	Mono	Neurto	Eosino	Baso
Plasma Zn (patients > 10.5 umol/l)	Pearson Correlation	.373*	.411*	.183	-.122	-.376*
	Sig. (2-tailed)	.035	.019	.315	.506	.034
	N	32	32	32	32	32
Plasma Zn (All patients)	Pearson Correlation	-.015	-.023	-.085	.044	-.240*
	Sig. (2-tailed)	.896	.849	.474	.712	.040
	N	74	74	74	74	74
Plasma Zn (patients < 10.5umol/l)	Pearson Correlation	-.120	.000	-.057	.198	-.163
	Sig. (2-tailed)	.454	1.000	.724	.214	.308
	N	41	41	41	41	41

*. Correlation is significant at the 0.05 level (2-tailed).

Mean lymphocyte count in the ASD cohort was 3.68 x10^9/L (SD 1.6). There was a significant correlation between total lymphocyte count and plasma zinc levels when zinc was over 10.5umol/l in the ASD cohort ($p < 0.04$). When zinc fell below 10.5 umol/L there was no direct correlation with lymphocyte count, although lymphocyte count was lower generally at a mean of 3.23 x10^9/L (SD 1.8). Lymphocyte counts were not available for the control group. Therefore the largest group (under 8yrs of age, n = 43) were analysed against population means. Mean lymphocyte count in the total under 8 group was 4.23 x10^9/L (SD 2.06) and this was significantly lower than the population mean of 5.0 x10^9/L (SD 1.65) (mean difference -0.767, CI -1.36 to -0.17, $p = 0.012$). In the low zinc group (n = 26) the mean lymphocytes were 4.11 x10^9/L (SD 2.03) (comparing to population means, the mean difference = -0.89, CI -1.65 to -0.13, $p = 0.023$). In the above 10.5 umol/l zinc group (n = 17) mean lymphocytes were 4.51 x10^9/L (SD 2.21), and this was not significantly lower than the population mean ($p = 0.37$) (see Table 33).

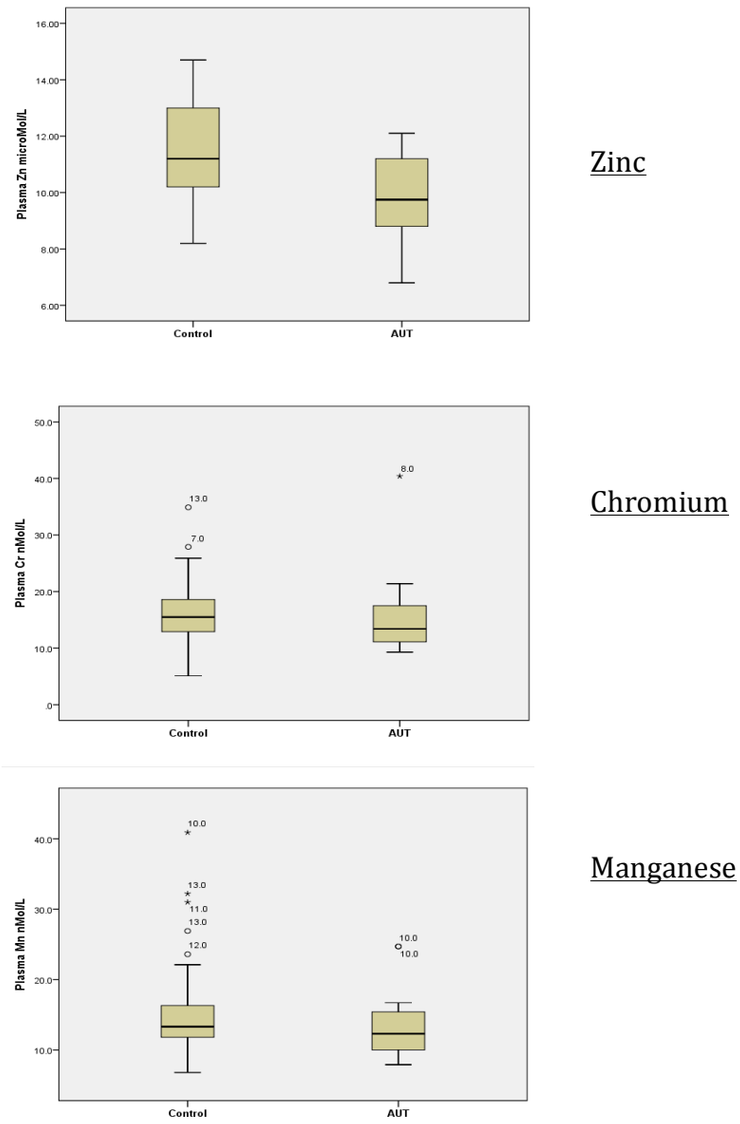

Figure 28 Box-whisker plots of serum zinc, chromium and manganese levels in ASD group (n=72) and control group (n=234).

Box whisker plots show the distributions of zinc, chromium and manganese in the ASD-group in comparison to controls, with visible differences in the zinc group versus the chromium and manganese groups (figure 29).

Confounders

Where reliable data was present, statistical analysis was undertaken for other variables that may have an impact on zinc status, such as age, sex and nutritional factors. There were no significant differences with age or gender in either the ASD-cohort or control group. Diet and supplementation were analysed for possible affects on zinc or white cell counts. The findings are summarised in table 34 (detailed statistical analysis can be found at appendix 10).

Table 34 Comparison of zinc status (and other plasma minerals and white cells) in ASD patients adhering to a gluten-free diet (n=22) and ASD patients not following a gluten-free diet (n=19), dairy-free diet (n = 24) and no dairy-free diet (n = 17), and those on supplements (n = 18) and those not on supplementation (n = 23). WCC = White Cell Count

	Gluten-free (n=22)	Not Gluten-free (n=19)	Dairy-free (n = 24)	Not Dairy-free (n = 17)	Supplements (n = 18)	No Supplements (n = 23)
WCC (x10^9/L)	8.89 (3.74)	8.14 (3.80)	8.63 (3.87)	8.42 (3.94)	9.13 (3.87)	8.09 (3.65)
Lymphocyte Count (x10^9/L)	4.68 (2.55)	3.02 (1.63)	4.34 (2.66)	3.31 (1.57)	4.59 (2.49)	3.38 (2.05)
Zinc (umol/l)	9.91 (1.61)	9.74 (1.45)	9.75 (1.51)	9.95 (1.59)	9.95 (1.74)	9.75 (1.38)
Manganese (umol/l)	17.12 (6.55)	12.37 (3.83)	17.11 (6.60)	11.79 (2.59)	17.72 (7.56)	12.96 (3.29)
Chromium (umol/l)	15.40 (3.63)	13.84 (3.80)	15.42 (3.60)	13.62 (3.79)	14.87 (2.72)	14.54 (4.44)

Of the 72 patients in the ASD-cohort, 41 patients had reliable medical records of whether the patient was or was not on a gluten-free diet. Of these, 22 patients were at the time of blood analysis maintaining a gluten-free diet, 19 were not. Zinc did not differ significantly between the two groups (Gluten-free diet = 9.96 umol/l (SEM 0.36), no gluten-free diet = 9.74 umol/l (SEM 0.36)). Lymphocyte count was higher in the gluten-free group versus the non-gluten free group (4.68 x10^9/L, SEM 0.54 vs. 3.02 x 10^9/L, SEM 0.37).

Of the 72 patients in the ASD-group, 41 patients had reliable record of presence or absence of a dairy-free diet. 24 patients were on a casein-free diet at blood analysis. Zinc did not differ significantly between the dairy-free and non-dairy free groups (9.75 umol/l SEM 0.31 vs. 9.95 umol/l SEM 0.4). Again lymphocyte count was higher in the dairy-free group than the non dairy-free group (4.34 x10^9/L, SEM 0.34 vs. 3.31 x10^9/L, SEM 0.38).

Of the 72 patients in the ASD-cohort, 41 had reliable record of supplement usage. 23 were taking nutritional supplementation. Zinc levels did not differ greatly between the supplemented and non-supplemented groups (9.95 umol/l SEM 0.42 vs. 9.75 umol/l SEM 0.29). Plasma manganese was significantly higher in the supplemented group (17.72 umol/l SEM 1.83 umol/l vs. 12.96umol/l SEM 0.69 umol/l).

Taken together these results demonstrate zinc deficiency is common in patients with autism versus age and sex-matched controls, and such differences were not explained by supplement use or diet.

CHAPTER 8
Discussion

8.0 Discussion

Variable Insult Model of ASD

The literature review is presented in chapter 1 'Introduction to autism'. Socialisation and speech are complex neurological functions. ASD may represent impairment in any system/faculty required to facilitate such complex neurological functions. There is likely ample opportunity for an environmental insult to disrupt one or more of the mechanisms leading to the impairment of the basic and higher order processes of social integration and along the way disrupt a number of other physiological mechanisms that may contribute or indeed cause the additional and variable behavioural manifestations within the spectrum. As troublesome as the notion may be, and granted there will be reasonable pathophysiological correlations identified particularly within sub-groups, the greatest commonality in ASD may be aetiological, and even then it may merely be a trend in human-environment relationship versus an exact noxious substance.

A number of publications have highlighted the possibility of variable types of insults conferring varying phenotypes of autism: e.g. Dietert et al., Hertz-Picotta et al, Goines and Ashwood 2013, and Unwin et al.,[15,79,240,241]. Such hypotheses are critical to providing a framework for identifying sub-types within the ASD group, building in some predictability both for researchers and for those faced with clinical management. Such sub-typing can be based on the dominant system pathophysiology involved or one can attempt clinical classification. Table 35 is an example of such an attempt at clinical sub-typing based on The Variable Insult Model of ASD.

Table 35 Broad clinical sub-categories of ASD. The number of crosses indicates the severity/frequency of each measure relative to exposure timing and duration. Early Acute Insult refers to a sudden or relatively sudden, usually marked exposure to xenobiotic, infection or other environmental stressor during the prenatal, antenatal and early infancy period. Early Chronic Insult refers to a sustained usually moderate level exposure over a period of months during the prenatal, antenatal and/or early infancy period. Late Acute Insult refers to a sudden or relatively sudden, usually marked exposure to xenobiotic, infection or other environmental stressor in infancy or early childhood. Late Chronic Insult refers to a sustained moderate level exposure over a period of months during infancy or early childhood.

	Early Acute Insult	Early Chronic Insult	Late Acute Insult	Late Chronic Insult
Congenital Abnormalities	++++	+++	+	+
Severe Dysmotility	++++	+++	++	++
Sudden Regression	+	++	++++	+++
Insidious Regression	++	+++	+	++++
Early Immune Related issues	+++	++++	++	++
Motor Delays	++++	+++	+	+
Family History of Autoimmunity	++	++	++++	+++
Prenatal, Gestational or Neonatal Exposure	++++	+++	+	+
Exposure in Infancy/Early Childhood	+	++	++++	++++

The sub-categories presented in Table 35 are broad and overlap considerably. More specific sub-typing seems probable, perhaps relating to the intensity of the insult and perhaps more specifically to the offending agent. Already abnormal RNA transcription has been identified in ASD children correlating with environmental toxicants versus controls with similar levels of toxicants[16,82]. The transcription abnormalities are specific to the toxicant, raising the possibility of different aetiological agents triggering different initial pathophysiological mechanisms sharing only the secondary consequences. The factors involved in the different gene expression in ASD, whether they be linked to genomic individuality, previous exposure, some kind of immunological priming, or

abnormal GI flora, raise interesting questions, but in these current considerations the mere difference in RNA transcription between different xenobiotics and also between ASD patients versus control groups raises important questions about accurate delineation of sub-types and the different pathophysiological mechanisms involved in the eventual ASD outcome.

Supported by the literature review presented in chapter 1, variable pathophysiological pathways leading to ASD seem likely, and recent advancements in scientific techniques provide the capacity to differentiate each pathway with relevance to the prevention and clinical management of the condition. For example, PCR analysis of GI microflora continues to reveal deeper insights into the common GI abnormalities prevalent in ASD[32–34]. Evidence demonstrates the importance of such microflora on immune and neurological function, and the evolution of GI microflora composition over the first few years of life[143,242]. The compositional dysbiosis discovered by Williams et al., in ASD patients may reflect another common manifestation of ASD due perhaps to similar complexities as is involved in social integration with genomic, neurological, immunological and neuroimmunological systems required to select and regulate the GI microflora. This may explain the frequently reported presence of abnormal species in ASD and the diversity of such abnormal microflora/pathogens - the selection and regulation processes are also part of the developmental process and are vulnerable to a variety of insults at a variety of levels. ASD diagnosis may be more scientifically sound should it move towards a formulation including environmental exposures, genomic vulnerability and the identification of the system(s) pathophysiology with treatment interventions based on such measurable criteria. Animal models can serve such ever increasing sub-categorisation, modelled to reflect each category and utilised to identify novel therapeutic interventions at specific groups.

The Variable Insult Hypothesis predicts diverse immune related abnormalities, probability of poor GI microflora regulation, and variable autonomic function with impaired/disordered autonomic reactivity and variable neuroanatomical findings. Further, The Variable Insult Model predicts that ASD animal models could be established through a variety of mechanisms. Valproate with an acute single dose administered gestationally at E12.5 leads to ASD symptomology in rats[243], as does valproate administered in a sub-chronic dosing given between post-natal days P6 - P12, albeit the sub-chronic dosing may have more sensory issues[244]. In another transgenic model of ASD butyl paraben was used. The ASD butyl paraben model (a chronic dosing administered between gestational day 1 and lactation day 21) demonstrated

similar social deficit in the stranger and exploration tests as the single insult valproate model. Whilst the butyl paraben model displayed such features consistent with an autism phenotype, there were significant differences to the valproate model, for example the butyl paraben model had significantly less impairment in the spatial recognition and memory tests in comparison to the Single acute dose valproate model of ASD. BDNF, amino acids and neurotransmitter abnormalities were present in both models in the same direction, but the abnormalities remained significantly different between models[245]. A number of other methods are utilised to generate transgenic ASD-like syndromes, including the maternal immune activation models[246].

Unwin et al proposed that homogeneity may be found by identifying and subtyping based on the aetiological agents, and presents the differences in associative symptomology in children with ASD who had either perinatal exposure to selective serotonin re-uptake inhibitor (SSRI) or who had low birth weight (LBW)[241]. The SSRI group seemed to have more gastrointestinal disturbance, although it wasn't clear whether this was a depression effect or drug effect, and the LBW group had more sleep and breathing disturbance. Within the Variable Insult Model of ASD, SSRI exposure may represent greater disruption to the peripheral autonomic nervous system, perhaps during neural crest formation and development, whereas the LBW group may have suffered more pronounced central autonomic disruption. Difficulties with sensory processing may then affect both groups through different mechanisms with similar social outcomes.

If we start to look at the aetiological agents contributing to the development of ASD, then perhaps we can start to find sub-groups. Such an approach brings more complexity to the clinical assessment of patients suffering ASD but perhaps such a complex condition requires greater effort and more complex formulations prior to predicting prognosis and response to medical or behavioural management. If we go further still and delineate the various pathophysiologies that can lead to ASD symptomology through animal and human experiments, then early identification of the system(s) requiring attention may be possible and may then better guide harm reduction strategies. Prevention remains the priority, but harm reduction through focused scientific investigation could reduce the burden of disease going forward. For example, in Unwin et al's., SSRI group early intervention relating to GI pathology could allow correction of the dysfunctional peripheral autonomic input and thus permit greater sensory integration, and thus improved developmental outcome. Equally addressing the breathing dysrhythmias in the LBW group early may improve autonomic reactivity improving

both sensory integration and neuroimmune responsiveness.

The Variable Insult Model would be nullified should a specific causation or genetic abnormality be found. This remains feasible, and indeed two of the modifiable environmental factors examined as part of this project – zinc deficiency and microflora – could represent single aetiological agents responsible for the development of autism. As it stands, based on the literature reviewed, The Variable Insult Model of autism remains a useful working model.

Microflora and autism

Urinary Metabolomics holds real promise as a potential biomarker for ASD. In the current cohort 90% of treatment naïve patients with confirmed ASD had at least one abnormality on a limited urinary organic acid profile. In the limited cohort (n = 49), against population means, succinic acid and 2-hydroxyhippuric acid were significantly elevated ($p = 0.007$ & $p = 0.001$ respectively), and either was raised in 79.1% of patients between 2 and 12yrs of age. These results suggest this limited urinary screen has a potential sensitivity for detecting autism of 92%, and just succinic acid and 2-hydroxyhippuric acid carries a 79.1% sensitivity in 2 to 12 yr. olds.

The specificity could not be determined on this study due to the lack of a control group. Equally it remains unknown whether such abnormalities are present early on in the disorder or indeed precede the visible autism symptomology, although it is worth noting that either succinic acid and/or 2-hydroxyhippuric acid were elevated in 80% of the two year olds in the initial cohort (n = 10), and either hippuric acid or propionic acid were raised in the other two cases (appendix 4). Additionally total number of abnormalities were similar between the ASD patients with and without GI symptoms suggesting that these markers may relate to an underlying autism pathophysiology as opposed to a co-morbid GI pathology.

Analysing the larger cohort of (n = 122) for mean levels of succinic acid and 2-hydroxyhippuric acid against population means confirmed the initial results. In the male 2 to 12yrs ASD group the mean succinic acid level was twice that of the expected normal population mean (Mean Difference 14.5 mmol/mol creatinine, $p < 0.0001$, 95% CI 10.1 to 18.8). 2-hydroxyhippuric acid was more than twice the expected mean level in the ASD-cohort (Mean Difference 0.97 mmol/mol creatinine , $p < 0.0001$, 95% CI 0.58 to 1.36). Females showed similar results with succinic acid more than twice the expected mean (mean difference 12.6 mmol/mol creatinine, $p < 0.0001$, 95% CI 7.2 to 18) and 2-hydroxyhippuric acid 2.5 times the expected normal mean level (mean difference 1.87, $p < 0.03$, 95% CI 0.23 to 3.51).

Combined either succinic acid or 2-hydroxyhippuric acid levels were classified as raised (above the 90th percentile of population norms) in over 60% of male patients with ASD aged 2 to 12yrs of age (n = 121).

The reference range of succinic acid is reasonably well established. Guneral et al

investigated levels of organic acids in children with succinic acid showing the following Means[247]:

- Newborn (n = 57) – 197.1 umol/L
- 1-6 months (n = 8) – 185.4 umol/L
- 2-6 yrs. (n = 66) – 10.9 umol/L
- 6-10yrs (n = 14) – 11.6 umol/L
- 10yrs (n = 16) – 7.7 umol/L

The metabolic pathways of succinic acid continues to be delineated, most notably succinic acid is a Krebs cycle metabolite, and as such is produced by both host and microflora. It is not possible to determine based on the limited urinary metabolomics utilized in this present study whether the elevated succinic acid emanates from the host or microflora.

Succinic Acid has been explored as augmentative therapy in viral and bacterial infections[248], to improve absorption of iron [249] and as an anxiolytic and anti-depressant (with sedative qualities) [250]. It has also been postulated to drive inflammation, correlating with damage to the GI mucosa, altered macrophage metabolism and has been associated with disease activity in autoimmune conditions such as UC and Crohns Disease [251]. Succinic acid is also increasingly used in food products as an acidity regulator. Further research into the metabolism and actions of succinic acid seems warranted.

2-Hydroxyhippuric acid has not been studied in any detail. It is considered a microbial derived metabolite, almost certainly a phenolic compound derived from salicylic acid and glycine [252]. It is endogenously produced and whilst no chronic toxicity studies have been produced, acute toxicity occurs over 1000mgs/kg and it seems unlikely 2-Hydroxyhippuric acid at the levels detected in the urine of humans is directly toxic (Data safety sheet). In saying that, 2-hydroxyhippuric acid has not been studied adequately enough to assert any assumptions about its chronic profile or human effects. It does though lend weight to the probability these urinary organic acids are linked to microflora given 2-hydroxyhippuric acid is absent in the serum or urine of patients without colons [252].

Bacterial metabolites emanating from the microflora detectable in the urine have previously been reported to be abnormal in ASD patients versus controls [157,253–255]. The

results presented here, despite the lack of controls and presence of confounders, support the assertion that gastrointestinal bacterial metabolites are higher in patients with ASD and this may have pathophysiological relevance. A larger, controlled trial seems warranted given these findings and may provide greater insight into the association between ASD and gut microflora, and may lead to a useful biomarker for ASD generally. In particular, should microflora abnormalities be involved in the aetio-pathogenesis of ASD, early detection of at least a sub-group of ASD through urinary metabolomics may be possible. A population based, longitudinal study of bacterial metabolites in infancy may be useful going forward.

There are other notable assertions worth considering following the urinary organic acid results discussed above. Reference ranges of the microbial metabolites differ according to sex and age. In the general population females maintain a significantly higher level of all microbial metabolites analysed here beyond the age of 12 yrs. Males demonstrate a fall in all microbial metabolites over time with an often 50% reduction. In this current cohort there was no decrease in mean levels of succinic acid in the male ASD-group aged 2 to 12yrs (n = 107), suggesting perhaps a loss of regulatory function over microflora compositions. However, in the female group (under 7yrs of age) there was a trend towards a positive association between mean 2-hydroxyhippuric acid levels and age, in opposition to the male. Gender differences are pertinent to autism given the marked male predominance. It may be females maintain a higher level of a certain microbial composition or permit a higher level of microflora activity, which could be related to the need for higher immune tolerance in preparation for conception and gestation. Subsequently, the mechanisms insuring a higher microbial composition (in the metabolites analysed here) may provide some protection in females over the acquisition of other microbes into the microbiota. Perhaps then, within the consideration of microflora in the aetio-pathogenesis of autism, the differences in composition between male and female could be a reason for the higher rates of autism in males versus females.

Succinic acid and 2-hydroxyhippuric acid did not correlate significantly with GI-symptoms in the ASD cohort. Raised 4-hydroxyphenylacetate was the only organic acid analysed here that showed a correlation with GI symptoms with a mean difference of 8.68 mmol/mol creatinine (CI 0.786 to 16.58, p = 0.033). 4-hydroxyphenylacetate is produced almost exclusively by bacteria, and has been identified as a biomarker for small intestinal bacterial overgrowth (SIBO)[256]. SIBO is a condition characterized by excess growth of bacteria in the small intestine where bacterial count is usually low or

absent. Symptoms can vary, but include abdominal distention, diarrhea or constipation, abdominal pain and/or nutrient deficiencies[257]. SIBO has been linked with functional bowel disorders such as Irritable Bowel Syndrome (IBS) and may be under-reported in both adults and children[258]. The prevalence of SIBO in patients with autism has been analysed in only one study, and it was not the primary outcome being measured[31]. Four out of the seven ASD-patients who underwent duodenal aspirate had evidence of SIBO. Based on this very limited study, SIBO may be more common in ASD, and certainly a failure to regulate microflora, when considered as a common/shared pathophysiology in autism or autism sub-type, would likely correlate to an increase of SIBO given similar microflora regulation is present in the small bowel and colon. In the current cohort of ASD patients with abdominal symptoms 29.4% had raised 4-hydroxyphenylacetate levels, suggesting patients with ASD may be more vulnerable to SIBO than the general population.

Quantitiative PCR Stool Analysis

QPCR stool analysis also shows promise in monitoring microflora compositions, and may have use in early detection of microflora abnormalities in ASD patients. In the present study, the qPCR analysis from 29 patients with ASD were compared with 7 unhealthy non-ASD controls. The mean firmicutes to bacteriodetes ratio was 66:34 (SD 8) in the ASD-cohort versus 54:46 (SD 11), and despite low numbers of controls and quite large standard deviations, significance between the groups was identified ($p = 0.007$). Further analysis on 159 cases supported these initial findings with the mean level of firmicutes to bacteriodetes in cases ($n = 147$) of 63:37 and in controls ($n = 12$) of 55:45 ($p = 0.005$), and this withstood correction for age and sex ($p = 0.009$). There was no significant correlation identified between the ASD group with GI symptoms and without ($n = 28$).

Firmicutes and bacteriodetes make up over 90% of GI flora[125]. In early life higher bacteriodetes is expected, and may confer a more anti-inflammatory local and systemic host environment[127,134,259]. Typically bacteriodetes gains a selective advantage when the diet is low in disaccharides and high in resistant starch. Firmicutes tends to gain advantage when the diet is high in disaccharides or when there is insufficiency in the enzymes controlling disaccharide content reaching the large bowel[260]. Firmicutes is thought to be more pro-inflammatory[134].

The results reported here support the most rigorous qPCR microflora study[34] in patients with ASD demonstrating elevated firmicutes to bacteriodetes ratio. These data will be

published shortly.

The role of microflora in ASD aetiology, pathophysiology, co-morbidity and disease evolution remains unclear. There is a growing body of evidence suggesting there are abnormalities in microflora composition in ASD patients. There is some limited evidence that altering microflora composition has an affect on ASD symptoms. As such it remains unknown if microflora is a modifiable factor in the disease process.

Autonomics, microflora and autism

Autonomic function was widely disturbed in this ASD-cohort. In this sub-group there were no clearly identifiable patterns of autonomic dysfunction. It was not possible to conclude that autonomic dysfunction is or is not central to the pathology of ASD, in particular due to the lack of dynamic assessment in the basic autonomic profiling utilized in the current investigation. Autonomic function is considered normal following an expected response to change in bodily function or following external stimulation. Given the autonomic function assessment here was a baseline investigation and not a fully dynamic assessment, a core deficit in autonomic function cannot be considered for exclusion.

The current data set (whilst lacking controls) does not support Ming et al's findings of reduced vagal tone in 80% of patients with ASD (which included controls)[115]. This disparity may be due to patient selection. Ming et al was a controlled trial where patients may have been more stable, whereas the current data is retrospective from patients who were referred for autonomic function investigations due to instability. It seems more probable that low vagal tone representing an under-developed parasympathetic response is a relatively common feature in ASD, merely difficult to detect with the other systemic pathologies or in patients who present as more severe with features of progression.

Autonomic profile was analysed for a potential relationship with microflora abnormalities. 13 treatment naïve patients were identified with both autonomic profile and qPCR analysis taken within one month of each other. There appeared to be a positive correlation between firmicutes to bacteriodetes ratio and vagal tone, but this did not reach significance. Removing one outlier (Firmicutes to Bacteriodetes level of 80:20) did increase the positive correlation parametrically (Pearson's correlation = 0.410, $p = 0.185$, $n = 12$), and this did reach significance under non-parametric testing (Spearman's Ro = 0.695, $p = 0.012$, $n = 12$). Removing a second outlier outlier

(bacteriodetes to firmicutes ratio of 56:44) achieved parametric and non-parametric significance (Pearson's Correlation = 0.630, p = 0.038, n = 11, and Spearman's Ro = 0.782, p = 0.004, n = 11). Whilst the numbers were low, there did appear to be a relationship between vagal tone and microflora composition.

There are many possible explanations for such a relationship. It may be that there is a *direct peripheral* monitoring of gut flora or gut lumen environment by the vagal nerve that then triggers a compensatory increase in vagal tone when excess or potentially pathogenic bacteria are detected. It is also feasible that the endotoxins produced by the microflora permeate the blood brain barrier (BBB) at the Circumventricular Organs (CVO's) causing a 'top-down' stimulation or irritation of the autonomic nuclei leading to a perceived increase in vagal tone. Another possibility considered here, mindful of the relatively convincing association between cytokines and autism symptomology/behaviours (see table 2), is that local immune response to the abnormal composition of microflora lead to systemic cytokines that also permeate the BBB at the CVO's leading to a compensatory 'anti-inflammatory' response through increasing the vagal tone. Differentiating and examining these possibilities should be relatively straight-forward utilizing a more extensive, dynamic autonomic profile test to examine higher autonomic functions, undertaking cytokine profiles and urinary metabolomics studies longitudinally and also following attempts at altering gut flora compositions. Analysis for association with behavioural outcomes in ASD may also yield clearer direction for exploring useful biomarkers for at least a sub-group of ASD.

Zinc and autism

There is increasing efforts to understand the aetio-pathogenesis, pathologies and evolution of ASD in a concerted effort by the scientific community to provide prevention, harm-reduction and treatment modalities for what can be a debilitating neurological disorder. Within these efforts there are several insights worth considering from the body of scientific investigations:

1. ASD is a multi-factorial condition.
2. There is significant variation in outcome[3,5,261]
3. The condition is highly heterogeneous.

Whilst it is expected that there are a number of factors leading to the development of ASD, there remains the possibility of identifying independent risk factors, and preventing the disorder or reducing the burden of disease. Exploring co-factor metabolism and micronutrient deficiency has proved successful in the past for developmental disorders, with notable examples being the identification of maternal iodine deficiency as the major cause of congenital hypothyroidism and folate supplementation as a preventative factor for Neural Tube Defects. Heritability estimates for Neural Tube Defects were also significant at 70%[262] until the introduction of prenatal folate. Exploring general health parameters in patients with ASD is also useful in the attempt to reduce the burden of co-morbid illness.

The current study examined plasma zinc levels in patients with ASD in comparison to a control group. Overall, 82% of patients with ASD were classified as deficient (< 11.5umol/L), 57% had plasma zinc levels below 10.5umol/L and over 25% had zinc levels below 8.80umol/L. The low plasma zinc level was having systemic effects with lymphocytes correlating with zinc levels above 10.5umol/L ($p < 0.04$), and below 10.5 umol/l total lymphocyte count was lower than the group mean, and lower than the population mean ($p = 0.023$)

In comparison to unhealthy controls, patients with ASD had significantly lower plasma zinc levels (10.01umol/L vs. 11.67umol/L ($p < 0.0001$)). The findings withstood correction for age and sex, and zinc levels in those with or without dietary modification or supplementation were similar.

Zinc deficiency is likely common in autism. Whether such deficiency is integral to the

pathophysiology, or indeed aetio-pathophysiology, or is a consequence of abnormal microflora or chronic illness remains unknown.

Is zinc deficiency a risk factor for the development of autism?

Given the diverse role of zinc throughout the body, zinc deficiency could go someway to explain the myriad of biological findings already identified in autism. Immune, neurological and gastrointestinal abnormalities have been reported in both prenatal/perinatal and infant zinc deficiency and reported separately in autism, and there is considerable symptom overlap.

Immune

Whilst zinc is known for its affects on immune function, our understanding of the extent and nature of prenatal, perinatal and infantile zinc deficiency on immune function in humans remains limited. Wong et al describe specific immune effects of prenatal zinc deficiency in a transgenic model following a maternal immune insult suggesting prenatal zinc deficiency may lead to epigenetic and immune effects responsible for maintaining a chronic inflammatory response[263]. Further transgenic work suggests that under zinc deficient conditions lysosomes containing inflammatory mediators lose integrity leading to a pro-inflammatory environment[202]. Such chronic inflammatory response has been reported in the elderly in response to zinc deficiency with an improvement of inflammatory cytokines following zinc supplementation [264]. Specifically microglial activation has been reported in-vitro in response to zinc deficiency [265], and reported in-vivo in patients with autism[8,12,266]. In animal models zinc has conferred protection against LPS induced maternal insult preventing aberrant behaviour in object recognition tasks[267], preventing abnormal sickness behaviour following immune challenge [268] and has recently been demonstrated to prevent communication deficits in an autism mouse model[269]. From an immunological standpoint, developing a zinc-based intervention may provide protection or reduce the harm associated with prenatal, maternal and paternal factors contributing to the development of autism.

Neurological

Neurological findings in zinc deficiency suffer similar limitations as immune findings. There are limited studies investigating the affects of prenatal, paternal or maternal zinc deficiency on neuropathological processes in human offspring. In a transgenic zinc deficiency model impaired glutathione metabolism was reported[270], perhaps explaining the protective affect of zinc supplementation on spatial and object memory following a maternal ethanol insult[271]. A significant reduction in transcription factors crucial for cell

differentiation and synaptic plasticity (AP-1, NF-KB and NFAT) were reported in an experimentally induced zinc deficiency[272]. Abnormalities of the cerebellum have been reported following postnatal zinc deficiency, specifically abnormal metabolism of Purkinje cells[273]. Cerebellum abnormalities including excess Purkinje cell loss in autism have been reviewed recently[274]. A number of environmental insults can alter glutathione metabolism, levels of transcription factors and neuronal cell loss. Perhaps then, zinc deficiency lowers the threshold for such environmental insults to lead to long-term neurodevelopmental disorders such as autism. Transgenic models have provided some evidence to suggest this may be the case. Pesticide induced neuropathology of the cerebellum and cerebrum was successfully reduced when zinc supplementation was given immediately following a 4-week exposure, and neurobehavioural abnormalities also improved[275]. 4-months of lithium-induced cerebrum and cerebellum lipid peroxidation was reduced following 4-months of zinc supplementation with improved levels of Glutathione-S-transferase[276]. Zinc administered together with a sub-acute organophosphate exposure over three days conferred complete protection over abnormalities in the Forced Swimming Test versus no zinc supplemented controlled exposure. There was also a corresponding protection against lipid peroxidation and impaired glutathione metabolism in the cerebral cortex, and protection against impaired glutathione metabolism in the hippocampus[277]. Zinc conferred a similar protective affect over aluminium-induced damage to the Blood Brain Barrier in an acute toxic insult model[278]. Following ten weeks of postnatal protein restriction 3 weeks of zinc supplementation improved oxidative stress markers and neurobehavioural deficits – specifically locomotor activity and memory and learning[279]. Zinc provided significant protection against Lead-induced neurotoxicity in a mouse model of postnatal and adult sub-acute insults via a reduction in oxidative stress[280] and improvement in monoamine metabolism[281]. As yet there are no studies exploring whether zinc confers a neuroprotective effect over exposure to air pollution or volatile organic chemicals.

Microflora

Zinc has been shown essential to microbiota composition[282], and maternal zinc deficiency has even been hypothesised to influence the development of the gastrointestinal tract in autism leading to an impaired gut-brain connectivity[283]. Beyond the immune and neurological effects of zinc deficiency discussed above, and the impact such effects will have on the normal mechanisms for microflora regulation, there are some direct microbiological effects of zinc. It appears that healthy microflora tend to have high affinity zinc transporters, capable of utilising low levels of zinc[284]. Certain

species of clostridia have been shown to increase fermentation in response to additional zinc[285], and the pathogenesis of Clostridium difficile has been reported as being zinc dependent[286]. This is consistent with the consensus of opinion that the majority of zinc absorption occurs in the small intestine, leaving lower levels of zinc available for the colon and hence microbiota, possibly limiting the growth of certain microflora such as clostridia. Zinc deficiency has also been suggested as a risk factor in the development of Environmental Enteric Dysfunction (EDD)[287]. Impaired absorption of zinc leading to increase transit of zinc to the colon may alter the composition of gut flora going forward. Equally the impairment of immune function found with zinc deficiency may be directly impeding the ability of the IEC's, Dendritic and T-regulatory cells immune regulation mechanisms. In this regard zinc may be a risk factor for the development of abnormal microflora in autism.

Equally zinc deficiency is expected in chronic disease, and more so where bowel symptoms predominate [282,284,288]. The zinc deficiency identified here may simply relate to the chronic ill-health or the established microflora compositional changes seemingly present in ASD patients. Given the majority of zinc is absorbed in the small intestine, it seems unlikely the principle cause of zinc deficiency is excessive consumption of zinc by the abnormal microflora present in the colon. Perhaps colonisation in the small intestine occurs or perhaps certain microbes gaining a slight selective advantage for another reason have the capacity to alter zinc transporter expression or pancreatic function in a bid to improve carbohydrate and zinc transition to the large bowel. A similar mechanism may be behind the lower level of disaccharidases previously identified[34].

Physical Co-morbidity

In adulthood the most likely cause of premature demise in ASD patients relates to seizure disorders with over 30 times increased risk of death from seizures regardless of co-existing intellectual disability[45]. Zinc deficiency has been explored as a risk factor for the development of seizures particularly intractable seizure disorders[218,231,289,290]. Cancer is another elevated risk for patients with ASD, and again there is likely increased risk through poor zinc status [288,291,292]. It seems likely poor zinc status will not only increase mortality rates in ASD, poor zinc status is likely to increase total morbidity and disease burden. There have been no studies investigating the relationship between zinc status and the level of morbidity in ASD.

Clinical Implications

This study represents the largest controlled trial of serum zinc levels in ASD patients. To date, all studies exploring zinc status in ASD have demonstrated a high probability of poor zinc status in ASD patients. The results presented here demonstrate none of the 72 patients examined reached national average levels for zinc, and, even accounting for selection bias, the current body of scientific opinion is that zinc deficiency is common in ASD patients. Whether this is cause or effect remains unknown, and largely clinically irrelevant when considering zinc status in patients with ASD. Clinicians should actively assess all patients with ASD, at diagnosis and throughout their life, for zinc deficiency. From the best available evidence it is likely adequate zinc status will reduce the burden of disease going forward. Supplementing zinc through a multi-vitamin is unlikely to be sufficient. Detailed analysis of the Cochrane reviews in this area reveal a loss of positive effect when zinc is given as part of a multi-vitamin formula[183,188,293]. Our results, whilst limited in number and detail of supplementation, suggest general supplementation alone is not adequate. Instead, oral zinc sulphate liquid titrated to serum response is recommended, preferably given in two divided doses daily. Clinicians should be aware of the potential impaired absorption caused by hypochlorhydria, small intestinal bacterial overgrowth, inflammatory bowel disease and excessive grain consumption. As per tolerance, zinc should be administered away from meals containing grains, and additional vitamin C may improve absorption.

Limitations

The urinary organic acid and qPCR stool data are of limited power. These investigations were undertaken within a clinic setting based on clinical suspicion. There is a selection bias in patients attending an outpatient clinic (those without physical symptoms or behavioural disturbance being less likely to attend). There is a further clinical selection bias. The lack of a control group for the urinary organic acids weakens the study further. Normal reference ranges are established for urinary organic acids and to a lesser degree qPCR stool analysis, and thus some inference can be drawn, in particular with the supporting scientific evidence. However, established reference ranges do not account for collection errors or alterations in equipment calibration. Controlled data for urinary organic acids and more control data for qPCR stool analysis would significantly increase the power of the study.

Comparison of autonomic function and qPCR analysis, despite the lack of healthy controls, carries more weight. There was a clear and distinct correlation between microflora composition and vagal tone, and even if this was not an autism specific association, the association is likely to withstand further investigation.

The zinc study carries reasonable power. To date this is the largest controlled trial of serum zinc levels in ASD versus controls. Clinical selection bias is likely to be present both in relation to the patient population attending the clinic and in the selection of patients to undergo serum zinc analysis. Similar bias will exist for the control group. A prospective longitudinal study of zinc levels in all patients presenting with ASD at diagnosis would correct for such bias. More details of supplementation would strengthen the study further, including differentiation with probiotics and zinc containing supplements, although the sub-group numbers would likely be insufficient to draw meaningful conclusions. The identification of an association between zinc and lymphocyte count is likely to withstand further analysis; it remains though unknown whether this is a finding specific to ASD. Analysis of lymphocyte counts in the control population would help to determine whether zinc status reflects lymphocyte count generally or is a specific finding in ASD. More detailed investigation of potential confounders in the control group would strengthen the study further. It was unknown whether the level of supplement intake, dietary modification or indeed underlying diagnosis affected zinc levels in the control group. Diet and supplements did not alter zinc levels in the ASD-group, and as such, discovery of an association in the control group would remain relevant, particularly in relation to possible absorption issues.

Certainly the results were significant enough here to generate the recommendation to undertake a larger controlled trial, a large prospective trial and to recommend clinicians actively consider zinc status in patients with ASD.

Future Studies

Despite the large number of studies currently underway pertaining to autism, there remains much speculation and uncertainty. There are reasonably substantial epidemiology studies underway specific to autism (e.g. CHARGE and SEED), and these are already identifying useful findings, particularly the CHARGE study, which may provide more cohesive bidirectional studies in ASD. As yet though, there are no population-based longitudinal studies exploring the evolution of disease, the factors associated with it and the potential protective factors. The main barrier to such a study is cost, given ASD – a common childhood disorder – still only represents around 1% of the population, and the associations requiring investigation would necessitate the testing or sample-preservation for numerous environmental factors and biological specimens. A large population study, beginning in the prenatal period remains though, the most important study to disentangle the cause and effect conundrum ASD has presented, providing a more coherent direction for ASD studies, research policies and treatment and prevention interventions.

Beyond the substantial public health considerations in the need to identify causative agents and modifiable environmental factors to prevent this costly condition there is a public health and clinical need in identifying modifiable factors and treatment modalities to reduce the severity, co-morbid illness and impact of the disease in those who already have it. In this regard, whilst supplemental zinc is promising and worthy of further study, treatment modalities aimed at altering the microflora composition in ASD are the most promising. Indeed given that such epidemiological studies are likely to take between five and ten years to reach fruition, treatment interventions are arguably more crucial to ease the suffering of individuals and society from an emotionally and financially costly condition. As such, the proposed next study is a Randomised Controlled Trial assessing the benefits of altering gut flora on autism symptomology. Ideally, cost permitting, such a study would include direct measures of gut flora composition such as qPCR, indirect measures such as metabolomics, mechanistic measures such as transcriptomics and cytokine profiles and evaluation of neurological function through autonomic profiles and possibly neuroimaging techniques. The design of the trial is included in appendix 3. Given the identification here of poor zinc status in the majority of ASD patients, more consideration will be given to zinc status during any attempts to modify microflora.

Whilst there appears sufficient evidence to warrant treatment trials aimed at altering

microflora composition, and such treatment success will depend on the degree of improvement achieved in psychosocial parameters not biomarkers, there remains a clinical utility in identifying reliable biomarkers. Metabolomics appears the most promising biochemical investigation in this regard. Future studies require a broad sampling from patients with ASD, not merely those attending outpatient clinics, and crucially require a substantive control population. Likely ASD is multi-factorial, with genomic individuality and environmental exposures somewhere in the mix. As such, it is possible other childhood conditions share similar alterations in microflora compositions (such as asthma, diabetes and allergies). A broad, large control population derived ideally from prospective population studies are likely needed to correct for the confounders of chronic disease. In this regard, again, a clinical trial would likely be the most useful follow-up study. Should there be an improvement in ASD symptomology measurable on psychosocial profiling following the successful implementation of a treatment protocol expected to alter microflora composition, then a concurrent improvement in metabolomics profile there would be greater validity to the use of metabolomics screening as a diagnostic aid for ASD.

A number of other suggestions inferred from the data presented here could be followed up through a larger, controlled study. A negative correlation between the total number of abnormal metabolites identified and the patient age if repeatable, could suggest alteration in microflora is an early feature of ASD. The gender differences noted above, in particular the converse increase in mean 2-hydroxyhippric acid levels in ASD females under 7 years of age, and the loss of expected relationship between age and microflora metabolites in male ASD patients, if investigated further with greater numbers and controls could provide some clarity over the strong gender differences apparent in ASD. The association between microflora composition (firmicutes %) and vagal tone would benefit from greater numbers. The addition of a control population may also provide some suggestion as to the uniqueness of any such correlation to ASD or not. Concurrent measurement of inflammatory cytokines, and other immune markers could be helpful in this regard also.

Certainly there is a suggestion here that the immune system and nervous system are both involved, and future studies may be able to unravel the order and evolution of such processes both in those with ASD who seem to stablise and those who seem to worsen. Bearing in mind the quite convincing association between neuroinflammation and ASD (see section 1.1.7) it may be in the case of ASD, the microflora composition maintains a more pro-inflammatory balance due to a failure or disruption to the mechanisms

controlling GI flora, and therefore it is the pro-inflammatory microflora composition that continues to drive such neuroinflammation. Exploring immune markers relating to microflora compositions over time may help delineate the mechanisms more.

There were insufficient serum zinc levels available in the patients analysed for stool qPCR and urinary metabolomics to ascertain what if any central role zinc may have in the pathophysiology of these processes. This may be worthwhile following up in relation to the potential positive or negative effects of corrective zinc supplementation. More, the results presented here suggest investigating zinc status prenatally and during pregnancy is a priority worthy of considerable attention.

CHAPTER 9
Conclusion

9.0 Conclusion

This project aimed to explore potential modifiable environmental factors associated with the aetiology, pathophysiology and co-morbid illness in Autism Spectrum Disorder, towards developing disease prevention, harm reduction and successful management strategies in this common condition. Specifically this project investigated the current body of scientific literature pertaining to environmental factors and pathophysiology in autism and undertook a retrospective analysis of microflora abnormalities, autonomic function and micronutrient status in an outpatient ASD population. The results of microflora abnormalities and micronutrient status are consistent with other studies, and consistent with the underlying hypothesis. The association between autonomic function and microflora composition via qPCR was a novel finding, and also consistent with the hypothesis.

From the clinical setting it appeared that patients with ASD form tangible sub-groups consistent with a Variable Insult Model. The supposition was that patients with autism have suffered an environmental insult(s) (toxin, infection or other stressor) to the developing immune, neurological and/or neuroimmune systems with diverse pathological sequelae and a shared phenotypic presentation of impaired socialisation. This hypothesis was generated following the experience of patients with ASD frequently suffering a broad range of physical illnesses, and the subsequent alteration in the patients' ability to socialize following successful management of such illnesses. An initial literature review supported this working model. The literature review and hypothesis was published in March 2014 "Neuro-immune Abnormalities in Autism and their association with the environment: A Variable Insult Model."[239].

The Variable Insult Model of ASD has not been established, and indeed may be too broad to be evidentially established. It is helpful nonetheless in focusing efforts on sub-grouping and researching treatment modalities. There is also a useful predictive value in using this model, predicting for example that gut flora abnormalities will be common due to the shared neurological and immunological networks required for both socialization and the selection of normal gut flora composition (e.g. appropriately dynamic vagal response). Subsequently, it was successfully predicted that the transgenic valproate insult model of autism causing disruption to autonomic nuclei would also cause disruption in gut flora composition and such disruption would not be linked to simply reduced neural innervation (as would be expected from a delayed development) but would be associated with disrupted neural innervation and increased nerve

innervation of the gut (as would be expected from an insult from an environmental provocation) (unpublished data). Some of these findings have been independently repeated and published recently[294].

Taking the model further, it should be possible following identification of the affected peripheral and system-wide pathology to predict the primary insult, certainly in terms of central region(s) affected, but also at what time of gestation or age the insult happened and perhaps even the likely environmental agent involved. Equally one should be able to predict the area affected and pathophysiological phenotype based on knowing the timing and nature of insult.

The appreciation of the environmental insult and the diversity of toxicants, infections and stressors that can lead to such an insult, across specific time periods moves away from considering a static, innate problem that will continue to be tangible and measurable throughout. More likely, from the body of scientific evidence, there is a transient insult that reduces or abates over time in most and the consequential pathology differs sufficiently depending on the timing and nature of the previous insult. Neuroinflammation may be a common shared pathophysiology or downstream and symptomatic effect, as may gut flora abnormalities, zinc deficiency, autonomic dysfunction and neurochemical abnormalities. Caution though remains in attributing an exact pathophysiology where the aetiological agent(s) remains unknown. The challenge it seems may be in the case of autism, the aetiological agent and its effects may need to be delineated in each patient or at least numerous sub-groups.

Gut microflora was examined as part of this project as a possible modifiable environmental factor involved in the aetio-pathogenesis of autism, disease evolution and/or the development of co-morbid illness. Clinical experience suggests a prominent role for microflora in the management of at least a sub-group of autism and perhaps even holds key insights towards prevention or harm reduction of the condition. It was observed that patients with small intestinal bacterial overgrowth (SIBO) or severe constipation achieved a seemingly stark improvement in autism symptomology – namely improved social engagement and skills – following resolution of these conditions. There are frequent reports also of developmental improvements following bowel preparation (using an osmotic laxative to 'clear-out' the bowel prior to colonoscopy), and such improvements whilst often transient appear to be more than would be expected form the positive effects of pain resolution. There is some evidence in the literature to support this assertion both in terms of abnormal gut flora

compositions discovered on investigation and also a clinical trial showing improvements in ASD symptomology on oral antibiotics for gut pathogens. Feingold et al, undertook a clinical trial in patients with autism and gut symptoms, examining specifically the hypothesis clostridial organisms produce endotoxins that disrupt neurochemistry causing the autism phenotype[168]. A non-absorbable antibiotic was given for six weeks and behavioural outcomes measured before and after treatment and then 12 weeks after cessation of treatment. Whilst there were relatively small numbers (n = 11), the results were significant with marked improvement in autism specific impairments following active treatment for clostridia. The trial has several limitations including focusing solely on patients with autism who also have gut symptoms, predominantly diarrhoea. It is difficult to extrapolate to autism generally but to say that, in some patients with autism gut bacteria seems to be having an adverse or causative effect in relation to autism symptomology. This supports the clinical experience of patients with autism.

Since the clinical trial of Feingold et al., there have been over a dozen investigations exploring gut flora compositions and species identification in autism spectrum disorder (see table 8). These preliminary studies are broadly supportive of the hypothesis that abnormal gut flora are present in autism. They implicate a variety of pathogens and commensal compositions, not just the clostridial group. In saying that, the only clinical trial exploring gut flora composition that corrected for confounders and had sufficient treatment naivety (Williams et al[34]) supported the initial hypothesis of Bolte et al[167], that there is abnormal clostridial species in patients with autism. It is worthy of note that again only patients with symptoms of bowel disorders were examined.

The results presented here give weight to the supposition that patients with autism have abnormal gut microflora and such compositional dysbiosis is involved in the pathophysiology of autism in some way. Metabolomics measures demonstrated increased urinary excretion of bacterial metabolites emanating from the GI tract in both patients with and without GI symptoms. A larger, controlled trial into gut flora composition in autism is keenly awaited.

Micronutrient status was also assessed here. There have been numerous studies supporting zinc deficiency in ASD, and the results of our moderately powered, controlled analysis support these findings. Indeed 82% of patients with ASD were characterized as deficient, and there was a significant difference with unhealthy controls ($p < 0.0001$). We did not find a strong association with age, but the numbers available in these sub-groups were probably not sufficient. Initially the observation of chronic

zinc deficiency in this patient group was attributed to a secondary effect of abnormal GI function. However, whilst this remains a likely possibility, due consideration must be given to the possibility that zinc deficiency (prenatal, maternal or early infantile) may be an independent risk factor for the development of autism. In future work the aim is to explore the protective affects of correct zinc supplementation on the development of ASD. Recently Kirsten et al have demonstrated a protective affect of zinc supplementation over communication impairments in a transgenic model of ASD[269].

This project also examined the potential in undertaking a clinical trial in altering gut flora in ASD and the affects this has on behavioural and physiological outcomes. Whilst the aetio-pathogenesis and pathophysiology of autism in general still requires much work, it is worthwhile pursuing a clinical trial targeting gut flora in ASD. Duration of antibiotic use, strictness of dietary modification and the use or not of augmentative therapies (such as probiotics) require more consideration, as does consideration pertaining to zinc status, but overall it seems such a trial would be the most effective use of time and resources in the bid to improve the outcome for patients with ASD.

In this project microflora abnormalities were identified in patients with autism, and such microflora abnormalities (namely qPCR compositional analysis) correlated with neurological function (i.e. autonomic function). Zinc deficiency was also identified as frequent in patients with ASD, and such deficiency was having an affect on immune function, lowering the lymphocyte count in patients with ASD. There is clinical use in remaining aware of the possibility that abnormalities of the GI tract may affect neurological function and zinc deficiency is common and may affect immune function in ASD, perhaps promoting a more proactive management of these areas (for example managing constipation until confirmed resolution). More powerful studies are needed before universal recommendations to alter these potential modifiable environmental factors in ASD can be made.

APPENDIX

Appendix 1

Critical Review of Microflora Studies in Autism

(See Chapter 1.2.5, Introduction)

Adams et al.,[162] used a culture based methodology to examine the common microflora species' (Bifido, Lacto, E.coli, Enterobacter) culturability in the stool of ASD children vs. healthy non-sibling controls. Difficulties with controlling for variables such as diet, probiotic use, fish and fish oil consumption was duly noted by the authors (ASD group had higher probiotic and fish oil usage and lower seafood consumption). Antibiotic use within one month was part of the exclusion criteria (versus 3 months for Williams et al 2011[34]). Results were consistent in regards to lower Bifidobacteria levels as other studies, and this correlation stood up to correction for known variables. However, like previous studies, there remains the lack of treatment naivety in the ASD group. *Lactobacillus* was also found at higher levels in the ASD group vs. controls ($p = 0.00003$), although there may have been confounding effects of seafood consumption after comparison of small sub-groups (lower levels of seafood consumption in the ASD group correlating with higher lactobacillus levels $p = 0.0007$).

Wang et al.,[166] used qPCR analysis of faecal samples to measure the levels of specific microflora in ASD patients versus sibling controls and 9 non-sibling controls. Sample collection was rigorous. The most statistically significant finding was of lower bifidobacteria levels in ASD versus controls ($p = 0.006$) and ASD versus siblings ($p = 0.032$). *Akkermansia muciniphila* (a member of the verrucomicrobia phyla) was also lower in ASD patients versus controls ($p = 0.029$). There was no comment on analysis for confounding factors such as diet or probiotic use. There were only two species belonging to the bacteriodetes phyla measured making a firmicutes to bacteriodetes ratio difficult to determine.

Fiengold et al.,[160] utilised pyrosequencing PCR analysis to measure a wide range of microflora in 33 ASD patients, 7 sibling controls and 8, presumably, healthy controls. Despite rigorous analytical methodology, measuring an extensive list of microflora with amplification and substantial statistical analysis, the study failed to report adequately on confounding factors. A 'number' of ASD patients were undertaking dietary modification, including disaccharide restriction, a known modality to alter microflora compositions. The refusal of patients to discontinue certain therapies also suggests on-going dietary and nutritional interventions. Therefore, many of the statistically significant differences may be as a consequence of treatment. Without detailed assessment of current and previous treatment modalities, the study may in fact be looking at peri- or post-treatment levels. The

additional correlation between severe autism and higher bacteriodetes versus non-sibling controls ($p = 0.044$), may in fact indicate a greater level of intervention and perhaps, following through on the microflora hypothesis of ASD, may indicate the presence of a more immunogenic bacterial population in severe autism versus a compositional alteration in mild and moderate autism. The identification of novel microflora associations with ASD in this study may then remain of significant interest. *Desulfovibrio* species were significantly elevated in those with severe ASD versus control group ($p = 0.01$), giving rise to the possibility eluded to in the paper that poor regulatory mechanisms in the bowel leave those with ASD more vulnerable to both abnormal compositions of gut microflora and perhaps even harmful, immunopathogenic strains of bacteria, and further, such differentiation may even confer degree of severity or specific pathology/symptomology. Overall, the findings of higher bacteriodetes versus firmicutes in ASD patients versus controls is limited in power due to the lack of correction for confounding factors, and specifically as the ASD-group continued with treatments (except antibiotics) during the study.

Parracho et al.,[33] used qPCR analysis to examine various strains of microflora in 58 ASD patients, 12 sibling controls and 10 unrelated healthy controls. Sample collection and methodology was rigorous. Confounders were recorded and analysed. Again the ASD group suffered a lack of treatment naivety with 65% undertaking a restricted diet, 53% on probiotics and 65% on other nutritional supplementation. The healthy control group had no dietary modification or supplementation for the preceding 6 months, were healthy and had no bowel related complaints. The *Clostridium histolyticum* group (a member of the firmicutes phyla) were present in higher numbers than in healthy controls ($p < 0.01$) and sibling controls ($p < 0.05$). As expected by the demographics there were correlations between *C. histolyticum* levels and probiotic use, as well as correlation between supplementation and *C. histolyticum* levels in ASD. Sub-group analysis was not reported. There were no correlations between restricted or unrestricted diets and *C histolyticum* levels. This study provides convincing evidence for some alteration in microflora compositions.

Song et al.,[32] utilised quantitative PCR analysis to analyse the presence of specific microflora (mainly clostridia groups) in 15 ASD subjects and 8 controls. There was limited description of study participants and no examination of potential confounders. The study reports: 46-fold increase in *Clostridium boltae* ($p = 0.01$), 9-fold increase in *Clostridium cluster I* ($p = 0.014$) and 3.5-fold increase in *Clostridium cluster XI* ($p = 0.004$). The low numbers, lack of examination for confounders, lack of patient demographics or selection criteria limit the inference to be drawn from this study.

Finegold et al.,[31] used culture based and qPCR techniques to analyse various microflora

compositions in both faecal and upper GI specimens in 7 ASD patients and 4 controls. All ASD patients had GI symptoms and had taken no antibiotics for 1 month. Treatment though remained active, and no assessment of confounding factors such as diet and probiotics occurred. Higher numbers of clostridia species were detected in the faeces of the ASD group versus non-ASD controls ($p = 0.0393$), and in the upper GI tract (gastric, duodenal and jejunal aspirates) non-spore forming anaerobic bacteria and microaerophilic bacteria were found in 4 out of 7 patients in the ASD group versus none of the control group. The lack of analysis on potential confounders particularly in an active treatment group weakens the power of this study.

Williams et al[163] utilised previous biopsy specimens from 15 ASD-GI patients and 7 control- GI patients together with 8 new ASD-GI patients and 2 control-GI patients to attempt delineation of the alcaligenaceae phyla discovered in around half of ASD-GI patients and none of the Control-GI patients in their previous study[34]. The Alcaligenaceae group was analysed via probe PCR and 4 distinct *Suterella* species were isolated in 46% of the initial cohort in both ileum ($p = 0.022$) and cecum ($p = 0.037$). Limited demographic information was available on the new subjects added to the trial and limited confounders were analysed. Western Blot analysis of *Suterella wadsworthinesis*, one of the four species isolated, was examined in all samples. 12 from the ASD-cohort showed significant immunoreactivity to *Suterella wadsworthensis*, 8 of whom tested positive to *Suterella spp*. on PCR analysis, and only one control-GI showed weak positivity. 65.2% of ASD-GI patients had evidence of *Suterella* at some time with PCR analysis and/or western blot analysis. The study reliably reports the presence of a unique species of bacteria in the GI tract of ASD subjects with GI symptoms versus atopic controls with GI symptoms. However, the inference from the immunoreactivity to suterrela is restricted as no other microbial western blot was undertaken, and no analysis of confounding variables were reported. Again no comparison to faecal PCR analysis was reported.

Appendix 2

Aetiological Considerations Relating to Abnormal Microflora

(See Chapter 8 – Discussion)

The mechanism behind the acquisition of abnormal GI flora

Our understanding of GI flora remains in its infancy. It is proposed that both 'top-down' regulation and 'bottom-up' regulation influences the microflora composition. 'Top-down' or host mediated mechanisms work at a basic level to limit damage to the intestinal mucosa and prevent invasive infections. 'Bottom-up' or microbe mediated mechanisms work at a basic level through competition methods to inhibit the growth of other bacteria to gain a selective advantage. Research is beginning to demonstrate much more complex and intricate mechanisms[126,133,134].

The acquisition of abnormal GI flora may be due to either or both 'top-down' and/or 'bottom-up' mechanisms. Disruption to immune function, for example in developmental immunotoxicity (DIT) could lead to abnormal responses to GI flora - one expects both underactivity and overactivity even within the same subject is possible. Disruption to the 'top-down' mechanisms may occur through direct neurological impairments or perhaps developmental neurotoxicity (DIN).

The Autonomic Nervous System is sensitive to environmental toxins[295,296]. Low vagal tone has been reported in farmers exposed to organophosphate pesticides[295]. It is possible an environmental toxin damages the peripheral or central autonomic nervous system and this then prevents the adequate development of higher autonomic areas. A key component of such pathology may include impairment in a proportionate autonomic response to somatosensory peripheral stimulation, including gut microflora.

If somatosensory input was impaired or disrupted then higher cortical areas may not adequately develop, as the development of higher cortical function depends on somatosensory input[116], which in turn depends on adequate autonomic responses[114]. Sensory impairment is a common and well known feature of ASD [297]. Often children have auditory hypersensitivity, olfactory dullness, altered visual processing, hypersensitivity to touch and visceral hypersensitivity. If the parasympathetic system is not in a position to respond normally, organs requiring high discriminatory power (e.g. sight and smell) may present as underactive, and linear senses (e.g. mechanical senses in the GI tract for example) may present as overactive. It is though a sensory dysfunction more than the level of activity that is fundamental. As suggested by Cohen et al in 1977: *some autistic children*

characteristically may be in a state of sensory rejection associated with generally higher levels of arousal or defense against environmental bombardment[298].

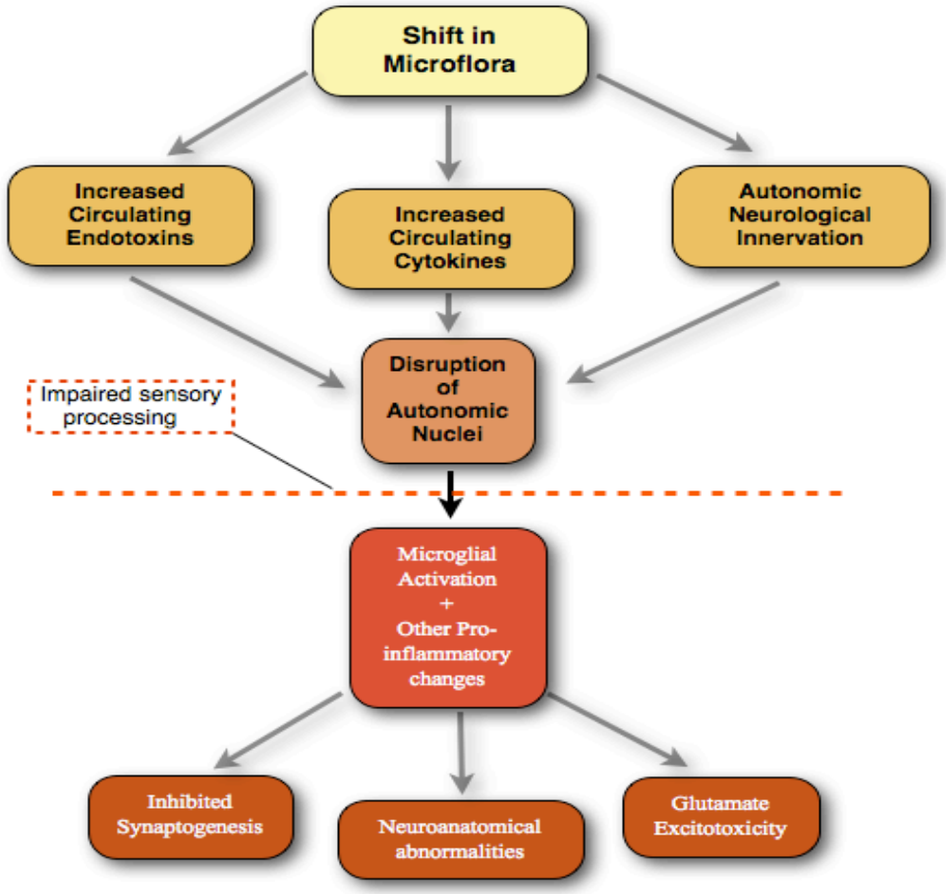

Figure 29 Mechanism of altered autonomic response in relation to shift in microflora composition. The key element is the disruption to sensory processing, which leads to delayed development of higher cortical areas. Down-stream neurological effects are variable and may be dependent on on-going compositional dysbiosis.

Aetiological considerations

Possibilities underpinning the aetiology of abnormal flora in ASD considered here can be divided as follows:

1. Eating habits
2. Ingestion of non-food products
3. General increase in toxic exposure
4. Diversity of pathogens
5. Multifactorial

1. Eating habits

Eating habits have changed dramatically since the early 1920's[299]. Specifically

carbohydrate consumption has increased significantly. Such a change in food source may confer improved survival for specific microflora within the 1000+ species discovered, thus posing significant pressure on the eventual 120 or so species eventually selected as the majority.

Alteration in dietary carbohydrate composition is known to affect species proliferation[260,300,301]. If excess carbohydrate consumption leads to a positive pressure for expansion of certain microbial colonies, it may then necessitate the 'top-down' pressure to increase and thus a more pro-inflammatory state may ensue.

2. Ingestion of non-food products

Ingestion of non-food products may lead to different selective pressures in the developing infant. Intensive farming and economic instability has lead to the use of chemicals to improve growth and prevent loss[299,302]. Generally pesticides cause damage to living cells, smaller organisms typically being more sensitive than larger organisms[88]. Colourings and preservatives are also frequently added to mass-produced food products to preserve shelf-life and increase pleasure derived from food for economic reasons. The commonly used preservative, azo-dyes were once used as antibiotics [299,302]. The intake of these products may be akin to consuming a consistent level of antibiotics, which may then lead to generally disrupted microflora.

3. General increase in toxic exposure

A general increase in toxic exposure may affect the microflora similar to the mechanisms hypothesized for increased carbohydrate consumption and ingestion of non-food products. Toxic exposure may though, directly affect the host, impairing 'top-down' regulation of the microbial environment through developmental immunotoxicity or developmental neurotoxicity. The abnormal microflora would then be a secondary effect of a wider pathophysiological state.

Host related toxicity is unlikely to be straightforward. Cytokine or antibody response to specific microbes, even if leading to an autoimmunity type state, is likely to be much more clearly defined and definable than chronic toxicity. Cytokines and antibodies have defined roles and specific sites of action and consequences. Toxins have no role in the body. Toxins can displace other essential nutrients, block enzymes, sensitize, affect conduction, disrupt hormones, alter gene expression, mimic hormones and neuropeptides and simply damage surface membranes [14,18,78,82,85,87–89]. An increase in toxic exposure would likely lead to the greatest diversity of symptoms, and necessitate a whole systems approach to its management.

4. Diversity of pathogens

Diversity of pathogens may have led to new pathogenicity's. *Streptococcus* infection is an apt example. *Streptococcus* can cause skin, ear, throat, chest and wound infections, in a quite typical fashion. *Streptococcus* can also cause Rheumatic fever, Sydenham's chorea and Paediatric Autoimmune Neurological Disorder Associated with Streptococcus (PANDAS). These are autoimmune conditions that will worsen in the presence of the offending streptococci. Rheumatic Fever damages heart valves, Sydenham's chorea damages the basal ganglia and PANDAS causes chronic neurobehavioural problems through several different autoantibodies.

Antibiotic use has exponentially increased worldwide since around the 1930's. Such a selective pressure may have led to unique species variations or simple mutations conferring more chronic manifestations. Microbes causing an acute infection are usually easily recognised by the immune system or prescribing physician and will be deselected. Microbes causing less obvious, chronic challenges to the body may not necessitate a strong response from the immune system or prescribing physician.

It is feasible that it is the pathogenicity of the microbes involved in ASD that cause the neurobehavioural manifestation, probably through the same autoantibody, cytokine response. If it is though, the nature of the microorganism that is different, then control of such microorganisms or control of the selective pressures on such microorganisms may allow control of the condition.

5. Multi-factorial

A singular causative factor would be convenient. It may be though, a series of events that give rise to ASD phenotypes. In-utero or early post-natal insults may disrupt the neurological, immunological and/or neuroimmunological development. This may set the system towards a certain state (e.g. pro-inflammatory, excitotoxicity, etc..). Such vulnerability may then only be realised where adverse environmental provocations continue. Within this framework some individuals may carry a very high probability of developing ASD and only require minimal environmental provocation, and others may carry a very low risk of developing ASD and it is a high level of environmental provocation that triggers the condition. It may be possible to delineate this variable vulnerability and variable provocation, thus forming aetiologically based sub-groups. This in turn may help to guide treatment interventions further. For example, those with low risk and high provocation may respond better to environmental control.

Appendix 3

Study Protocol: altering gut flora in patients with ASD

(See Chapter 8 – Future Studies)

Study Introduction

The aim is to evaluate neurological, immunological, biochemical and behavioural parameters pre-treatment, and sequentially following instigation of dietary modification.

Clinical experience suggests a positive outcome in neurological, biochemical and behavioural parameters. If the current project confirms positive treatment effect, then funding to support a larger RCT will be sought.

Summary of Study Rationale

A high power study conducted by Stanford University demonstrated lower twin concordance than previously reported[6]. This supported previous and subsequent reports from the Centre for Disease Control that there is a true rise in ASD cases, including more severe cases[40].

Neurological, immunological and gastrointestinal abnormalities are consistently reported. In 2004 Reading University discovered abnormal species of commensal flora in ASD patients with bowel symptoms versus neurotypical children with bowel symptoms[33]. In 2010 Imperial discovered abnormal bacterial metabolites in the urine of ASD patients aged between 3yrs and 9yrs[157]. In 2011 Harvard Medical School identified abnormal commensal flora in an ASD cohort and this was consistent regardless of bowel symptoms[34].

A low powered study demonstrated a marked improvement in ASD symptoms with a six-week trial of oral vancomycin[168]. The treatment was intended to reduce the level of abnormal flora. Within six to twelve weeks following discontinuation of treatment relapse occurred. For a review of this groups study series see[303].

Harvard University's ASD research group also studied the RNA expression and levels of disaccharidases produced by ASD patients versus controls. There were reduced levels of disaccharidases, and atypical RNA expression. Adiposity index (the ratio of firmicutes to bacteroidetes) was also found in reverse ratios from neurotypical, suggesting the composition of gut flora favours disaccharide's[34].

Autonomic abnormalities have long been described in patients with ASD. Low vagal tone was identified in 80% of patients with ASD[115]. Clinically, an improvement in ASD

symptoms following successful intervention often corresponds to an improvement in autonomic function.

It is proposed that controlled disaccharide restriction will achieve significant improvement in symptoms of ASD in around half of cases. By reducing the disaccharide supply to GI flora environmental regulation of GI flora may be possible. A treatment response depends on the patient's abnormal flora gaining a selective advantage through a diet high in disaccharides. Such flora is then sensitive to disaccharide restriction. The mechanism for improvement could be either an improvement in autonomic function, a reduction in chronic endotoxaeima (reduction in toxins produced by abnormal flora) or by a reduction in immunological response (reducing cross reactive antibodies and/or excessive pro-inflammatory cytokines).

Dietary composition affects microbial flora signature in the GI tract[304]. In this study adiposity index changes will be correlated with immune profiles, autonomic profiles and behavioural assessments, to ascertain whether, following dietary modification, there are significant improvements in core autism symptomology and whether such improvements correlate with alterations in adiposity index, immune and/or autonomic functions. Such analysis may help delineate the reason for any positive response as immunological (e.g. reduced pro-inflammatory microflora), autonomic (e.g. reduced sympathetic drive) or something else. This may guide future work and also contribute to greater understanding of the underlying pathophysiology

Dietary Manipulation of Gut Microbiota

It is estimated that about 60% of gut flora are significantly influenced by dietary factors[301]. Humanized mice models showed significant alterations in gut flora composition when transitioned from high-fibre, low fat diet to a low fibre, high sugar diet[301]. A further study in healthy volunteers demonstrated a significant alteration in species proportions when comparing diets high in resistant starch (RS) versus diets high in non soluble polysaccharides (NSP)[260].

Low-FODMAPs (Fermentable, Oligo-, Di-, Mono-saccharides and Polyols) diet has been in use since 2005. By starving the pathogenic or dysbiotic flora the low FODMAPs diet reduces bacterial fermentation and thus overgrowth. It is thought to also reduce the endotoxic effects of excess fermentation. Low-FODMAPs dietary restriction is now frequently used for Irritable Bowel Syndrome (IBS), and has been used in Inflammatory Bowel Disease (IBD)[305].

The Ketogenic Diet (KD) or an adapted version of it is used to treat neurological and neurometabolic conditions, leading to a reduction in seizure activity in typically over 50% of drug refractory epilepsy cases[306]. The KD typically restricts carbohydrates, sometimes as low as 4% with more emphasis on increased fat consumption. The mechanism for its action remains unknown. There has already been an open trial of a modified version of the Ketogenic Diet in Autism showing substantial improvements in the core behavioural symptoms in autism[307]. Again the reason for this is unknown. A mouse model for autism also showed significant improvements on a ketogenic diet, researchers showing the positive effects were not attributable to improved seizure control or any anti-epileptic effects[308].

It is estimated that 20-60 grams of starch makes it to the large intestine daily. Meals containing high levels of starch tend to be a risk factor for undigested starch reaching the large intestine. Processing of starch also increases the risk due to cross-bonding and loss of natural disaccharidases[309,310].

Hypothesis

The key hypothesis to be tested is:

Controlled disaccharide intake will improve core autistic features, autonomic abnormalities and markers for GI microbial overgrowth.

Aims

1. Identify whether or not there is a meaningful positive response to disaccharide restriction in a sub-group of ASD
2. Further establish whether or not intestinal flora is central in the pathophysiology associated with ASD
3. Provide further insight into autonomic, biochemical, immunological and behavioural parameters in ASD
4. Provide further insight into autonomic, biochemical, immunological and behavioural parameters in ASD over time
5. Improve interdisciplinary collaboration

Objectives

1. To measure behavioural outcomes before and after dietary intervention.
2. To measure adiposity index and urinary bacterial metabolites, and correlate to

behavioural measures.

3. To measure and record autonomic profile, urinary bacterial metabolites, cytokine array and behavioural assessments in a controlled setting in ASD patients.
4. To repeat the measures of autonomic profile, urinary bacterial metabolites, cytokine array and behavioural assessments after six months in the treatment group to ascertain change in such parameters following dietary intervention.
5. To repeat the measures of autonomic profile, urinary bacterial metabolites, cytokine array and behavioural assessments after six months in non-treatment control groups to ascertain change in such parameters over time.
6. To correlate each measure of neurological, immunological, gastrointestinal, biochemical and behavioural function following disaccharide restriction.

Study Protocol

Project design

A randomised one-way crossover study is proposed as the initial investigation.

Number of participants

80 patients will be selected based on inclusion and exclusion criteria. 40 patients in each arm.

Duration of study

The first phase is projected to last six months. A one-way crossover for a further six months. Final assessment for all patients at 12 months.

Methodology

Following ethics approval, patients will be recruited from outpatient clinics, charity groups and through advertisement in relevant media. An initial assessment will be undertaken to ascertain suitability for trial. Upon inclusion, patients will be randomised to either placebo arm or treatment arm. Autonomic, Biochemical, Immunological and Behavioural assessments will be undertaken within one month of each other. Placebo group will be assigned an 'anti-inflammatory' diet. Patients will undergo monthly review by dietician/nutritionist and working-hours access on-demand to dietician or physician. GPs will be informed. Physician assessment will take place if there are any health concerns and at three monthly intervals. The physician only becomes aware of the dietary protocol - anti-inflammatory vs. disaccharide restriction -, if there are concerns regarding health impacts of diet. Co-morbid illness will be treated if and when they arise, and this will not exclude patients from the study unless co-morbid health condition is protracted. Re-

assessment is intended to occur initially at three months, then six months and after crossover at one year.

Clinical Assessment and Inclusion/Exclusion Criteria

1.Patients will have a confirmed ASD diagnosis
2.Patients will be 5 years of age or younger
3.Patients will be from any ethnicity, gender or socio-economic class
4.Disaccharides will be present in the diet
5.Patients will not have a significant genetic disease
6.Patients will not be on a disaccharide restriction.
7.Patients will have undergone a full clinical assessment.

Biochemical assays

LC/MS-MS Tandem Mass Spectrometry is used to identify a metabolomics profile including Succinic acid, 2-Hyrdoxyhippuric acid, Hippuric acid, p-Hydroxyphenylacetate and Phenyl-propionate acids in urine.

The Adiposity Index is derived by using DNA probes that detect multiple genera of the phyla Firmicutes and Bacteriodetes in the stool. A percentage ratio is then calculated.

Autonomic Profiling

The non-invasive index Cardiac Vagal Tone (CVT) is measured on a continuous beat-to-beat (R-wave to R-wave) basis using the NeuroScope. R-R variability is a non-invasive measure of vagal tone (inferred to represent parasympathetic tone). By recording a continuous ECG the R wave on the ECG can be monitored, and the variability of the length of one R wave to the next R wave can be calculated against normal predictable variations (such as those of breathing). An increase in Vagal Tone is inferred by detecting increased R-R variability. Combining these measures with Blood Pressure and Transcutaneous gas measurements the Linear Vagal Tone can be determined. This gives a numerical number with 0 being a fully atropinesed control.

Immune Assays

Multiplex bead-system will be the primary measure of cytokines and chemokines. Microbeads with antibodies against specific cytokines and chemokines are automatically mixed with the serum sample. The beads are then put through a flow cytometer and the number of beads with antibody-antigen specific to each cytokine are counted giving a

quantitative value. Two other cytokine measures will also be used including cytokine array and western blotting.

Behavioural Assessments

The ADOS-2 (Autism Diagnostic Observation Schedule) is a semi-structured, standardized assessment of communication, social interaction, play, and restricted and repetitive behaviors. It presents various activities that elicit behaviors directly related to a diagnosis of ASD. By observing and coding these behaviors, one can obtain information that informs diagnosis, treatment planning, and educational placement. The ADOS-2 provides a highly accurate picture of current symptoms, unaffected by language.

Quantitative Checklist for Autism in Toddlers (Q-CHAT) is a diagnostic and monitoring parent-led questionnaire for ASD. 25 multiple choice questions completed within 15-20 minutes allows frequent completion and has been established as an accurate and relatively subtle marker of ASD traits[311].

The ATEC is a one-page form designed to be completed by parents, teachers, or caregivers. It consists of 4 subtests: I. Speech/Language Communication (14 items); II. Sociability (20 items); III. Sensory/ Cognitive Awareness (18 items); and IV. Health/Physical/Behavior (25 items). The questionnaire was designed specifically to monitor treatment response.

One-way cross over

ASD is a developmental condition. Measuring change in development during a rapid developmental period is fraught with difficulties. Often there remains the question as to whether such improvement would have occurred without intervention. Given that developmentally, of ASD patients, 20% worsen, 20% improve and 60% remain static between diagnosis at 2 years of age and reassessment at 4.5 years of age, the assessment becomes even more challenging[50]. Added to this variability in disease progression are the wide-ranging heterogeneity of ASD and the subjective nature of the assessment of the behavioural manifestations of the condition. To account for these assessment issues we have taken a reasonable number of participants, and by crossing-over the control group to the treatment arm we achieve greater numbers and hence statistical power. The multiple objective measures (autonomic profile, immune profiling and biochemical assays) will also serve to bolster the power of the study.

Additionally, there are ethical considerations if the treatment arm shows meaningful results, particularly in a developmental condition. It is expected, reasonably, that earlier intervention will yield greater harm-reduction in the developing brain. Crossing-over the patient group is one may to mitigate this ethical dilemma. Further, we will assess the initial

10 subjects at three months. If a positive meaningful result is obtained, altering the time-scale for crossover from 6-months to 3-months will be considered.

An anti-inflammatory diet has been chosen as the placebo arm versus no treatment due to the additional compounders of dietary modification in ASD. ASD can involve control, routine and repetition. It is plausible that merely altering the diet improves the rigidity by means of reconditioning and altering parent-child interaction. There are also the numerous other known and unknown placebo effects that can be mitigated by choosing an active treatment control group. Dietician will be blinded to the intervention, and by using an active treatment in the control arm, and given there is reasonable rationale that an anti-inflammatory diet could help the neuroinflammation of autism, there is expected to be reasonable commitment to the control arm.

Insuring Patient Safety

All patients will be initially assessed by dietician and clinician. The inclusion and exclusion criteria will be applied. Clinical opinion will guide the suitability for the initial screening tests. Urine Proteomics and Faecal Adiposity Index involve urine and stool respectively, which can be collected with very little disruption. There is no venipuncture required for the initial assessment. Those selected will be randomised and then undergo blood tests, autonomic profiling and behavioural analysis. Information sheets and consent forms will be provided and a further information sheet will be provided about the dietary modification. There will be a dedicated time for a dietician to discuss the risks and benefits, and consent for treatment will be signed with the clinician. All patients and patient guardians will have access to dietician or clinician within working-hours, and the GP will be informed of the intervention, its risks and benefits. Ethics approval will be in place prior to any aspect of the study progressing.

Data Collection/analysis

Patients will be randomized to treatment arm or placebo arm by an administrator. Dietician will receive instruction to commence patient on either the anti-inflammatory diet or disaccharide restriction. The dietician will not be aware of which arm is the treatment arm. Clinician will be blinded to the selection.

Patients will be instructed to not discuss the details of the diet with any technicians or assessors. Patients will be assigned a numerical number. A copy of all assessment reports will be added to the patient's records and then a copy will be sent to the study administrator and will be assigned a numerical number. Data will be analyzed by an external statistician, and again at the end of the study by the clinical lead/principle investigator.

Physician review occurs three monthly. Diet will only be discussed if problems encountered (e.g. weight-loss, reflux, etc.).

Limitations

Assessing development during a developing period poses several challenges. To mitigate the background development that may occur without intervention a control group has been used. A no treatment control group could be used; however there may be a positive effect of dietary control merely due to the control aspect. This is particularly relevant given a common symptom of ASD is controlling behaviour. Providing an avenue to essentially down-regulate this behaviour through alteration in dietary preferences may yield measurable differences particularly in behavioural assessments.

Compliance is probably the greatest limitation. Dietary modification relies on patient compliance and involves more effort than taking medication, for example. The disaccharide restriction is slightly more challenging in this regard than the anti-inflammatory diet, and hence there may be less of a positive effect in the treatment arm. Monitoring compliance is also troublesome, as it depends on patient/guardian reports and patient/guardian knowledge regarding the approved foods. There is also the possibility for unsupervised infringements. This is mitigated somewhat by having regular nutritional assessments, and by using younger children.

There are no imaging studies of cortical activity in this study. CSF samples are also avoided here, again leading to greater distance to the assessment of central pathological processes. By combining autonomic and behavioural assessments this distance is mitigated somewhat.

Appendix 4

Raw data – urinary metabolomics in ASD patients (n=49)

Patient ID	Gut Symp-toms (y/n)	Sex	Proprionic	Hippuric	4-Hydroxy-phenyl-acetate	2-Hydroxy-hippuric	Succinic	3-oxyglutaric	Age	No. abnorm-alities	Supplements	Diet	Meds	Severity
a18	**0**	**1**	398	524	41	4.5	16	0	3	3	0	0	0	2
a27	**0**	**1**	51	560	31	3.2	23	0.14	4	4	0	0	0	5
a33	**0**	**1**	106	312	12	1.5	25	0.37	4	2	0	0	0	6
a5	**0**	**1**	48	1740	12	0.93	33	0	12	2	1	0	0	3
a8	**0**	**1**	27	532	31	0.46	0.72	0	6	1	1	0	0	6
a39	**0**	**1**	60	337	9.2	2.2	1.7	0	13	1	1	0	1	5
a1	**0**	**1**	237	67	53	4.3	4.6	0.22	6	3	1	1	0	3
a22	**0**	**1**	45	61	12	2	26	0.26	5	2	1	1	0	5
a30	**0**	**1**	74	534	7.2	1.6	15	0	13	1	1	1	0	5
a31	**0**	**1**	228	344	18	1.9	27	0.19	2	2	1	1	0	8
a34	**0**	**1**	236	401	12	3	45	1.1	10	3	1	1	0	4
a36	**0**	**1**	534	779	41	1.4	16	0.18	4	4	1	1	0	4
a24	**0**	**1**	21	195	15	0.57	58	1.1	11	1	1	1	1	3
a6	**0**	**1**	19	128	9.1	0.36	14	0	11	0	1	GFCF	0	7
a37	**0**	**1**	56	140	11	0.59	5.5	0	19	0	1	GFCF	0	6
a46	**0**	**2**	28	182	18	0.11	22	0	5	0	0	0	0	6
a49	**0**	**2**	27	91	5.4	0.59	5.2	0.06	10	0	0	0	0	4
a45	**0**	**2**	51	849	14	0.86	12	0.024	3	1	1	0	0	2
a47	**0**	**2**	56	857	10	1.4	49	0.28	5	3	U	U	U	U
a19	**1**	**1**	164	491	90	3.3	37	0.93	2	3	0	0	0	7
a29	**1**	**1**	297	150	25	3	24	0.19	5	2	0	0	0	7
a40	**1**	**1**	312	94	8.2	0.75	34	0.07	8	2	0	0	0	5
a10	**1**	**1**	24	130	38	3.5	7.7	0.21	2	2	0	1	0	7
a12	**1**	**1**	355	93	33	1.5	11	0.56	4	3	1	1	0	5
a17	**1**	**1**	32	318	24	0.47	55	0.22	8	2	1	1	0	8
a20	**1**	**1**	18	148	14	1.1	21	0.17	4	1	1	1	1	7
a32	**1**	**1**	0.31	137	34	1.6	145	0.88	3	3	1	1	1	7
a26	**1**	**1**	64	220	19	0.02	21	0.15	4	0	0	GFCF	0	8
a44	**1**	**2**	36	46	11	1.5	18	0	4	1	0	0	0	4
a48	**1**	**2**	1375	1594	11	1.4	32	1.8	2	4	0	1	0	3
a41	**1**	**2**	20	693	20	6.4	19	0.85	10	2	1	1	0	7
a42	**1**	**2**	24	181	16	0.42	48	0.27	9	1	1	1	0	3
a9	**1 (d)**	**1**	316	261	13	1.9		0.07	2	1	0	0	0	6
a23	**1 (d)**	**1**	402	1186	56	2.2	29	0.63	2	5	0	0	0	7
a35	**1 (d)**	**1**	348	209	17	0.35	14	0.29	3	1	0	0	0	4
a11	**1 (d)**	**1**	472	335	8.8	0.49	37	0.13	3	2	1	1	0	6
a28		**1**	92	2.2	17	0.85	29	0.24	2	1				
a7		**1**	64	484	24	1.5	14	0.11	5	1				
a13		**1**	40	358	23	0.64	27	0.32	4	1				
a15		**1**	44	106	18	1.1	17	0.17	5	1				
a16		**1**	132	787	17	0.29	18	1.7	2	1				
a2		**1**	47	902	14	3.1		1.6	8	2				
a3		**1**	112	77	29	1.2	7.1	0	11	2				
a4		**1**	711	993	213	3.3	31	0.13	4	5				
a14		**1**	54	282	9.4	0.84	33	0	11	1				
a21		**1**	172	1.2	12	0.83	73	0.08	6	1				
a25		**1**	688	186	18	1.1	15	2.2	2	2				
a38		**1**	21	174	9	5.2	6.8	0.13	15	1				
a43		**2**	198	349	35	0.74	19	0	9	1				

Appendix 5

Metabolomics in Male ASD patients aged 2 to 12yrs (n = 122)

Urinary Succinic Acid Levels and 2- Hydroxyhippuric Acid levels in Male ASD patients aged 2-12 yrs of age (2013-2014) {outliers are marked in light grey for succinic acid and dark grey for 2-hydroxyhippuric acid}

Boys 2-12yrs (2013 to 2014)

person_id	AGE	Succinic Acid (mmol/mol creatinine)	2-Hydroxyhippuric Acid (mmol/mol creatinine)
164	3	0.83	2.3
136	3	1.8	0.24
60	2	2.8	1.9
25	3	5.2	0.24
3	10	5.2	0.59
79	4	5.3	0.29
31	4	5.9	1.4
148	8	6.3	0.69
67	6	8	2.2
158	5	8.1	0.6
41	9	8.1	0.17
9	6	8.8	2.3
162	3	8.9	0.51
73	3	9.4	0.39
100	5	9.4	0.56
27	7	9.7	1.5
115	9	10	0.88
133	2	12	1.4
13	5	12	0.22
167	6	12	1.1
183	3	13	4
142	5	14	0.71
49	6	14	0.45
90	6	14	0.86
176	6	14	1.9
107	10	14	0.36
106	2	15	1.1
178	3	15	1.1
170	6	15	1
130	5	16	1.6
35	2	19	6.2
97	10	19	2
161	5	20	3.5
77	4	21	0.33
39	5	21	3.1
43	5	21	1.4
83	6	21	0.34
169	7	21	1.5
150	7	22	0.7
132	8	22	0.48
40	4	23	1.2
166	6	23	11
23	3	24	3.1
137	2	25	1.1

99	6	25	0.32
156	9	25	0.69
80	4	26	0.63
96	5	26	1.2
78	4	27	1.9
6	6	29	1.1
5	5	32	2.3
92	4	33	2
149	4	33	0.43
48	7	34	1.2
61	7	37	0.89
28	3	38	0.81
33	5	38	1.3
180	4	46	0.55
18	9	46	3.4
95	2	47	1.4
157	4	49	1.3
22	4	61	0.75
140	3	63	2.2
152	3	63	0.91
154	9	64	0.86
65	2	66	1.7
68	6	66	10
112	10	68	0.64
62	9	69	1.3
181	5	74	0.48
165	2	76	1.6
108	4	88	6.7
126	4	134	2.3
51	3		1.1
53	3		1.2
74	3		3
86	3		3.8
145	3		0.51
17	4		2.2
81	4		1.9
88	5		0.87
174	6		1.9
70	7		4.3
4	8		17
45	9		0.67
76	10		1

Appendix 6

Metabolomics in Female ASD Patients aged 2 to 12yrs (n = 20)

Urinary Succinic Acid Levels and 2- Hydroxyhippuric Acid levels in Female ASD patients aged 2-12 yrs of age (2013-2014) {the outlier is marked in dark grey for 2-hydroxyhippuric acid}

person_id	AGE	SEX	Succinic Acid (mmol/mol creatinine)	2-Hydroxyhippuric acid (mmol/mol creatinine)
144	12	f	7.1	0.42
160	11	f	6.8	0.16
87	11	f	13	2.9
36	9	f	14	0.73
153	9	f	22	1.8
119	7	f	16	0.73
182	6	f	7.1	22
50	6	f	26	2.2
175	6	f	43	4.1
177	6	f		7.2
59	5	f	5.5	2.7
159	5	f	13	1.3
16	5	f	26	1.7
89	4	f	5.5	6.4
135	4	f	21	1.3
11	4	f	23	2
93	4	f	44	0.09
163	4	f		1.7
151	3	f	12	1.4
38	2	f	38	1.1

Appendix 7

QPCR stool analysis ASD and control groups (2012) n = 61

ID	Case 1 Control 0	Age	Sex	Firmcutes	Bacteroidetes	Severity	Abdo Symptoms	Supplements	Diet	Meds
a1	1	5	2	67	33					
a2	1	9	2	80	20	2	0	0	0	0
a3	1	5	1	65	35	5	0	1	1	0
a4	1	16	1	48	52	6	1	0	0	0
a5	1	4	2	62	38	3	1	0	0	0
a6	1	6	1	63	37	8	1	1	1	0
a7	1	10	1	64	36					
a8	1	6	2	60	40	5	0	0	0	0
a9	1	6	1	65	35					
a10	1	9	2	68	32	2	0	0	1	0
a11	1	13	1	62	38	6	1	1	1	0
a12	1	4	2	64	36	5	1	0	0	0
a13	1	11	2	51	49					
a14	1	6	1	69	31	8	1	1	1	1
a15	1	4	1	60	40					
a16	1	8	1	53	47					
a17	1	13	1	64	36					
a18	1	11	1	53	47					
a19	1	7	1	69	31	7	0	u	u	0
a20	1	4	1	75	25	7	0	0	0	0
a21	1	3	1	67	33	5	0	0	0	0
a22	1	4	2	76	24	7	0	0	0	0
a23	1	3	1	71	29	7	1	0	0	0
a24	1	5	1	57	43	5	0	0	0	1
a25	1	3	1	53	47					
a26	1	5	2	64	36					
a27	1	3	1	58	42	6	1	0	0	0
a28	1	5	1	65	35	7	1	1	1	0
a29	1	8	1	58	42					
a30	1	8	2	71	29					
a31	1	4	1	61	39					
a32	1	14	2	48	52	9	1	0	0	1
a33	1	7	1	69	31					
a34	1	6	1	62	38					
a35	1	10	2	65	35	5	1	1	1	1
a36	1	6	1	53	47					
a37	1	3	1	52	48					
a38	1	3	1	53	47					
a39	1	4	1	64	36	4	1	0	0	1
a40	1	5	1	57	43					
a41	1	5	1	65	35					
a42	1	14	1	67	33	5	1	1	1	1
a43	1	5	1	66	34	6	1	1	1	0
a44	1	6	1	62	38					
a45	1	5	1	70	30	7	1	1	1	0
a46	1	13	1	63	37					
a47	1	3	1	85	13	7	1	0	0	1
a48	1	2	1	68	32	4	1	1	1	0
a49	1	2	1	67	33	8	1	1	1	1
a50	1	5	2	68	32	8	1	1	1	1
a51	1	8	1	54	46					
a52	1	2	2	62	38	5	1	1	1	0
a53	1	6	1	68	32					
a54	0	2	1	61	39		1	0	1	0
a55	0	1	1	67	33		0	0	0	0
a56	0	16	2	40	60		1	1	1	1
a57	0	11	1	59	41		1	1	0	0
a58	0	7	2	39	61		1	0	0	0
a59	0	2	1	61	39		1	0	1	0
a60	0	12	1	52	48		1	1	0	0
a61	0	56	2	59	41		1	1	1	1

Appendix 8

QPCR Stool Analysis in ASD patients (n= 147)

	Case 1 Control 0	Age	Sex	Firmcutes	Bacteroidetes	Severity	Abdo Symptoms	Supplements	Diet	Meds
c7	0	1	1	67	33		0	0	0	0
c2	0	1.2	1	67	33					
c4	0	1.4	2	60	40					
c6	0	2	1	61	39		1	0	1	0
c11	0	2	1	61	39		1	0	1	0
a48	1	2	1	68	32	4	1	1	1	0
a49	1	2	1	67	33	8	1	1	1	1
a52	1	2	2	62	38	5	1	1	1	0
a80		2	1	59	41					
a86		2	1	61	39					
a93		2	1	63	37					
a105		2	1	68	32					
a110		2	1	69	31					
a114		2	1	70	30					
a117		2	1	71	29					
a118		2	1	72	28					
a121		2	1	74	26					
a124		2	1	77	23					
a137		2	2	62	38					
a21	1	3	1	67	33	5	0	0	0	0
a23	1	3	1	71	29	7	1	0	0	0
a25	1	3	1	53	47					
a27	1	3	1	58	42	6	1	0	0	0
a37	1	3	1	52	48					
a38	1	3	1	53	47					
a47	1	3	1	85	13	7	1	0	0	1
a54		3	1	44	56					
a56		3	1	46	54					
a62		3	1	52	48					
a65		3	1	53	47					
a76		3	1	58	42					
a77		3	1	58	42					
a78		3	1	58	42					
a97		3	1	64	36					
a126		3	1	78	22					
a127		3	1	79	21					
a130		3	1	81	19					
a131		3	1	87	13					
a134		3	2	56	44					
a142		3	2	66	34					
a5	1	4	2	62	38	3	1	0	0	0
a12	1	4	2	64	36	5	1	0	0	0
a15	1	4	1	60	40					
a20	1	4	1	75	25	7	0	0	0	0
a22	1	4	2	76	24	7	0	0	0	0
a31	1	4	1	61	39					
a39	1	4	1	64	36	4	1	0	0	1
a55		4	1	45	55					
a58		4	1	48	53					
a60		4	1	51	49					

a63		4	1	52	48					
a64		4	1	52	48					
a68		4	1	55	45					
a70		4	1	56	44					
a72		4	1	57	43					
a73		4	1	57	43					
a90		4	1	62	38					
a98		4	1	64	36					
a111		4	1	69	31					
a119		4	1	72	28					
a139		4	2	64						
a146		4	2	76	24					
a1	1	5	2	67	33					
a3	1	5	1	65	35	5	0	1	1	0
a24	1	5	1	57	43	5	0	0	0	1
a26	1	5	2	64	36					
a28	1	5	1	65	35	7	1	1	1	0
a40	1	5	1	57	43					
a41	1	5	1	65	35					
a43	1	5	1	66	34	6	1	1	1	0
a45	1	5	1	70	30	7	1	1	1	0
a50	1	5	2	68	32	8	1	1	1	1
a57		5	1	47	53					
a69		5	1	55	45					
a74		5	1	57	43					
a81		5	1	59	41					
a83		5	1	60	40					
a87		5	1	61	39					
a94		5	1	63	37					
a101		5	1	65	35					
a106		5	1	68	32					
a120		5	1	73	27					
a123		5	1	75	25					
a140		5	2	64	36					
a143		5	2	67	33					
a144		5	2	70	30					
c5	0	6	2	60	40					
a6	1	6	1	63	37	8	1	1	1	0
a8	1	6	2	60	40	5	0	0	0	0
a9	1	6	1	65	35					
a14	1	6	1	69	31	8	1	1	1	1
a34	1	6	1	62	38					
a36	1	6	1	53	47					
a44	1	6	1	62	38					
a53	1	6	1	68	32					
a61		6	1	51	49					
a75		6	1	57	43					
a82		6	1	59	41					
a84		6	1	60	40					
a88		6	1	61	39					
a89		6	1	61	39					
a95		6	1	63	37					
a96		6	1	63	37					
a102		6	1	65	35					

a103		6	1	65	35					
a125		6	1	77	23					
a128		6	1	79	21					
a132		6	2	51	49					
a138		6	2	62	38					
c3	0	7	2	39	61					
c10	0	7	2	39	61		1	0	0	0
a19	1	7	1	69	31	7	0	u	u	0
a33	1	7	1	69	31					
a99		7	1	64	36					
a112		7	1	69	31					
a115		7	1	70	30					
a136		7	2	57	43					
a16	1	8	1	53	47					
a29	1	8	1	58	42					
a30	1	8	2	71	29					
a51	1	8	1	54	46					
a66		8	1	53	47					
a79		8	1	58	42					
a91		8	1	62	38					
a107		8	1	68	32					
a122		8	1	74	26					
a2	1	9	2	80	20	2	0	0	0	0
a10	1	9	2	68	32	2	0	0	1	0
a92		9	1	62	38					
a108		9	1	68	32					
a116		9	1	70	30					
a135		9	2	56	44					
a141		9	2	65	35					
a147		9	2	80	20					
c1	0	10	1	52	48					
a7	1	10	1	64	36					
a35	1	10	2	65	35	5	1	1	1	1
a85		10	1	60	40					
a109		10	1	68	32					
c9	0	11	1	59	41		1	1	0	0
a13	1	11	2	51	49					
a18	1	11	1	53	47					
a100		11	1	64	36					
a133		11	2	53	47					
a145		11	2	75	25					
c12	0	12	1	52	48		1	1	0	0
a11	1	13	1	62	38	6	1	1	1	0
a17	1	13	1	64	36					
a46	1	13	1	63	37					
a71		13	1	56	44					
a113		13	1	69	31					
a129		13	1	79	21					
a32	1	14	2	48	52	9	1	0	0	1
a42	1	14	1	67	33	5	1	1	1	1
a104		14	1	67	33					
c8	0	16	2	40	60		1	1	1	1
a4	1	16	1	48	52	6	1	0	0	0
a59		17	1	48	52					
a67		21	1	53	47					
c13	0	56	2	59	41		1	1	1	1

Appendix 9

Zinc, Manganese and Chromium levels, age and sex in control population under 16yrs of age (n = 231)

Case/Control	AGE	SEX	SEX_01	Plasma Zinc	Plasma Chromium	Plasma Manganese
0	1	M	0	11	15.5	10.8
0	2	M	0	11.2	17.2	13.9
0	2	F	1	10.9	12.4	12.2
0	2	M	0	9.8	10.4	10.6
0	2	M	0	13.4	13.8	24.3
0	2	F	1	8.4	10.9	12
0	3	M	0	13	18.5	18.6
0	3	F	1	11.6	13.6	22.5
0	3	M	0	12.9	14.8	19.2
0	3	F	1	12.4	25	10.6
0	3	M	0	9.4	19.9	19.1
0	3	M	0	11.8	13	14.9
0	3	F	1	15.9	19	28.7
0	3	M	0	7.7	12.1	12.1
0	3	M	0	11.8	13.9	20.4
0	4	M	0	11.9	16.1	12.6
0	4	M	0	11.4	16.9	10.8
0	4	M	0	9.8	14	12.7
0	4	M	0	9	15.3	14
0	4	M	0	9.8	25.4	12
0	4	M	0	13.3	13	20.1
0	4	M	0	11.5	17.6	18.1
0	4	M	0	11.9	9.7	18
0	4	M	0	12.7	8.4	12.6
0	4	M	0	12.2	8.5	11.4
0	4	M	0	10.8	22.6	16.6
0	4	F	1	11.7	11.6	9
0	4	M	0	9.6	12.8	13.1
0	4	M	0	9.8	14.7	13.1
0	4	M	0	10.9	20.3	18.1
0	4	M	0	11.2	11.2	27.9
0	4	M	0	11	11.9	9.3
0	5	F	1	10.6	95.1	15.1
0	5	M	0	9.6	11.9	8.5
0	5	M	0	12.5	14.8	10.6
0	5	M	0	10.2	21.2	14.2
0	5	M	0	11.2	21.3	14.3
0	5	M	0	14.1	14.2	14.1
0	5	M	0	9.5	17.5	14.2
0	5	F	1	14.2	11.5	9
0	5	M	0	15.2	13.2	20.1
0	5	M	0	13.1	14.8	18.9
0	5	M	0	12.2	14.9	17.8
0	5	M	0	9.8	10.9	14.6

0	5	F	1	9.7	13.2	15.2
0	5	M	0	11.5	12	12.2
0	6	M	0	10	15.8	10.1
0	6	F	1	11.3	17.4	14.9
0	6	M	0	10.8	14.2	21.5
0	6	M	0	10	15.1	11.6
0	6	F	1	9.7	15.4	38.2
0	6	M	0	20.7	15.1	12.6
0	6	F	1	16.3	17.3	12.9
0	6	M	0	11.5	16.4	15.6
0	6	M	0	9.8	21.2	14.6
0	6	F	1	10.5	13	12.4
0	6	M	0	9.7	15.7	8.7
0	6	M	0	8.1	15.2	14.9
0	6	M	0	12.8	25.9	13.8
0	6	M	0	14.6	19.1	13.7
0	6	M	0	11.4	15.3	20
0	6	F	1	20.4	11.7	6.5
0	6	M	0	12.7	14.8	10.9
0	6	M	0	9.8	15.1	12.6
0	6	M	0	14	10	15.1
0	6	F	1	9.9	13.6	9.7
0	6	M	0	11.9	15.8	7.8
0	6	F	1	11.7	16.3	12.5
0	6	M	0	10.7	15.3	12.3
0	7	M	0	8.31	19.8	16.3
0	7	F	1	10.8	16.7	12.6
0	7	F	1	10.4	14.5	9.6
0	7	F	1	10.2	13.9	12.2
0	7	F	1	10.1	20.6	10.4
0	7	M	0	10.6	27.9	20
0	7	F	1	13	14.8	13.6
0	7	M	0	9.8	11.8	13.3
0	7	M	0	11.1	18.5	15.7
0	7	F	1	11.9	18.7	11.3
0	7	M	0	12.9	13.8	9.2
0	7	M	0	10.1	13.6	10.9
0	7	M	0	14.1	12.9	19.3
0	7	M	0	12.5	12.9	13.2
0	7	F	1	12.7	14.4	18.6
0	7	M	0	9.3	14.6	14.3
0	8	M	0	10.5	22.1	12.9
0	8	F	1	10.8	23.3	14.9
0	8	M	0	13	14.7	15.2
0	8	M	0	9.5	18.1	10.3
0	8	F	1	13.2	21.5	22.1
0	8	M	0	10.5	11.9	11.9
0	8	F	1	10.4	11.5	12.6
0	9	F	1	13.7	16.6	17
0	9	F	1	9.4	13	7.7
0	9	F	1	11.6	19.6	19.8
0	9	F	1	12.5	20.4	18.9
0	9	M	0	9.3	16.7	16.6
0	9	F	1	8.5	14.1	11.8

0	9	M	0	8.5	15.8	17.2
0	9	M	0	10.6	14.6	11.4
0	9	M	0	12.4	11.4	16.3
0	9	M	0	9.6	12.4	11.2
0	9	M	0	12.3	12.6	8.4
0	9	F	1	11.7	10	13.7
0	9	M	0	13.4	11.8	10.9
0	9	F	1	9.47	10.4	12.7
0	10	M	0	10.9	18.2	12.5
0	10	F	1	13.3	13.5	15.6
0	10	F	1	9.8	18.1	18.1
0	10	F	1	12	19.1	16.6
0	10	F	1	13.1	18.8	15.4
0	10	M	0	9.6	21	8.2
0	10	F	1	10.5	16.5	12
0	10	F	1	13	24.3	13.1
0	10	M	0	11.9	9.6	12.5
0	10	M	0	13.6	18.5	18.9
0	10	M	0	11	15.4	40.9
0	11	M	0	20.5	14.9	15.4
0	11	F	1	11.3	12.5	11.8
0	11	M	0	11.1	5.1	13.7
0	11	F	1	11	18.2	12.1
0	11	F	1	14.7	19.4	31
0	11	F	1	9.8	15.4	12.3
0	11	F	1	12.9	11.8	10.7
0	11	F	1	14.5	19.9	16.3
0	11	F	1	12.7	18.6	16.2
0	11	F	1	11.8	13.3	12.5
0	11	M	0	9	12.9	12.5
0	11	F	1	13.8	16.4	12.5
0	11	M	0	13.1	15.8	11.6
0	11	F	1	12.3	13.5	12.2
0	11	F	1	10.4	17.3	19.3
0	11	M	0	9.3	17.8	6.8
0	11	M	0	13	11.9	13.5
0	11	M	0	10.8	8.6	12.4
0	12	F	1	13.4	16.4	17.8
0	12	F	1	12.5	13.2	14.5
0	12	F	1	11.5	13.6	11.3
0	12	M	0	10	18.5	23.6
0	12	F	1	11.7	15.8	9.4
0	12	F	1	13.6	21.6	21.2
0	12	F	1	9.6	25.4	12.6
0	12	M	0	12.7	25.9	16.9
0	12	F	1	10.5	19.2	10.2
0	12	F	1	14.3	13.7	21.4
0	12	M	0	9.1	11.8	7.9
0	12	M	0	11.4	14.2	10.4
0	13	F	1	11.2	15.6	11.6
0	13	F	1	10.4	15.5	14
0	13	M	0	9.4	19	16.1
0	13	M	0	13.3	16.3	11.7
0	13	F	1	10.2	13.6	32.2

0	13	M	0	13.1	20.8	16.2
0	13	M	0	14.6	22.6	26.9
0	13	M	0	10.8	15.5	12.7
0	13	F	1	9	12.1	15.5
0	13	F	1	14.3	11.5	13.9
0	13	M	0	14.3	10	13.4
0	13	F	1	13.2	10.9	14.8
0	13	M	0	8.2	16.8	14.5
0	13	F	1	12.4	15.1	20.4
0	13	F	1	11	8.2	10.8
0	13	M	0	11.2	34.9	13.1
0	14	F	1	13.3	20.8	12.8
0	14	F	1	10.3	12.9	11.1
0	14	F	1	11	5.8	10
0	14	F	1	12.1	19	12.6
0	14	F	1	14.8	22.1	12.8
0	14	M	0	15.4	17.5	25.6
0	14	F	1	12.6	14.1	13.6
0	14	F	1	14.2	18.6	15.3
0	14	F	1	10.2	19.8	13.7
0	14	F	1	10	22.6	11.4
0	14	F	1	11.8	15.6	26.8
0	14	M	0	14.6	8.6	11
0	14	M	0	10.8	12	16.3
0	14	M	0	10.4	9.9	14.4
0	15	F	1	14.6	15.1	22.1
0	15	F	1	13	23.1	16.7
0	15	M	0	13.9	20.2	13.9
0	15	M	0	10	17.4	14.9
0	15	F	1	14.6	23.2	16.4
0	15	M	0	11.5	24.4	17.1
0	15	F	1	10.3	11.9	7.94
0	15	F	1	12	13.9	19.9
0	15	F	1	9.8	11.6	9.1
0	15	F	1	11.9	14.2	14.1
0	15	M	0	11	17.6	12.6
0	15	F	1	12.2	17.9	13.6
0	15	F	1	11.5	22.6	16.3
0	15	M	0	11.8	14.1	12.7
0	15	F	1	11.9	11.5	17.3
0	15	M	0	16.7	16.8	16.5
0	15	F	1	11.6	12.3	9.5
0	15	F	1	11.6	18.3	13.3
0	15	F	1	21.2	50.7	9.7
0	15	M	0	14.8	26.6	14
0	15	F	1	14.3	9.2	14.9
0	15	F	1	13.3	18.8	17.6
0	15	F	1	12.8	9.5	9.8
0	15	M	0	11.8	12.1	12.6
0	15	M	0	14.9	15.8	13.6
0	16	F	1	13.2	16.4	10.9
0	16	F	1	18.4	22.1	12.9
0	16	F	1	12.7	13.5	15.6
0	16	F	1	11.6	13.9	11.3

0	16	F	1	11.2	23.9	16.7
0	16	M	0	8.7	18	16.1
0	16	F	1	10.4	14.2	9.9
0	16	M	0	13.5	12.5	11.3
0	16	F	1	10	16.2	8.6
0	16	F	1	10.3	14	8.3
0	16	F	1	11.8	16.6	11.1
0	16	M	0	9.7	16.3	19.2
0	16	M	0	14.8	18.5	19.4
0	16	M	0	11.5	17	12.5
0	16	M	0	10.8	21.2	15.6
0	16	M	0	10.2	17.8	11
0	16	M	0	9.9	17.3	21.2
0	16	M	0	13.9	15.5	22.6
0	16	F	1	11.5	17	6.6
0	16	M	0	10.2	24	15.8
0	16	M	0	14.8	21	13.6
0	16	F	1	9.1	19.5	8.5
0	16	F	1	9.3	18.8	11.5
0	16	M	0	11.6	17.6	11.5
0	16	M	0	11.5	14.7	10.9
0	16	M	0	10.9	16.2	11.7
0	16	M	0	10.5	11.9	12.3
0	16	F	1	10.4	7	8.6
0	16	M	0	13.9	16.7	14.3
0	16	M	0	11.7	12.9	9.3
0	16	M	0	10.5	19.9	20.8
0	16	F	1	11.9	13.9	9.1

Appendix 10

Zinc, chromium, manganese and total lymphocyte count in ASD patients (<16yrs) n = 72

Person Id	SEX	AGE	WCC	Lymph	Mono	Neurto	Eosino	Baso	Plasma Cr	Plasma Mn	Plasma Zn
	M	15	4.16	1.69	0.36	1.93	0.14	0.04	25.4	6.9	11
173	M	14	5.05	2.8	0.4	1.68	0.13	0.04	15.4	10.4	10.8
47	M	14	4.13	1.99	0.25	1.68	0.17	0.04	13.5	19.7	8.6
47	M	14	5.74	2.55	0.42	2.43	0.29	0.05	13.5	19.7	8.6
	M	14	6.4	2.78	0.47	2.92	0.17	0.06	15	12.2	11
37	M	14	6.01	1.85	0.44	3.35	0.31	0.06	20	20.8	9.4
	F	14	7.9	3.18	0.51	3.88	0.2	0.08	12.6	8.6	10.9
122	M	14	10.02	2.92	0.47	6.4	0.17	0.06	15	12.2	11
105	M	13	7.09	3.11	0.39	3.4	0.16	0.03	10.7	9	12.1
	M	13	5.53	2.37	0.45	2.15	0.5	0.06	10.8	15.7	11.7
	F	12	7.44	2.39	0.34	4.53	0.15	0.03	12.8	11.2	9.6
	F	12	4.7	0.9	0.69	3.09	0	0.03	11.1	8.1	9.5
	F	11	5.5	2.41	0.4	2.49	0.08	0.07	15.3	7.9	10.6
	F	10	8.7	3.62	0.41	3.53	1.01	0.11	15	12.2	10.6
131	M	10	8.31	3.38	0.61	4.06	0.15	0.11	18.7	14.9	8.8
	F	10	8.2	3.16	0.5	4.41	0.07	0.1	12.8	15.4	11.4
115	M	10	6.64	2.55	0.45	3.31	0.22	0.11	19.1	24.7	9.7
	M	10	7.2	2.54	0.49	3.83	0.18	0.12	19.1	24.7	9.7
32	M	10	6.01	1.85	0.44	3.35	0.31	0.06	11.9	7.9	9.8
118	F	9	2.65	1.64	0.24	0.7	0.05	0.02	11.4	9.8	11.7
	M	9	10.76	3.62	0.87	5.32	0.87	0.09	16.5	12	8.8
	M	9	8.5	2.17	0.66	5.43	0.18	0.1	9.3	13.4	7.8
4	M	8	5.72						17.5	15.7	8.5
132	M	8	7.22	3.25	0.46	3.23	0.25	0.03	16.3	12.2	8.5
62	M	8	8.29	3.22	0.35	4.5	0.14	0.08	9.8	14.1	8.5
	M	8	13.8	5.21	0.75	7	0.66	0.18	13.9	16.7	9.8
	M	8	6.54	2.42	0.51	2.95	0.59	0.07	40.4	10	10.8
	M	7	5.65	2.43	0.5	2.63	0.07	0.02	18.1	28.8	22.8
	M	7	8.29	3.32	0.51	3.38	1.03	0.05	21.4	12.2	11.3
70	M	7	10.5	3.88	0.67	5.24	0.55	0.16	12.9	14.7	6.8
176	M	7	5.47	1.71	0.38	3.18	0.19	0.01	10.5	12.4	11.2
	F	6	6	3.57	0.46	1.86	0.08	0.05	13.7	13.8	11.2
	M	6	7.18	3.62	0.81	2.48	0.25	0.02	19.9	14.1	9.7
67	M	6	7.39	3.55	0.37	2.76	0.61	0.1	24.7	8	10.3
90	M	6	7.78	3.54	0.27	3.24	0.69	0.04	14.7	20.7	9.8
111	M	6	9.81	4.45	0.65	3.89	0.69	0.13	11.9	9.2	10.7
134	M	6	5.67	0.46	0.56	3.98	0.42	0.25	12.8	12.1	10.5
20	M	6	4.27	0.15	0.32	3.55	0.19	0.06	10	16.8	10.4
	M	5	7.94	5.34	0.43	1.5	0.62	0.05	14.2	16.9	12.1
100	M	5	6.69	4.15	0.41	1.9	0.13	0.1	14.1	14.4	10.6
139	M	5	7.29	4.28	0.46	2	0.41	0.14	17.8	9.4	10.4
	M	5	9.17	5.22	0.51	3.26	0.13	0.05	18	22.5	7.3
108	M	5	15.5	8.65	0.82	5.38	0.53	0.12	18	22.5	7.3
181	M	5	7.06	3.93	0.39	2.55	0.1	0.09	13.3	11.9	10.5
9	M	5	6.92	3.37	0.46	2.74	0.2	0.15	13.5	12	11.9
130	M	5	9.69	4.5	0.54	3.67	0.93	0.05	14.2	12.4	12
	M	5	8.1	3.35	0.48	4.1	0.04	0.13	12.3	9.7	7.7
	M	5	9.1	3.69	0.88	4.32	0.19	0.02	14.5	8.6	10
	M	5	9.7	3.91	0.66	5.03	0.12	0.01	13.6	18	10.1
145	M	5	8.2	3.11	0.5	4.13	0.41	0.05	13.9	10.3	9.8
	M	5	9.78	3.63	0.73	5.01	0.36	0.05	16.3	26.7	9.7

	M	4	10.43	6.15	0.73	3.25	0.25	0.04	12.2	13.7	9.8
	M	4	7.81	4.57	0.47	2.27	0.44	0.06	13	12.3	8.6
	M	4	5.4	3.12	0.27	1.88	0.1	0.02	14.9	10.7	10.6
	M	4	3.1	1.6	0.49	0.87	0.1	0.04	13.2	12.4	12
126	M	4	9.35	4.65	0.84	3.44	0.26	0.16	13	12.1	9.5
	M	4	8.33	3.93	0.69	3.53	0.14	0.03	16.2	16.8	13.5
	M	4	8.49	3.76	0.57	3.94	0.2	0.02	13	10.1	9.2
104	F	4	4.03	1.44	0.47	2	0.04	0.08	9.4	12.7	7.8
	F	4	10.04	3.01	0.55	6.38	0.08	0.02	17.2	25.5	8.21
64	F	4	20.25	4.68	1.17	12.98	1.24	0.18	11.7	12.6	9.2
73	M	3	14.18	9.08	0.72	4.14	0.17	0.07	19.1	37.8	12.6
15	M	3	7.74	4.85	0.56	2.06	0.11	0.16	20.6	20.1	7.8
73	M	3	13.37	8.09	0.6	4.36	0.27	0.05	18.8	22.1	9.9
106	M	3	8.33	4.66	0.83	2.72	0.09	0.03	13.2	24.8	11.5
74	M	3	17.89	9.36	1.09	6.37	1.02	0.05	15.8	17.4	11.6
152	M	3	9.83	5.14	0.69	3.82	0.12	0.06	15.2		
	M	3	10.38	4.99	0.71	4.58	0.06	0.04	23.3	24.6	13.3
157	M	3	7.5	2.96	0.68	3.59	0.26	0.01	8.8	11.8	10.1
136	M	2	13.52	9.58	0.53	3.08	0.21	0.12	23.4	16.9	8.4
129	M	2	12.39	7.06	0.69	4.35	0.27	0.02	14.6	12.3	11.9

Appendix 11

Group statistics and SPSS tables for zinc group analysis of casein-free diet, supplements and gluten free diet

Group Statistics (Casein Free Diet)					
CF Current		N	Mean	Std. Deviation	Std. Error Mean
WCC	Y	21	8.79	3.84	0.84
	N	20	8.29	3.71	0.83
Lymph	Y	21	4.44	2.82	0.62
	N	20	3.35	1.47	0.33
Mono	Y	21	0.57	0.21	0.05
	N	20	0.51	0.21	0.05
Neurto	Y	21	3.44	1.37	0.30
	N	20	3.98	2.45	0.55
Eosino	Y	21	0.26	0.22	0.05
	N	20	0.37	0.32	0.07
Baso	Y	21	0.08	0.06	0.01
	N	20	0.08	0.05	0.01
Plasma Zn	Y	21	9.60	1.51	0.33
	N	19	10.09	1.54	0.35
Plasma Cr	Y	21	15.51	3.85	0.84
	N	20	13.80	3.51	0.79
Plasma Mn	Y	21	17.48	6.78	1.48
	N	19	12.22	3.15	0.72

Independent Samples Test (Caesin Free Diet)									
	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper

WCC	Equal variances assumed	.735	.396	.429	39	.670	0.51	1.18	-1.88	2.89
	Equal variances not assumed			.430	38.989	.670	0.51	1.18	-1.88	2.89
Lymph	Equal variances assumed	7.818	.008	1.543	39	.131	1.09	0.71	-0.34	2.53
	Equal variances not assumed			1.566	30.489	.128	1.09	0.70	-0.33	2.52
Mono	Equal variances assumed	.114	.737	.897	39	.375	0.06	0.06	-0.07	0.19
	Equal variances not assumed			.897	38.949	.375	0.06	0.06	-0.07	0.19
Neurto	Equal variances assumed	.587	.448	-.878	39	.385	-0.54	0.62	-1.79	0.71
	Equal variances not assumed			-.867	29.531	.393	-0.54	0.62	-1.82	0.73
Eosino	Equal variances assumed	4.627	.038	-1.279	39	.208	-0.11	0.09	-0.28	0.06
	Equal variances not assumed			-1.267	32.983	.214	-0.11	0.09	-0.28	0.07
Baso	Equal variances assumed	1.296	.262	.317	39	.753	0.01	0.02	-0.03	0.04
	Equal variances not assumed			.319	37.543	.751	0.01	0.02	-0.03	0.04
Plasma Zn	Equal variances assumed	.000	.986	-1.036	38	.307	-0.50	0.48	-1.48	0.48
	Equal variances not assumed			-1.035	37.466	.307	-0.50	0.48	-1.48	0.48
Plasma Cr	Equal variances assumed	1.659	.205	1.491	39	.144	1.72	1.15	-0.61	4.05
	Equal variances not assumed			1.495	38.931	.143	1.72	1.15	-0.61	4.05
Plasma Mn	Equal variances assumed	7.909	.008	3.091	38	.004	5.26	1.70	1.82	8.70
	Equal variances not assumed			3.195	28.882	.003	5.26	1.65	1.89	8.63

Group Statistics (Supplements)					
Supp Current		N	Mean	Std. Deviation	Std. Error Mean
WCC	Y	9	8.74	3.04	1.01
	N	32	8.49	3.95	0.70
Lymph	Y	9	4.45	1.82	0.61
	N	32	3.76	2.43	0.43
Mono	Y	9	0.58	0.18	0.06
	N	32	0.53	0.22	0.04
Neurto	Y	9	3.31	1.12	0.37
	N	32	3.82	2.15	0.38
Eosino	Y	9	0.33	0.31	0.10
	N	32	0.30	0.27	0.05
Baso	Y	9	0.07	0.04	0.01
	N	32	0.08	0.06	0.01
Plasma Zn	Y	8	10.59	1.55	0.55
	N	32	9.64	1.48	0.26
Plasma Cr	Y	9	14.64	2.68	0.89
	N	32	14.68	4.03	0.71
Plasma Mn	Y	8	15.93	6.94	2.45
	N	32	14.75	5.76	1.02

Independent Samples Test (Supplements)										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
WCC	Equal variances assumed	.620	.436	.173	39	.864	0.25	1.43	-2.64	3.13

	Equal variances not assumed			.200	16.445	.844	0.25	1.23	-2.36	2.85
Lymph	Equal variances assumed	1.011	.321	.782	39	.439	0.68	0.87	-1.08	2.45
	Equal variances not assumed			.921	16.969	.370	0.68	0.74	-0.88	2.25
Mono	Equal variances assumed	.015	.904	.614	39	.543	0.05	0.08	-0.11	0.21
	Equal variances not assumed			.686	15.348	.503	0.05	0.07	-0.10	0.20
Neurto	Equal variances assumed	.859	.360	-.685	39	.497	-0.51	0.75	-2.02	1.00
	Equal variances not assumed			-.962	25.956	.345	-0.51	0.53	-1.61	0.58
Eosino	Equal variances assumed	1.037	.315	.295	39	.769	0.03	0.10	-0.18	0.24
	Equal variances not assumed			.275	11.737	.788	0.03	0.11	-0.22	0.28
Baso	Equal variances assumed	.833	.367	-.237	39	.814	-0.00	0.02	-0.05	0.04
	Equal variances not assumed			-.292	18.593	.774	-0.00	0.02	-0.04	0.03
Plasma Zn	Equal variances assumed	.249	.620	1.596	38	.119	0.94	0.59	-0.25	2.14
	Equal variances not assumed			1.555	10.460	.150	0.94	0.61	-0.40	2.29
Plasma Cr	Equal variances assumed	2.019	.163	-.028	39	.978	-0.04	1.43	-2.94	2.86
	Equal variances not assumed			-.035	19.350	.973	-0.04	1.14	-2.43	2.35
Plasma Mn	Equal variances assumed	1.743	.195	.497	38	.622	1.18	2.37	-3.62	5.97
	Equal variances not assumed			.443	9.549	.667	1.18	2.66	-4.78	7.14

Group Statistics (Gluten Free Diet)

GF Current		N	Mean	Std. Deviation	Std. Error Mean
WCC	Y	19	9.11	3.92	0.90
	N	22	8.06	3.60	0.77
Lymph	Y	19	4.85	2.70	0.62
	N	22	3.10	1.55	0.33
Mono	Y	19	0.57	0.22	0.05
	N	22	0.51	0.19	0.04
Neurto	Y	19	3.33	1.28	0.29
	N	22	4.03	2.39	0.51
Eosino	Y	19	0.28	0.22	0.05
	N	22	0.33	0.32	0.07
Baso	Y	19	0.08	0.05	0.01
	N	22	0.08	0.06	0.01
Plasma Zn	Y	19	9.76	1.65	0.38
	N	21	9.90	1.44	0.32
Plasma Cr	Y	19	15.50	3.90	0.90
	N	22	13.96	3.54	0.75
Plasma Mn	Y	19	17.54	6.74	1.55
	N	21	12.67	4.00	0.87

Independent Samples Test (Gluten Free Diet)

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
WCC	Equal variances assumed	.620	.436	.173	39	.864	0.25	1.43	-2.64	3.13
	Equal variances not assumed			.200	16.445	.844	0.25	1.23	-2.36	2.85
Lymph	Equal variances assumed	1.011	.321	.782	39	.439	0.68	0.87	-1.08	2.45
	Equal variances not assumed			.921	16.969	.370	0.68	0.74	-0.88	2.25
Mono	Equal variances assumed	.015	.904	.614	39	.543	0.05	0.08	-0.11	0.21
	Equal variances not assumed			.686	15.348	.503	0.05	0.07	-0.10	0.20
Neurto	Equal variances assumed	.859	.360	-.685	39	.497	-0.51	0.75	-2.02	1.00
	Equal variances not assumed			-.962	25.956	.345	-0.51	0.53	-1.61	0.58
Eosino	Equal variances assumed	1.037	.315	.295	39	.769	0.03	0.10	-0.18	0.24
	Equal variances not assumed			.275	11.737	.788	0.03	0.11	-0.22	0.28
Baso	Equal variances assumed	.833	.367	-.237	39	.814	-0.00	0.02	-0.05	0.04
	Equal variances not assumed			-.292	18.593	.774	-0.00	0.02	-0.04	0.03
Plasma Zn	Equal variances assumed	.249	.620	1.596	38	.119	0.94	0.59	-0.25	2.14
	Equal variances not assumed			1.555	10.460	.150	0.94	0.61	-0.40	2.29
Plasma Cr	Equal variances assumed	2.019	.163	-.028	39	.978	-0.04	1.43	-2.94	2.86
	Equal variances not assumed			-.035	19.350	.973	-0.04	1.14	-2.43	2.35
Plasma Mn	Equal variances assumed	1.743	.195	.497	38	.622	1.18	2.37	-3.62	5.97
	Equal variances not assumed			.443	9.549	.667	1.18	2.66	-4.78	7.14

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