THE PERIPHERAL IMMUNOPHENOTYPE IN NEURODEVELOPMENTAL DISORDERS

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A THESIS

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Abstract

The factors contributing to the severity of the neurodevelopmental disorders autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) are largely unknown. Previous studies have indicated immune abnormalities in these disorders, such as increased inflammation and altered immune cell numbers. We, in collaboration with the Province of Ontario Neurodevelopmental Disorder (POND) Network, analyzed markers of intestinal permeability and inflammation in children diagnosed with ASD or ADHD, as well as typically developing controls. Plasma from these participants was used to investigate levels of soluble inflammation, denoted by circulating acute phase proteins, as well as circulating levels of markers of intestinal epithelial damage and bacterial translocation. Peripheral blood mononuclear cells were isolated from these participants and used to construct an immunophenotype of ASD and ADHD, focusing on monocytes and monocyte activation and maturation. These data were then compared with scores of behaviour severity to identify associations between inflammation and behaviour in these disorders.

We identified increased soluble inflammation in ASD, indicated by increased circulating C-reactive protein. We associated this inflammation with intestinal permeability, indicated by increased circulating LPS. Classical monocyte frequency was significantly lower in ASD and these monocytes displayed an altered migratory phenotype, indicated by a reduction in CCR2 expression. Furthermore, we have identified potential maladaptive monocyte responses to soluble inflammation in both ASD and ADHD, with altered monocyte phenotypes in response to inflammatory mediators compared to typically developing controls. Finally, we identified that changes in monocyte phenotype are associated with more severe behaviours in both ASD and ADHD. These findings imply that inflammation and immune abnormalities contribute to the severity of neurodevelopmental disorders.

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Abbreviations

ASD	Autism spectrum disorder
ADHD	Attention deficit hyperactivity disorder
TD	Typically developing
PBMCs	Peripheral blood mononuclear cells
APPs	Acute phase proteins
CRP	C-reactive protein
tPA	Tissue plasminogen activator
I-FABP	Intestinal fatty acid binding protein
LPS	Lipopolysaccharide
MDP	Muramyl dipeptide
GI	Gastrointestinal
ELISA	Enzyme-linked immunosorbent assay
ABAS	Adaptive behaviour assessment system
CBCL	Child behaviour checklist
ADOS	Autism diagnostic observation scale
ADI-R	Autism diagnostic interview – Revised
VABS	Vineland adaptive behaviour scale
NF-κB	Nuclear factor kappa B
TLR	Toll-like receptor
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
PAMP	Pathogen associated molecular pattern
DAMP	Damage associated molecular pattern

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Chapter One

Introduction

1.1) Neurodevelopmental disorders:

Neurodevelopmental disorders are identified by cognitive and behavioural deficits, typically accompanied by genetic and brain abnormalities. They occur early in development and are caused by a combination of genetic and environmental factors[1]. The two neurodevelopmental disorders investigated in this thesis are autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD).

1.2) Autism spectrum disorder

1.21) Characteristics of autism spectrum disorder

ASD is a heterogeneous disorder characterized by severe impairments in social interaction and communication, as well as restrictive and repetitive behaviours[2]. Approximately 1:68 children are diagnosed with autism and ASD prevalence has increased in recent decades, potentially due to improved diagnosis[3]. The disorder is 4.5 times more common in males than in females[3]. The reason for this male dominance is not fully understood. One hypothesis is that females are under identified when using traditional diagnostic methods, which are aimed to recognize a male ASD phenotype[4]. Another hypothesis is that females are better at "masking" their ASD behaviours, thus they are not recognized as having a disorder[5].

ASD manifests in many ways, with symptoms ranging from mild social impairments to severe mental disability. The etiology of ASD remains elusive. A combination of genetic and environmental factors contribute to the risk of ASD; however, no single gene is associated with an ASD phenotype. Due to the heterogeneity of the disorder, it is believed that there are multiple etiologies, with many genes and environmental factors associated with an elevated risk[6]. The

wide variety of phenotypes complicates studies of the underlying causes of the disorder. There is currently no widely used treatment for ASD outside of behavioral modification programs.

1.22) The immune system in ASD

There is some evidence that the immune system contributes to the risk of developing ASD. After the rubella epidemic in the United States in 1964, researchers noticed an increase in autism prevalence in children that contracted congenital rubella after their mothers were infected during pregnancy[7]. Furthermore, a study conducted using the Danish Medical Birth Register indicated a link between maternal viral infection requiring hospitalization in the first trimester of pregnancy and ASD development[8]. Similar observations have been made using mouse models. When pregnant mice were infected with the human influenza virus mid-gestation, the offspring had behavioural abnormalities such as deficiencies in social interaction and exploratory behaviour, as well as heightened sensory sensitivity[9]. Similarly, when pregnant mice were infected with synthetic double stranded RNA poly (I:C), which mimics viral infection, offspring displayed several autism-like behaviours such as deficits in communication and social interaction[10]. These mice also have elevated circulating levels of the pro-inflammatory cytokine IL-6[11]. It is hypothesized that maternal infection during pregnancy could lead to dysregulation of the fetal and newborn immune system during key developmental windows.

There is evidence of immune changes or immune abnormalities in individuals with ASD. In 1977, Stubbs and Crawford observed depressed function of lymphocytes from ASD subjects after *in vitro* stimulation with a mitogen[12]. Over the years, it has been found that individuals with ASD have higher levels of circulating pro-inflammatory cytokines[13–15] as well as increased cytokine production by peripheral blood mononuclear cells (PBMCs) from ASD subjects after stimulation in vitro[13,16], however, due to small sample size and inconsistency with characterizing

patients, many of these observations have not been replicated. Others reported differences in the numbers or proportions of leukocytes in ASD, such as increased circulating monocytes, myeloid dendritic cells, NK cells and B cells[3,18,19]. These studies have also been difficult to replicate. For example, some studies have found that monocyte numbers increased in ASD while others have found no difference[17,20,21].

T cells have also been of interest. Studies have found no difference in ratios or numbers of CD4+ and CD8+ T cells or absolute numbers of CD3+ T cells[19]. Although the numbers of T cells were not significantly different, studies have shown that T cells from ASD patients express more HLADR, an activation marker[20]. Additionally, TGF-β, a cytokine that is produced at high levels by regulatory T cells (Tregs) and regulates immune cell responses, is reduced in ASD.[14] Consistent with this observation, studies have found lower frequencies of Tregs in ASD cohorts.[22,23] If ASD patients have fewer Tregs, this could lead to dysregulated immune responses.

1.23) Gastrointestinal symptoms in ASD

Individuals with ASD more frequently report gastrointestinal (GI) symptoms. ASD patients experience constipation, food selectivity, chronic diarrhea, chronic reflux/vomiting, chronic abdominal pain, foul smelling stool, and others more frequently than those of neurotypical controls[24–26]. Despite these increased reports of GI symptoms, individuals with ASD do not have increased frequencies of GI disorders such as celiac disease or ulcerative colitis[26,27].

Although there is no evidence of gross physiological differences, the presence of GI distress indicates a potential disruption of intestinal barrier function. The intestinal barrier is made up of epithelial cells connected by tight junctions and a mucosal layer. The purpose of this

barrier is to regulate absorption of nutrients, electrolytes and water, as well as prevent the entry of pathogens[28]. Below the epithelial layer is the lamina propria. This is a layer of connective tissue rich in immune cells that help maintain barrier function[29,30]. It has been proposed that an increase in intestinal permeability contributes to the symptoms of certain diseases. Increased intestinal permeability could be due to inflammation or damage, leading to the translocation of bacterial products into the bloodstream and subsequent immune activation[28]. This process is thought to occur in ASD. An increase in intestinal permeability in ASD patients, as well as their first-degree relatives was observed using the lactulose/mannitol sugar intestinal permeability test, with a high ratio indicating increased intestinal permeability[24,31,32]. This data suggests a genetic component to barrier function deficit in these patients[32]. A consequence of barrier dysfunction is the translocation of bacteria and bacterial products into the bloodstream. Serum endotoxin levels were found to be significantly higher in patients with severe ASD[33]. Endotoxin, also known as lipopolysaccharide (LPS), is a component of the cell wall of Gram negative bacteria and it is released during bacterial lysis. It binds to toll-like receptor (TLR)-4, which initiates inflammatory signalling pathways[28]. An increase in circulating LPS could be indicative of increased intestinal permeability

The immune system and the central nervous system form a bidirectional communication network. This communication is mediated by the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic nervous system, and cytokines[34]. This immune-brain connection serves as one of the paths through which the gut can communicate with the brain. Chronic gastrointestinal inflammation has been shown to induce anxiety like behaviour[35]. The communication system between the gut, immune system and the brain has implications for ASD. Adams et al. found GI severity to be strongly correlated with ASD severity[37]. A meta-analysis has also indicated that GI

symptoms put ASD patients at a higher risk for severe or problem behaviors.[38] ASD coupled with GI symptoms is associated with more severe irritability, social withdrawal and anxiety.[39,40]

1.3) Attention deficit hyperactivity disorder

ADHD is characterized as a persistent pattern of inattention, hyperactivity and impulsivity that leads to various degrees of functional impairments[41]. ADHD affects up to 1:20 children and is approximately 2 times more prevalent in males than in females[42]. Prevalence of ADHD has been increasing, which, like ASD, is thought to be due to increased awareness and improved diagnostic criteria[41]. The etiology of ADHD is unknown, however, it is thought to be caused by a combination of genetic and environmental factors.

Less is known about immune involvement in ADHD than in ASD. Buske-Kirschbaum et al. speculate, but have not yet experimentally shown, that increased levels of pro-inflammatory cytokines triggered by an allergic or autoimmune contribute to the development of ADHD[43] due to higher rates of eczema and asthma in individuals with ADHD[44]. Furthermore, Schmidtt et al. identified higher rates of ADHD among eczema patients[45]. The role of allergic or autoimmune involvement in ADHD remains unclear.

1.4) Diagnosis and behaviour in ASD and ADHD

The method of diagnosis of ASD and ADHD is primarily, if not entirely, based on behaviour, as there are no biomarkers for these disorders. Both are diagnosed by a clinician based on the Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) criteria[46]. In ASD, these criteria are;

1) Persistent deficits in social communication and social interaction across multiple contexts.
2) Restricted and repetitive patterns of behaviour, interests or activities.

3) Symptoms are present in the early development period.
4) Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.
5) These disturbances are not better explained by intellectual disability or global developmental delay.

In ADHD, these criteria are;

1) A persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with functioning or development.
2) Several inattentive or hyperactive-impulsive symptoms are present prior to age 12 years.
3) Several inattentive or hyperactive-impulsive symptoms are present in two or more settings.
4) There is clear evidence that symptoms interfere with or reduce the quality of social, academic, or occupational functioning.
5) The symptoms do not occur exclusively during the course of schizophrenia or another psychiatric disorder and are not better explained by another mental disorder.

A diagnosis of ADHD using these criteria is often accompanied by a label of mild, moderate or severe and followed by an intervention therapy, with no further behavioural phenotyping. However, a diagnosis of ASD using the above criteria is often followed by autism specific diagnostic and phenotyping tests. Two such tests are the Autism Diagnostic Observation Scale (ADOS) and the Autism Diagnostic Interview – Revised (ADI-R). The ADOS is an observation-based test that involves interacting with the child and observing how they interact with others and respond to pre-determined situations. The test administrator can then determine

the severity of the child's communication, social interaction and restricted and repetitive behaviours. The ADI-R is an interview with the child's primary caregiver. From this interview, the administrator can infer the severity of the child's symptoms in the same three domains; communication, social interaction and restricted and repetitive behaviours.

Neurodevelopmental disorders are often associated with behavioural deficits not directly specified in the DSM-5. For example, neurodevelopmental disorders are associated with more severe problem and adaptive behaviours. Problem behaviours can be identified with the Child Behaviour Checklist (CBCL). This is a report completed by the child's primary caregiver, which groups various problem behaviours into internalizing behaviours and externalizing behaviours. Internalizing behaviours includes anxious, withdrawn and depressed behaviours, while externalizing behaviours includes categories such as rule breaking, aggressive behaviour and attention problems. Adaptive behaviours are behaviours that allows a person to complete everyday skills and move through the day with the least amount of conflict with others. These behaviours can be measured using the Adaptive Behaviour Assessment System (ABAS). This examines four main domains of adaptive functioning; conceptual, social, practical and general. In general, those with more severe forms of ASD or ADHD will have worse problem behaviours and less adaptive functioning, or maladaptive functioning.

1.5) The immune system and behaviour:

The inflammatory response is tightly tied to the febrile response, defined as the elevation of core body temperature by behavioural or physiological means[47]. Furthermore, this response is associated with behaviours such as lethargy, anhedonia, irritability and reduced socialization. These are behaviours that aid the individual in clearing the infection, by re-routing energy resources to fighting the infection, as well as preventing the spread of infection[48]. Studies have

shown that these behaviours are a result of the release of pro-inflammatory cytokines such as IL-6, TNF-α and IL-1β. The brain monitors the peripheral innate immune response through multiple pathways. Firstly, cytokines are able to activate afferent nerves to send information to the brain[49,50]. Secondly, toll-like receptors (TLRs) on cells in circumventricular organs detect pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). These cells then produce pro-inflammatory cytokines[51]. Finally, pro-inflammatory cytokines can be transported through the blood-brain barrier[52,53]. With this information about the peripheral inflammatory state, the brain changes our behaviour to facilitate resolution of infection.

Some studies have shown an association between the inflammation and behaviour in ASD. Increases in plasma levels of IL-6,[22,54] TNF-α,[54] and IL-4[56] have been linked to ASD like behaviours, such as those outlined in the DSM-5 criteria. In addition, IL-1β and GM-CSF has been associated with behaviour regression.[56] This data indicates that inflammation may be influencing behaviour in ASD, specifically worse behaviour.

1.6) Model

These data are consistent with a gut-immune-brain pathway that contributes to behavioural changes in neurodevelopmental disorders. An increase in intestinal permeability, potentially due to GI distress, would allow for the translocation of bacteria and other antigens from the gut into the circulation. The presence of these antigens could illicit an inflammatory response, leading to the recruitment of immune cells and release of inflammatory mediators. If this intestinal permeability and inflammation is systemic, it could lead to more severe behaviours and lower overall functioning in these disorders (Figure 1.1).

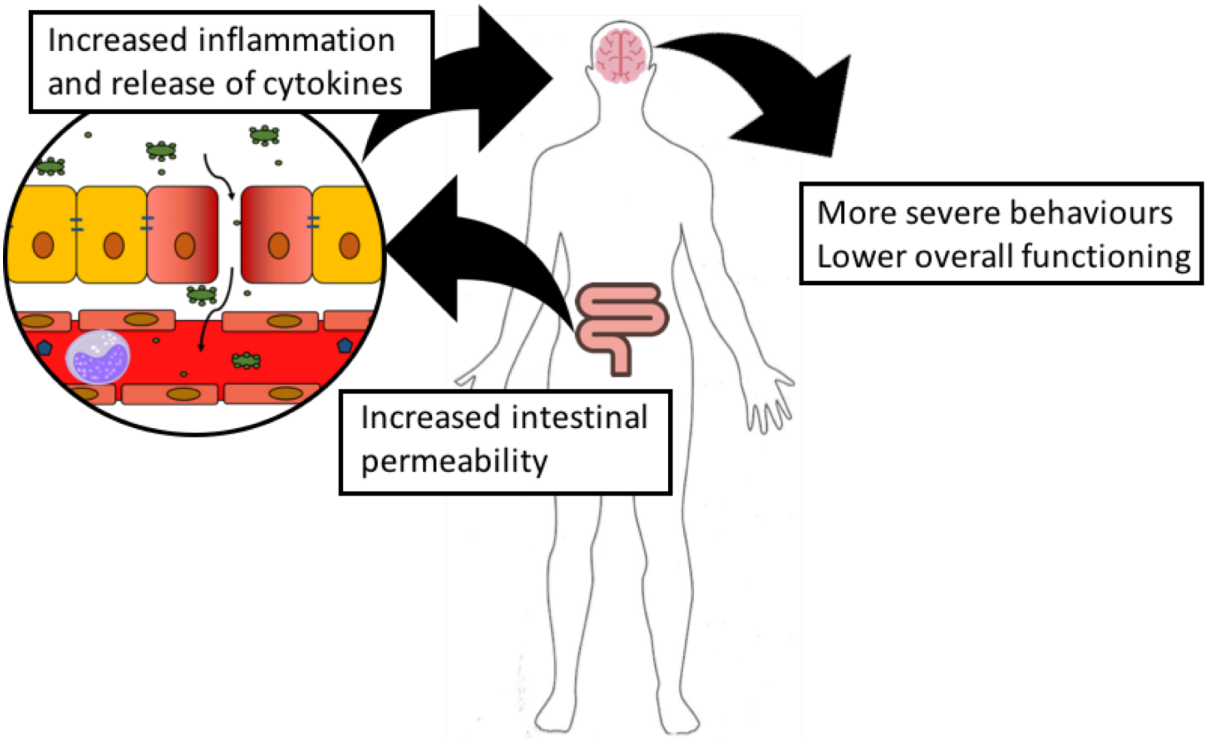


Figure 1.1) *Model of intestinal permeability and inflammation leading to increased severity in neurodevelopmental disorders.*

1.7) Hypothesis

Children and young adults with neurodevelopmental disorders (ASD, ADHD) will have an increase in circulating markers of soluble and cellular inflammation. These will be proportionate to behavioral severity. These inflammatory markers will correlate with an increase in markers of intestinal permeability.

1.8) Soluble markers

1.81) Acute phase proteins

To investigate peripheral inflammation, we will measure acute phase proteins (APPs) in the circulation. These are proteins, most of which are synthesized in the liver, that are released during the acute phase of inflammation. APPs have a variety of functions aiding the

inflammatory response such as activating complement, trapping pathogens, aiding or deterring blood coagulation and activating enzymes and immune cells needed for the inflammatory response[57]. We chose to study APPs as opposed to cytokines because they represent a clinically relevant measurement of inflammation. Some APPs such as C-reactive protein (CRP) are measured in clinical settings to indicate infection. APPs can be secreted during both chronic and acute inflammation. Production of these proteins is triggered by cytokines produced at the site of infection or inflammation that travel to the liver. The acute phase response has been shown to resolve 4-7 days after it is first initiated[58]. However, the acute phase response can become chronic if there are repeated stimuli. By measuring the concentrations of APPs in neurodevelopmental disorders, we can gain insight into the inflammatory status of these patients. For the purpose of this study, we measured a total of seven acute phase proteins; C-reactive protein (CRP), serum amyloid A, serum amyloid P, haptoglobin, α-2-macroglobulin, ferritin and tissue plasminogen activator (tPA).

1.82) Intestinal permeability

To investigate intestinal permeability, we measured circulating concentrations of intestinal fatty acid binding protein (I-FABP) and the bacterial products lipopolysaccharide (LPS) and muramyl dipeptide (MDP). I-FABP is a cytosolic intestinal epithelial protein that is increased in the circulation after intestinal epithelial damage.[59] Measuring the concentrations of this protein in the circulation indicates the level of damage, potentially due to gastrointestinal distress. LPS is a molecule present on the outer membrane of Gram negative bacteria, and MDP is a component of the cell wall of both Gram positive and negative bacteria. Under homeostatic conditions, these two molecules should be at very low levels or completely undetectable in the

circulation. An increase of these markers in the circulation could indicate increased intestinal permeability.

1.9) Cellular markers of inflammation

Monocytes are a versatile cell population that are precursors to macrophages and some dendritic cells. They have various circulating functions, including cytokine production and phagocytosis. Monocytes can be divided into three sub-populations; classical, intermediate and non-classical. These subsets are identified by their surface expression of CD14 and CD16. Classical monocytes, identified as $CD14^{++}$ $CD16^{-}$ are the most recent emigrants from the bone marrow. They are involved in phagocytosis, anti-microbial responses and chemotaxis to infection sites. Intermediate monocytes, identified as $CD14^{++}$ $CD16^{+}$, are dubbed inflammatory monocytes as their primary function is to secrete pro-inflammatory cytokines in response to PAMPs and DAMPs[60]. Non-classical monocytes, identified as $CD14^{+}$ $CD16^{+}$, patrol the vascular endothelium and are considered the most differentiated form of blood borne monocytes[61]. The frequency of these three monocyte populations can give insight into the inflammatory status of the individual. Monocyte egress from the bone marrow is increased in response to acute infection or injury. These monocytes then traffic to the site of inflammation[61,62]. Circulating monocyte populations are also altered in response to chronic inflammation[63,64]. Studies of chronic conditions associated with increased inflammation such as rheumatoid arthritis, osteoarthritis and advanced age have shown changes in monocyte frequency and phenotype[63–65].

Monocytes express a variety of surface receptors that dictate the activation or maturation status of the cell. Two such receptors are CCR2 and CX_3CR_1. CCR2 is the receptor for CCL2, also known as monocyte chemoattractant protein-1 (MCP-1). CCR2 activation is necessary for monocyte egress from the bone marrow[66,67]. The binding of CCR2 to its ligand facilitates

monocyte chemotaxis to sites of infection or inflammation[67]. CCR2 is expressed at higher levels on classical monocytes than on any other subset (Appendix Figure 5). CX_3CR_1 is the receptor for CX_3CL_1, also known as fractalkine. This binding facilitates chemotaxis, as well as leukocyte adhesion to the vascular endothelium[68]. CX3CR1 is expressed at higher levels on non-classical and intermediate monocytes (Appendix Figure 5).

Monocyte expression of CD16 can also give insight into monocyte activation status. CD16 is an Fc receptor called FcRIII. This binds to the Fc region of IgG antibodies and is necessary for antibody dependent cellular cytotoxicity by monocytes[69]. CD16 expression increases on monocytes as they become more activated, and expression is highest on intermediate and non-classical monocytes. Expression of CD16 is also increased in inflammatory events like sepsis[70].

We have chosen to measure monocyte expression of three other surface markers to gain insight into the maturation and activation status of the monocyte populations. We will measure changes in the expression of CD115, CD13 and CD64 (Appendix Figure 6). CD115 is the receptor for colony stimulating factor-1 (CSF-1), a key growth factor in the differentiation of monocytes and macrophages. CSF-1 was shown to cause an increase in monocyte surface expression of CD16, driving the maturation of monocytes[71]. CD13 is expressed on monocytes in different levels at different stages of development. This receptor facilitates adhesion and phagocytosis[72]. Expression of this receptor can increase with maturation, and is also upregulated after LPS stimulation[73]. CD64, or FCγRI, is a high affinity IgG receptor. Monocytes with increased CD64 have increased phagocytic ability. Upregulation of this receptor is induced by inflammatory cytokines[74]. By constructing this monocyte immunophenotype, we can gain insight

into the inflammatory status of neurodevelopmental disorders and investigate how these cellular markers associate with behaviour.

Surface Receptor	Classical Monocytes	Intermediate Monocytes	Non-Classical Monocytes
CD14	++	+	+/-
CD16	-	+	+
CCR2	++	+	+/-
CX_3CR_1	+/-	++	++
CD115	+	++	+
CD13	++	++	++
CD64	++	+	+/-

Table 1.1) Chart of monocyte surface expression of maturation, activation and migration markers.

Chapter Two

Materials and Methods

2.1) Study participants

Participants were recruited through the Province of Ontario Neurodevelopmental Disorder (POND) Network, from which a cohort of children and young adults with neurodevelopmental disorders as well as typically developing participants were enrolled.

Eligible participants were males and females between the ages of 1 and 21 years with a diagnosis of autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), intellectual disability (ID), or typically developing (TD) controls. Recruitment and blood collection was carried out at four sites across Ontario; Queen's University (Kingston), The Hospital for Sick Children (Toronto), McMaster University (Hamilton) and Lawson Health Research Institute (London).

Exclusion criteria was as follows; fever greater than 38°C in the preceding 3 days, vaccination in the preceding 7 days, oral or intravenous steroids in the preceding 14 days, oral or intravenous antibiotics in the preceding 21 days, chemotherapy, intravenous immunoglobulins of monoclonal antibodies in the preceding 12 months, known primary or secondary immunodeficiency.

2.2) Cryopreservation of blood

2.21) Serum collection

BD vacutainer serum collection tubes (BD #367815) were centrifuged at 3100 rpm for 10 minutes at room temperature. Serum was aliquoted into cryovials and stored at -80°C.

2.22) Plasma collection

Heparinized (BD #367874) and ACD treated BD vacutainer blood collection tubes were centrifuged at 1500 rpm for 5 minutes at room temperature. Plasma was aliquoted into cryovials and stored at -80°C.

2.23) Peripheral blood mononuclear cell separation and cryopreservation

After centrifugation and removal of plasma, the cellular fraction was mixed 1:1 with warm PBS in a biological safety cabinet. An equal volume of Ficoll-Paque Plus medium (VWR, CA95038-168L) was pipetted below the blood. The PBMC layer was separated by centrifuging the solution at 1500 rpm for 25 minutes at room temperature with the brake off. The PBMC layer was collected, washed with PBS and frozen in a solution of 10% DMSO in human AB serum. Cells were stored at -80°C.

2.3) Flow cytometry

To quantitate immune cell populations within the PBMC fraction, 3 surface stains were used for analysis with flow cytometry. Fluorochrome conjugated antibodies against CD45 (BV510), CD3 (APCef780), CD4 (PerCPCy5.5), CD8 (PECy7), CD19 (AF700), NKp46 (PE) and CD56 (PE) were used to identify lymphocyte populations. In order to identify monocytes, fluorochrome conjugated antibodies against CD45 (BV510), HLADR (PerCPCy5.5), CD11b (APC), CD14 (BV421), CD16 (PECy7), CD3 (AF700), CD19 (AF700), CD56 (AF700) were used. Fluorochrome conjugated antibodies against CCR2 (PE), CX_3CR_1 (FITC), CD115 (APC), CD64 (BV605) and CD13 (PE) were used to identify maturation and activation of monocyte populations. Gating strategies are outlined in Appendix Figures 1-3. Immunophenotyping stains outlined in Appendix Figure 4.

PBMCs were thawed in warm RPMI medium and washed with FACS wash (0.5% (w/v) BSA, 5 mM EDTA (pH 7.4-7.6), for 500 mL 2.5 g BSA, 5 mL of 0.5 M EDTA) prior to staining.

FC receptor blocking was done with 20% human AB serum in FACS wash. 500,000 cells were stained per antibody cocktail for 30 minutes at room temperature, then fixed using 1-step Fix/Lyse solution (1X) for 10 minutes. PBMCs were washed with FACS wash and analysed on an LSR II flow cytometer.

2.4) Cell culture

HEK-Blue™ TLR4 cells (Invivogen, CA, US, hkb-htlr4) are a cell line that stably express TLR4, MD2 and CD14 and are transfected with the pNifty-SEAP (Invivogen, CA, US, pnifty2-seap) reporter plasmid. These cells were cultured in DMEM medium supplemented with 10% (v/v) FBS, 1% penicillin-streptomycin and 1% L-glutamine and incubated at 37°C in a 5% CO_2, 95% humidity incubator. They were cultured under antibiotic selection using HEK-Blue selection (Invivogen, CA, US, hb-sel).

HEK-Blue™ mNOD2 cells (Invivogen, CA, US, hkb-mnod2) are a cell line that stably express the NOD2 receptor and are transfected with the pNifty-SEAP (Invivogen, CA, US, pnifty2-seap) reporter plasmid. These cells were cultured in DMEM medium supplemented with 10% (v/v) FBS, 1% penicillin-streptomycin and 1% L-glutamine and incubated at 37°C in a 5% CO_2, 95% humidity incubator. They were cultured under antibiotic selection using blasticidin and zeocin.

2.41) HEK-Blue detection assay

HEK-Blue™ cells were seeded in a 96 well plate at a density of 4,000 cells/well and allowed to adhere overnight. Serum samples were diluted 1:10 with PBS and Milliq H_2O. Diluted samples were then heat inactivated at 75°C for 5 minutes. HEK-Blue cells were treated with diluted serum and HEK-Blue Detection medium (Invivogen, CA, US, hb-det3) and

incubated for 24 hrs at 37°C. 50ng/mL of LPS and 1ug/mL of MDP were used as a positive control. After 24 hours, absorbance was measured at 650nm using a SpectraMax plate reader.

2.5) ELISA

2.51) Acute phase proteins

Measurement of acute phase proteins α-2-macroglobulin, C-reactive protein, haptoglobin, serum amyloid P, serum amyloid A, ferritin and tissue plasminogen activator was done by Eve Technologies (Calgary, Alberta) using the Featured Human Acute Phase Array 9-Plex (HACUTE-09-01).

2.52) I-FABP ELISA

For the detection of circulating intestinal fatty acid binding protein, the human FABP2 ELISA kit was purchased from Abcam (ab193700). The assay was performed according to manufacturer's instructions.

2.53) Tissue plasminogen activator ELISA

For detection of circulating tissue plasminogen activator, the human tissue type plasminogen activator ELISA kit was purchased from Abcam (ab119563). The assay was performed according to manufacturer's instructions.

2.54) Ferritin ELISA

For the detection of circulating ferritin, the human ferritin ELISA kit was purchased from Abcam (ab108837). The assay was performed according to the manufacturer's instructions.

2.55) C-reactive protein ELISA

For the detection of circulating C-reactive protein, we used an in-house ELISA. ELISA well strips (VWR, CA62409-134) were coated with 1ug/mL of human CRP capture antibody (abcam8279) diluted in coating buffer (1.5 g Anhydrous Na_2CO_3 + 2.93 g Anhydrous $NaHCO_3$ +

1 L distilled water, pH 9.6). This was incubated at 4°C overnight. The liquid was removed and the plates were blocked with blocking buffer (PBS + 1% w/v BSA, filter-sterilized) for 1 hour at room temperature. The liquid was removed and plasma samples, diluted 1/500 in blocking buffer, were added to the plates in duplicate and incubated for 2 hours at room temperature. The plates were washed three times in wash buffer (PBS + 0.05% v/v Tween-20) the incubated with 1ug/mL of human CRP detection antibody (abcam24462) diluted in blocking buffer for 1 hour at room temperature. The plates were washed five times in wash buffer, then developed with TMB substrate (BioLegend 421501). The reaction was stopped with 2N H_2SO_4 and the plates were read at 450nm using a SpectraMax plate reader.

Chapter Three

The Association of Acute Phase Proteins and Intestinal Permeability with Behaviour in Autism Spectrum Disorder

3.1) Introduction

Evidence of inflammation in ASD is accumulating. Many studies have found increases in circulating inflammatory cytokines as well as a decrease in regulatory cytokines such as TGF-β[13–15,75,76]. Furthermore, some of these studies have linked pro-inflammatory cytokines to behavioural alterations. Ashwood et al. found that IL-4 was associated with impaired communication and IL-6, IL-1β and IL-8 were associated with increased stereotyped behaviours[77]. Another study from the same group found that a decrease in TGF-β was associated with more severe ASD behaviours, measured using the Autism Behaviour Checklist, and worse social interaction[76]. Although most of these studies measured circulating cytokines, we will measure acute phase proteins as markers of inflammation. These are measures of systemic inflammation that have less person to person and day to day variability. They are also often used in clinical practice. With these data as a starting point, we aimed to measure circulating acute phase proteins and identify behaviour correlates with these proteins.

In 2012, the Province of Ontario Neurodevelopmental Disorder (POND) network published a study that tested if omega-3 fatty acid supplementation improved behavioural outcomes in children with ASD.[78] Blood was collected and plasma was cryopreserved as part of this study. We measured acute phase proteins and markers of intestinal permeability in plasma from 32 subjects aged 2-5 years (male=25, female=7). Additionally, the original study collected behaviour data from these subjects. Behaviour tests included the Autism Diagnostic Observation Scale (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R) to quantitate the severity

of ASD behaviours. The Vineland Adaptive Behaviour Scale (VABS) was used to quantitate adaptive behaviours.

We hypothesized that intestinal permeability allows for the translocation of bacteria and bacterial products from the gut into the circulation leading to an inflammatory response. Soluble inflammation was determined by measuring circulating concentration of acute phase proteins. We also measured circulating levels of intestinal fatty acid binding protein (I-FABP) to indicate intestinal epithelial damage, and lipopolysaccharide (LPS) to indicate translocation of bacterial products.

3.2) Results

The circulating concentrations of seven acute phase proteins were quantified; α-2-macroglobulin, C-reactive protein, haptoglobin, serum amyloid P, serum amyloid A, ferritin and tissue plasminogen activator (tPA). Concentrations of I-FABP were quantitated with a sandwich ELISA. Reference ranges for this age group are represented by dotted lines[59,79–85]. Overall, the concentrations of the acute phase proteins were within the reference range (Figure 3.1). Many of the males had levels of tPA above the reference range (Figure 3.1G) and most subjects are above the reference cut-off for circulating levels of I-FABP (Figure 3.1H). There were no significant sex differences in the concentrations of α-2-macroglobulin, C-reactive protein, haptoglobin, serum amyloid P, serum amyloid A, I-FABP and LPS. Levels of ferritin were higher in males (mean=16760 pg/mL ± 9651) than in females (mean=7380pg/mL ± 4042). Tissue plasminogen activator (tPA) was also higher in males (mean=4575 ±1742) than in females (mean=1981 ±1636) (Figure 3.2).

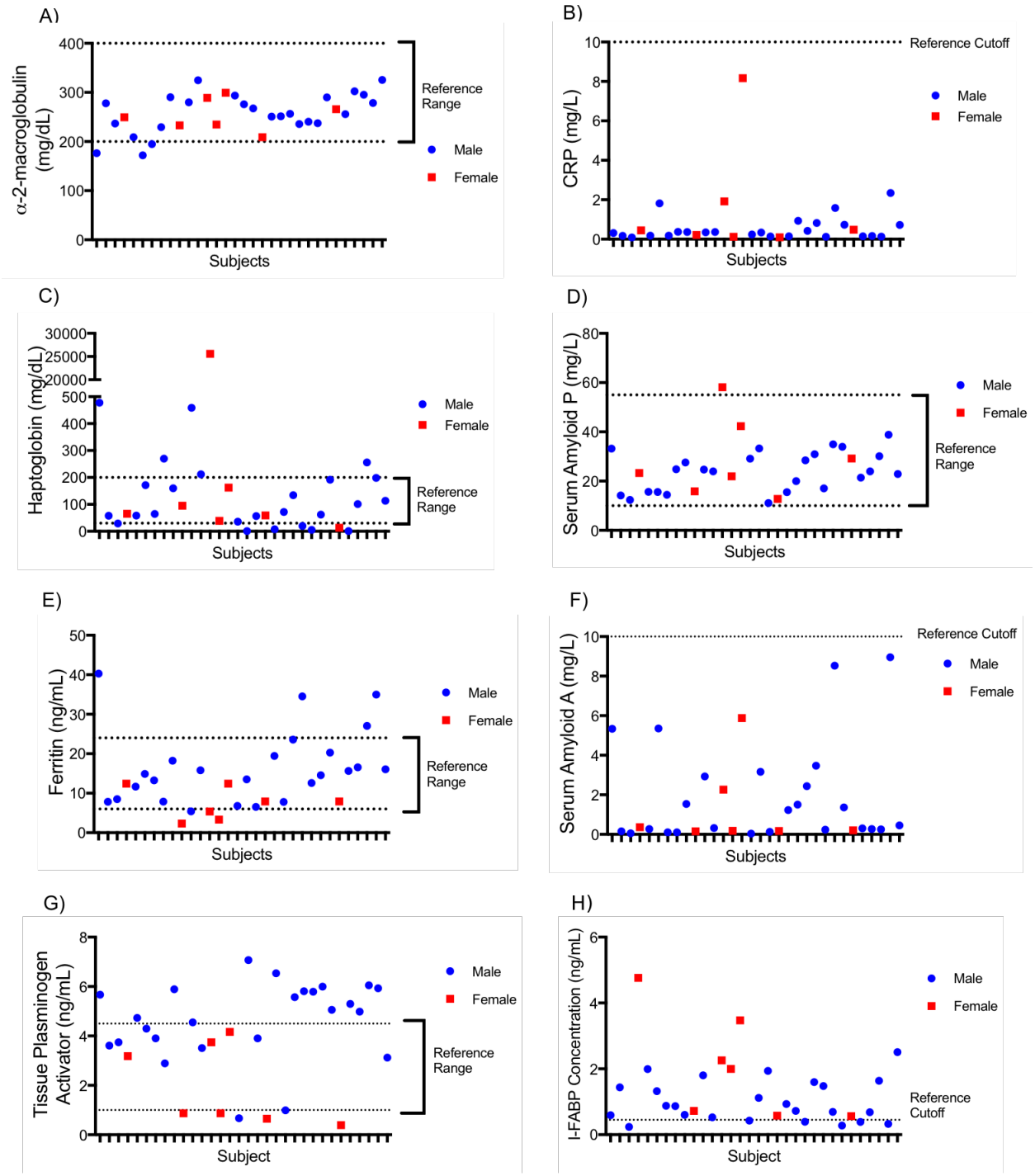


Figure 3.1) *Concentrations of circulating markers of inflammation and intestinal permeability in children with ASD*. Seven acute phase proteins were quantified by multiplex ELISA and I-FABP was quantified by sandwich ELSIA. Reference ranges are marked by dotted lines. Each point represents an individual.

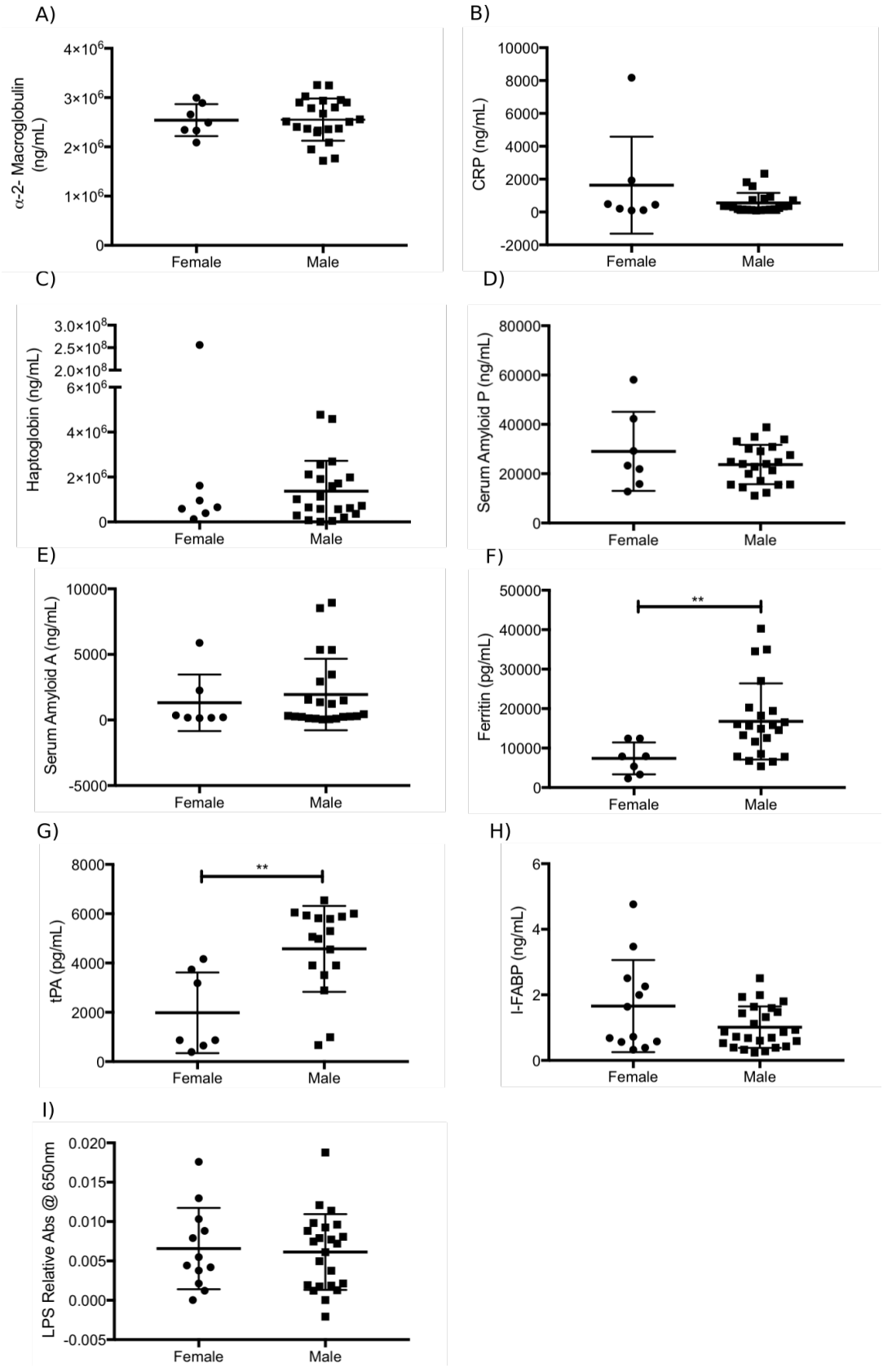

A)
α-2- Macroglobulin (ng/mL)
4×10^6
3×10^6
2×10^6
1×10^6
0
Female
Male
B)
CRP (ng/mL)
10000
8000
6000
4000
2000
0
-2000
Female
Male
C)
Haptoglobin (ng/mL)
3.0×10^8
2.5×10^8
2.0×10^8
6×10^6
4×10^6
2×10^6
0
Female
Male
D)
Serum Amyloid P (ng/mL)
80000
60000
40000
20000
0
Female
Male
E)
Serum Amyloid A (ng/mL)
10000
5000
0
-5000
Female
Male
F)
Ferritin (pg/mL)
**
50000
40000
30000
20000
10000
0
Female
Male
G)
tPA (pg/mL)
**
8000
6000
4000
2000
0
Female
Male
H)
I-FABP (ng/mL)
6
4
2
0
Female
Male
I)
LPS Relative Abs @ 650nm
0.020
0.015
0.010
0.005
0.000
-0.005
Female
Male

Figure 3.2) *Sex differences in the circulating concentrations of acute phase proteins and markers of intestinal permeability*. Concentrations of seven acute phase proteins, I-FABP and relative absorbance of LPS were separated by sex and their means were compared using a non-parametric Mann-Whitney test. Significant sex differences were identified in the concentrations of ferritin (p=0.0052) (F) and tissue plasminogen activator (p=0.0031) (G).

I-FABP and LPS were measured in the plasma to indicate intestinal permeability. Concentrations of I-FABP positively correlated with circulating LPS in females (r=0.8669, p=0.0115), indicating intestinal epithelial damage potentially leading to translocation of bacterial products (Figure 3.3A). The acute phase protein tPA was found to positively correlate with both I-FABP (r=0.8783, p=0.0093) and LPS (r=0.8608, p=0.0129) in females (Figure 3.3B-C).

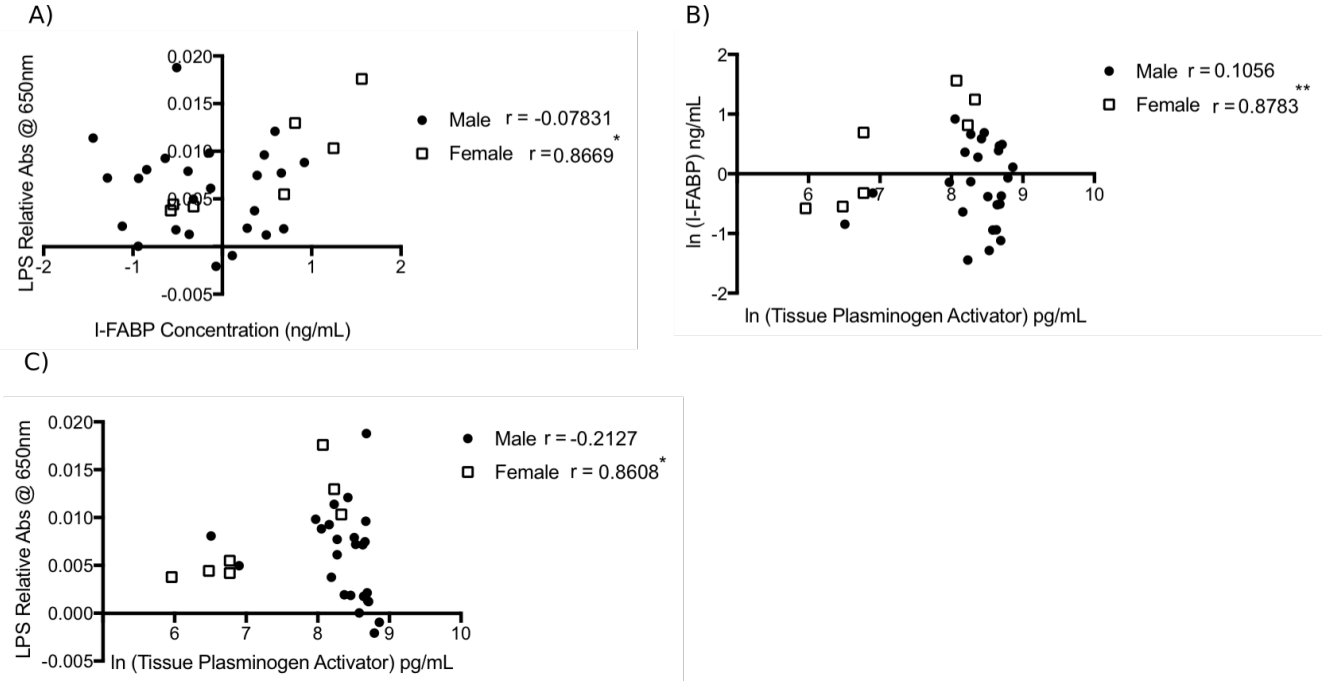


Figure 3.3) *Evidence that intestinal permeability correlates with elevated tissue plasminogen activator.* Pearson correlations were performed to identify associations between acute phase proteins. A) Concentrations of I-FABP positively correlated with LPS in females. (p=0.0115). B) Concentrations of tissue plasminogen activator positively correlated with I-FABP concentration (p=0.0093). C) Concentrations of tissue plasminogen activator positively correlated with circulating LPS in females (p=0.0129).

In order to determine if sub-clinical inflammation was associated with behaviour, correlations between acute phase proteins and measures of ASD behaviour severity were investigated. In females, tPA significantly correlated with more severe VABS communication score (r=0.8203, p=0.0455), more severe ADOS social interaction score (r=0.8647, p=0.0102) and less severe ADI play score (r=0.8773, p=0.0095) (Figure 3.4). No other significant correlations were identified between acute phase proteins and behaviour.

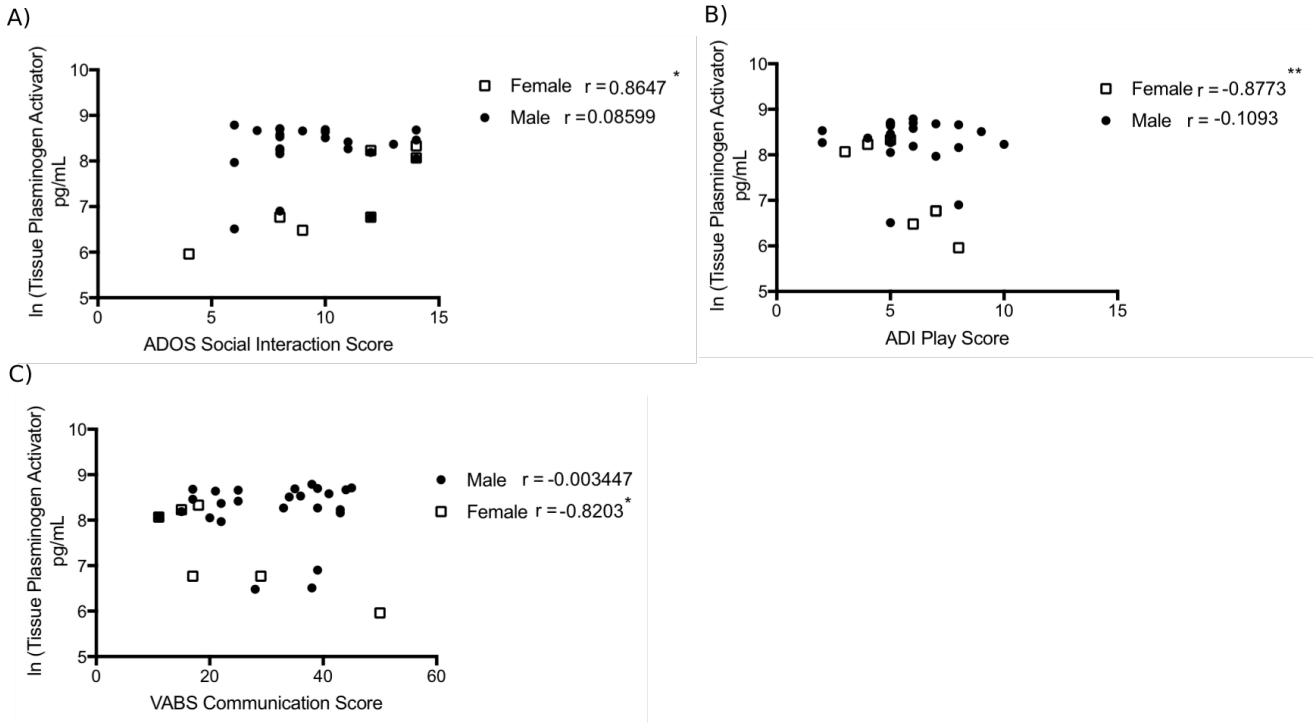


Figure 3.4) *Female ASD behaviours correlate with the acute phase protein tissue plasminogen activator.* Pearson correlations were performed to identify associations between acute phase proteins and behavior. Concentrations of tissue plasminogen activator (tPA) correlate with more severe social interaction (A) (p=0.0102), communication (C) (p=0.0455) and less severe play behaviours (B) (p=0.0095).

Relationships between markers of intestinal permeability and measures of ASD behaviour severity were investigated. In females, an increase in LPS correlated with worse ADOS communication score (r=0.7563, p=0.0491) and less severe ADI play score (r=-0.9505, p=0.0010) (Figure 3.5 A-B). Also in females, an increase in I-FABP concentration correlated with a more severe VABS motor skills score (r= -0.8127, p=0.0263) and less severe ADI play

score (r=-0.7875, p=0.0355) (Figure 3.5 D, F). In males, an increase in I-FABP correlated with more severe ADOS social interaction (r=0.4333, p=0.0389) and VABS communication (r=-0.4172, p=0.0476) (Figure 3.5 C, E).

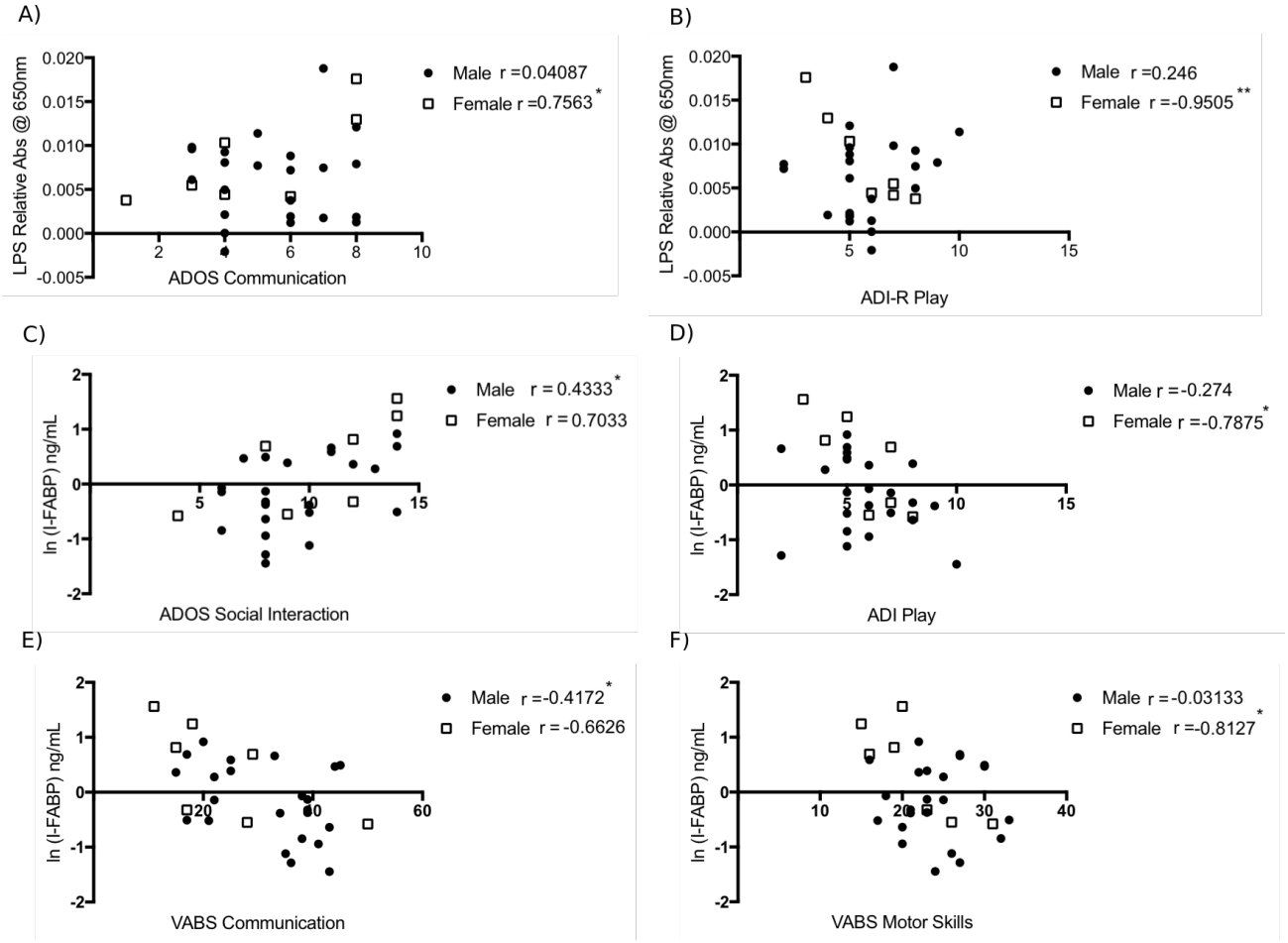


Figure 3.5) *Markers of intestinal permeability correlate with ASD behaviours*. Pearson correlations were performed to identify associations between behaviour and intestinal permeability. LPS correlated with more severe communication (A) (p=0.0491) and less severe play behaviours in females (B) (p=0.0010). In males, I-FABP concentration correlated with more severe social interaction (C) (p=0.0389) and communication (E) (p=0.0476). In females, I-FABP concentration correlated with more severe motor skills (F) (p=0.0263) and less severe play behaviors (D) (p=0.0355).

3.3) Discussion

Acute phase proteins (APPs) aid in the inflammatory process by carrying out a variety of functions such as trapping pathogens, assisting or deterring blood coagulation and activating

enzymes and immune cells needed for the inflammatory response. These proteins can be secreted during both chronic and acute inflammation.[57] In this cohort, α-2-macroglobulin, C-reactive protein, haptoglobin, serum amyloid P, serum amyloid A and ferritin levels were all within the reference range, with the exception of one subject with a very high concentration of haptoglobin. Many of the male subjects had levels of tPA outside of the reference range. Additionally, in this cohort, we have identified that elevated levels of the APP tissue plasminogen activator (tPA) correlates with behaviour.

tPA is a serine protease involved in the breakdown of blood clots by converting plasminogen to plasmin.[86] It is released from endothelial cells in response to circulating thrombin, another serine protease involved in blood clotting[87]. In addition to its protease functions, tPA has cytokine-like functions. When it binds to its membrane receptors, LRP-1 and Annexin A2, it can initiate the translocation of the inflammatory transcription factor NF-kB[88]. TPA has different functions when it is released in the brain than in the periphery. In the central nervous system, tPA has been shown to activate microglia and increase the permeability of the blood brain barrier during cerebral ischema[89,90].

Sex differences were identified in the concentrations of both tPA and ferritin, with lower levels of these proteins in females than in males. Whether these observed sex differences are unique to ASD is unclear. No sex differences in tPA have been previously documented. Ferritin is an iron carrier protein. An increase in circulating ferritin corresponds to an increase in circulating iron[91]. Studies have shown increased incidence of iron deficiency in children with ASD,[92–94] however sex differences in this age group have not been documented. These findings indicate that in future studies, we should consider splitting the cohort by sex or using sex as a covariate to account for inherent differences between males and females.

Intestinal fatty acid binding protein (I-FABP) was increased above the reference cut-off in almost all subjects. This indicated that these subjects had increased intestinal epithelial damage, potentially due to GI distress which is common among those with ASD[25,26,95–97]. One limitation of this finding is that there is no clinically relevant reference range for I-FABP. The reference cut-off indicated was from a study that measured I-FABP in 61 healthy individuals.[59] We also found that I-FABP positively correlated with LPS in the circulation in females. These observations indicate intestinal epithelial damage may lead to intestinal permeability in these subjects. We also found that levels of I-FABP and LPS positively correlated with tPA. Finally, we identified that LPS and I-FABP correlated with behaviour changes. These data imply a relationship in this ASD cohort between markers of intestinal permeability, soluble inflammation, indicated by the acute phase protein tPA, and behavioural outcomes.

With this ASD cohort, we have found that we can quantify markers of soluble inflammation and intestinal permeability in plasma. Furthermore, we have shown that these markers correlate with ASD behaviour severity. There are some limitations to these findings. Firstly, there was a small sample size (n=32). Larger sample sizes are desirable when working with a heterogeneous cohort such as an ASD cohort. This sample size was further reduced when the data was separated by sex, with 25 males and only 7 females. These correlations will need to be confirmed with a larger sample size to improve the statistical power.

Chapter Four

Associations of Behaviour and Immunophenotype in Neurodevelopmental Disorders

4.1) Introduction

Previous evidence indicates that the immune response in neurodevelopmental disorders may be altered. Many studies have found increases in circulating inflammatory cytokines as well as a decrease in regulatory cytokines such as TGF-β[13–15,75,76]. There is also evidence of alterations in circulating immune cell populations that may indicate a dysfunctional immune response[3,18,19]. We know from studies of sickness behaviour that peripheral inflammation can influence behaviour. This has been demonstrated with data from the Omega-3 cohort, shown in Chapter 3. With this cohort, we found that the acute phase protein tissue plasminogen activator (tPA) correlated with behavioural alterations (measured using the ADOS, ADI-R and VABS), and in some cases more severe behavioural outcomes. To reproduce these findings in a larger cohort with a broader age range we accessed cryopreserved plasma collected as part of the Province of Ontario Neurodevelopmental Disorder (POND) Network's immune platform. To expand upon these findings, we investigated cellular changes in neurodevelopmental disorders using cryopreserved peripheral blood mononuclear cells (PBMCs) also collected as part of the POND Network's immune platform.

As per our previous study, we measured circulating levels of the acute phase proteins tPA, ferritin, and C-reactive protein (CRP). Ferritin and tPA were chosen to replicate the findings in the Omega-3 cohort (Chapter 3). CRP is used as a measure of inflammation in clinical settings. These markers of inflammation were chosen to identify clinically relevant alterations in the inflammatory state.

To investigate cellular changes in neurodevelopmental disorders, we analyzed immune cell frequency and phenotype using PBMCs isolated from peripheral blood of children with neurodevelopmental disorders, as well as typically developing controls. Frequencies of B cells, T cells, NK cells, monocytes and the three subsets of monocytes (classical, non-classical and intermediate) were measured as a percentage of all leukocytes. Monocyte surface expression of activation and maturation markers (CD115, CD13, CD64, CX_3CR_1, CCR2 and CD16) was determined by the mean fluorescence intensity (MFI) using a geometric mean calculation. With this information, we can identify changes in immune cell frequency and phenotype across diagnosis.

In addition to identifying immune cell alterations between diagnosis, we want to explore soluble and cellular correlates with behaviour. To investigate immune associations with behaviour across diagnosis, we used scores of adaptive behaviours, measured by the Adaptive Behaviour Assessment System (ABAS) and scores of problem behaviours, measured using the Child Behaviour Checklist (CBCL). To replicate findings from the Omega-3 cohort, we used ASD-specific behaviour data collected using the Autism Diagnostic Interview-Revised (ADI-R) and the Vineland Adaptive Behaviour Scale (VABS).

4.2) Results

4.21) POND Immune cohort

Details of the cohort, including diagnosis, age and sex are outlined in Table 4.1 and Figure 4.1. The diagnosis of intellectual disability (ID) was removed from analysis, as the number of subjects was too low for proper analysis.

Diagnosis	N (Male)	N (Female)	N (Total)
ASD	31	7	38
ADHD	18	2	20
ID	1	1	2
TD	10	10	20

Table 4.1: Number of POND Immune subjects separated by diagnosis.

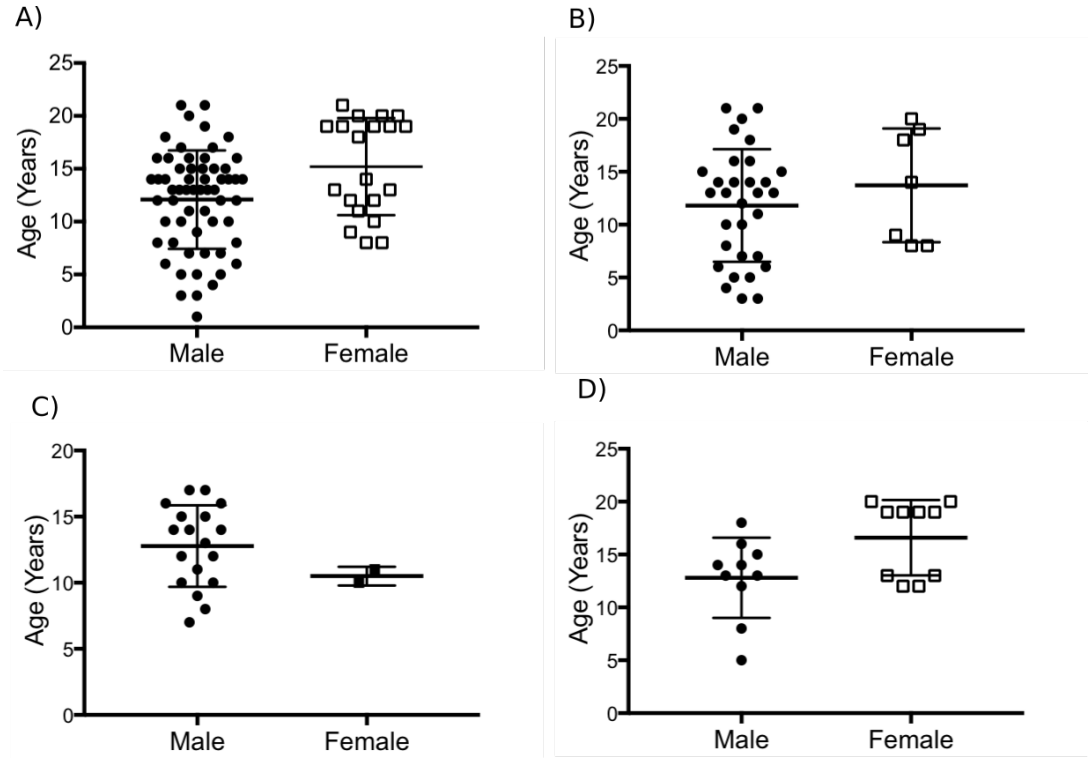


Figure 4.1: *Age in years of POND Immune subjects.* A) Age in years of all subjects. B) Age in years of subjects with an ASD diagnosis. C) Age in years of subjects with an ADHD diagnosis. D) Age in years of typically developing subjects.

PBMCs from all 80 subjects were accessed for flow cytometry. Eight samples were removed prior to flow cytometry due to poor cell recovery following the freeze-thaw process

(Figure 4.2A). Eleven samples were removed after flow cytometry because the samples were not of sufficiently high quality (Figure 4.2B).

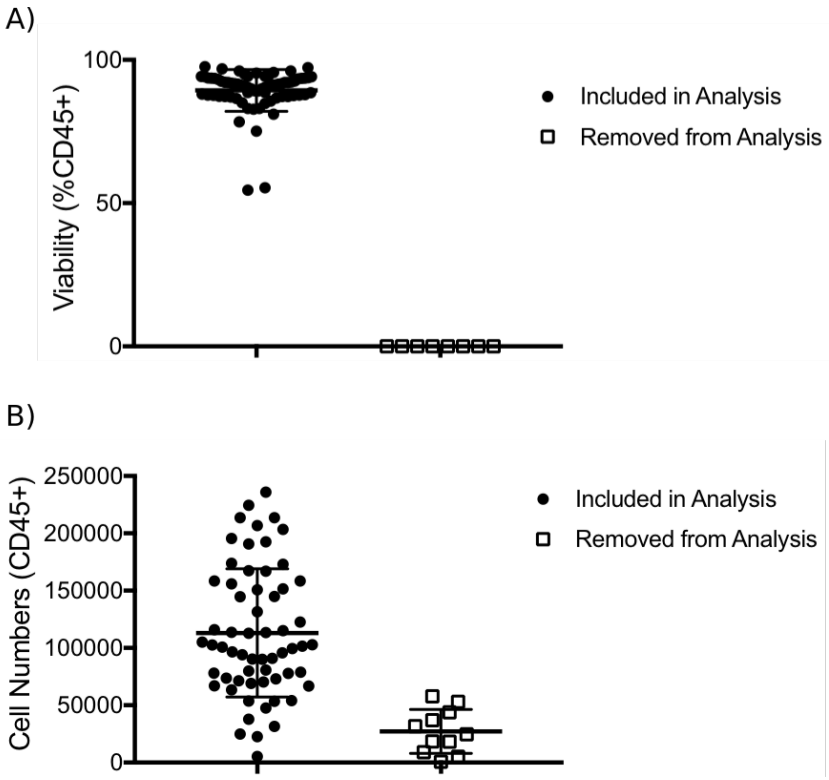


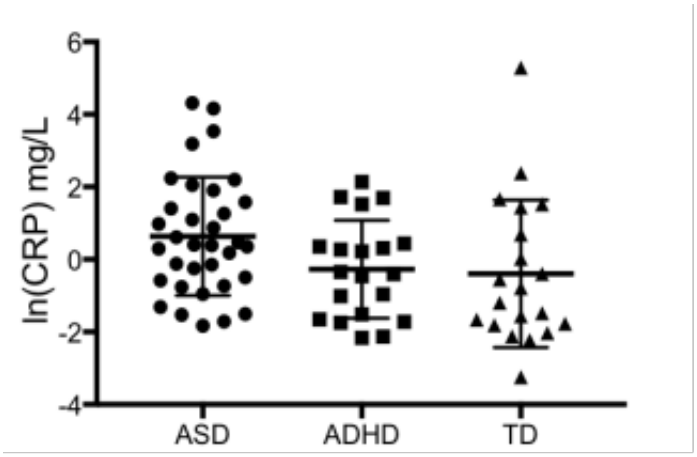
Figure 4.2: *Immune cell viability.* A) Viability of POND Immune PBMCs as a percentage of the CD45+ population, quantified using Zombie Red™ staining. B) Event recovery after flow cytometry.

4.22) Soluble inflammation in neurodevelopmental disorders

To investigate differences in circulating levels of inflammation across diagnosis, we quantitated the APPs ferritin, tPA and CRP in the plasma of children with neurodevelopmental disorders (Table 4.2). Multiple linear regression was used to compare concentrations between diagnosis, controlling for sex. No significant differences concentrations of acute phase proteins in the TD or ADHD groups were identified. Circulating concentrations of CRP were found to be significantly higher in the ASD cohort than in ADHD or TD (Figure 4.3).

	ASD			ADHD			TD		
Variable	N	Mean	SD	N	Mean	SD	N	Mean	SD
ln (Ferritin) ng/mL	34	3.705	0.863	20	3.663	0.534	20	3.564	0.423
ln (tPA) ng/mL	35	0.100	0.720	20	0.035	0.685	19	0.040	0.832
ln (CRP) mg/L	34	0.634	1.634	20	-0.271	1.347	20	-0.393	2.034

Table 4.2: Descriptive statistics of concentrations of acute phase proteins.



Dependent: ln(CRP) mg/L				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	1.291	0.377	**0.008***
	ADHD	0.267	0.069	0.625
	Sex	0.523	0.137	0.257
ADHD	ASD	1.091	0.319	**0.02***
	Sex	0.486	0.127	0.785

Figure 4.3: *Comparison of CRP concentration across diagnosis*. Circulating concentrations of CRP were found to be higher in the ASD group than in the ADHD or TD group using multiple linear regression analysis.

4.23) Cellular inflammation in neurodevelopmental disorders

Monocytes are very sensitive to changes in inflammation, thus we hypothesized that changes in circulating acute phase proteins would be reflected in monocyte frequency and phenotype. To investigate these changes, we measured in immune cell frequencies as well as markers of monocyte activation and maturation across diagnosis. Frequencies of immune cell

populations were calculated as a percentage of the CD45+ population (Table 4.3). Monocyte surface expression of the receptors CD115, CD13, CD64, CX_3CR_1, CCR2 and CD16 was determined by the mean fluorescence intensity (MFI) using a geometric mean calculation (Table 4.4). Multiple linear regression was used to compare immune cell frequencies and MFIs across diagnosis, controlling for sex.

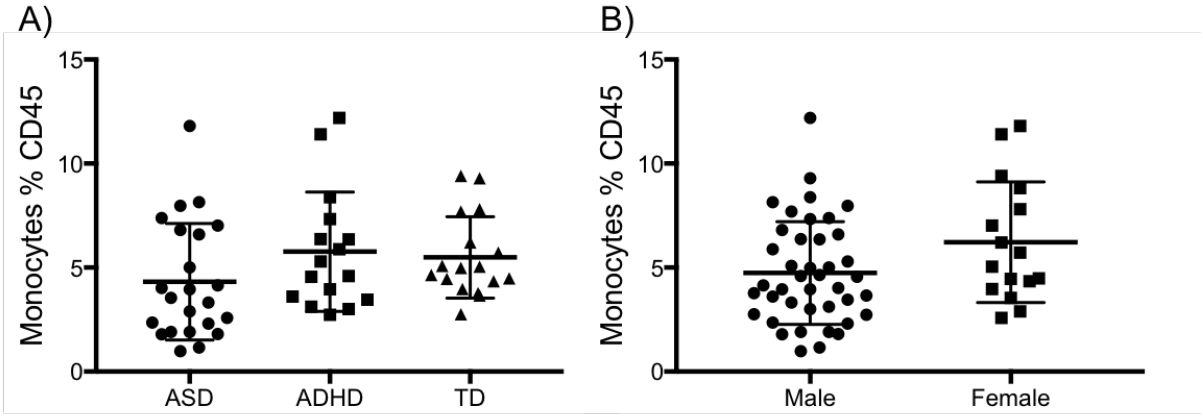
	ASD			ADHD			TD		
Variable	N	Mean	SD	N	Mean	SD	N	Mean	SD
Monocytes % CD45	23	4.322	2.800	16	5.766	2.867	17	5.489	1.960
CD14++ CD16+ % Monocyte	23	2.869	1.970	16	3.098	1.058	17	3.012	0.929
CD14++ CD16- % Monocyte	23	65.543	17.399	16	77.325	8.087	17	80.041	6.471
CD14+ CD16+ % Monocyte	23	4.811	2.962	16	3.962	1.961	17	3.348	2.108
NK % CD45	25	12.358	6.991	17	10.368	4.110	17	10.708	5.343
B Cells % CD45	25	7.239	3.567	17	6.826	2.290	17	5.481	1.616
T Cells % CD45	25	70.440	8.852	17	70.129	6.909	17	71.841	7.207
CD4+ % CD45	25	59.047	14.231	17	61.300	7.010	17	61.547	7.919
CD8+ % CD45	25	28.196	5.794	17	27.994	5.684	17	27.871	6.513
CD4+ CD8+ % CD45	25	1.624	0.941	17	1.695	0.967	17	1.267	0.629

Table 4.3: Descriptive statistics of the frequency of immune cell populations.

Variable	ASD			ADHD			TD		
	N	Mean	SD	N	Mean	SD	N	Mean	SD
Monocyte MFI CD115	24	259.45	257.73	16	254.39	140.06	17	213.33	142.79
Monocyte MFI CD13	24	3860.60	3244.11	16	4551.83	2628.69	17	6083.25	2872.04
Monocyte MFI CD64	24	1766.49	1034.72	16	2335.45	772.03	17	2349.89	729.22
CD14++CD16+ MFI CD115	24	118.35	85.06	16	47.54	51.34	17	327.86	681.96
CD14++CD16+ MFI CD13	24	11170.77	7506.00	16	9795.71	6594.04	17	13647.37	7518.02
CD14++CD16+ MFI CD64	24	2138.70	1188.17	16	1608.14	690.87	17	1682.71	523.30
CD14++CD16- MFI CD115	24	313.43	368.60	16	275.18	159.79	17	234.84	154.22
CD14++CD16- MFI CD13	24	8475.02	5311.57	16	7499.96	4316.62	17	10195.14	5261.41
CD14++CD16- MFI CD64	24	4303.49	1639.25	16	4047.83	1026.53	17	4011.24	1097.51
CD14+CD16+MFI CD115	24	339.41	379.22	16	229.06	136.32	17	160.18	83.34
CD14+CD16+MFI CD13	24	5127.91	3965.63	16	5114.16	4462.84	17	6455.49	4712.68
CD14+CD16+MFI CD64	24	373.71	214.40	16	428.08	181.55	17	342.83	104.10
Monocyte MFI CX3CR1	24	475.80	276.40	16	530.17	209.94	17	545.56	248.08
Monocyte MFI CCR2	24	4796.52	2719.41	16	6635.71	2262.31	17	6868.60	2708.84
Monocyte MFI CD16	24	34.22	35.01	16	21.79	16.87	17	17.27	11.48
CD14++CD16+ MFI CX3CR1	24	1909.57	923.00	16	2060.79	622.13	17	2211.97	716.41
CD14++CD16+ MFI CCR2	24	2399.27	1837.04	16	1971.59	2105.16	17	1268.72	785.59
CD14++CD16+ MFI CD16	24	1456.23	1490.49	16	1743.42	820.93	17	2101.90	976.73
CD14++CD16- MFI CX3CR1	24	779.07	390.99	16	570.92	154.55	17	590.44	232.45
CD14++CD16- MFI CCR2	24	15344.44	3738.06	16	13412.90	2094.26	17	13423.45	3491.24
CD14++CD16- MFI CD16	24	7.14	5.94	16	5.71	9.99	17	2.79	2.43
CD14+CD16+MFI CX3CR1	24	1809.90	647.25	16	2139.11	627.61	17	1896.32	453.23
CD14+CD16+MFI CCR2	24	112.15	158.14	16	76.96	100.39	17	78.37	79.31
CD14+CD16+MFI CD16	24	7068.73	4120.17	16	8617.36	3903.56	17	8658.14	3069.87

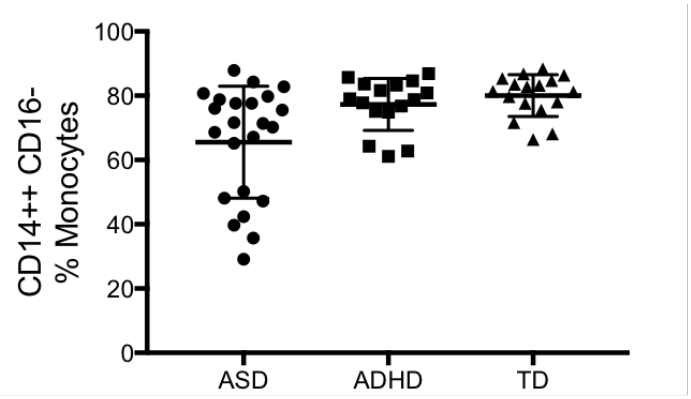
Table 4.4: Descriptive statistics of the mean fluorescence intensity of maturation and activation markers.

No significant changes in cell frequency or surface receptor expression in the TD or ADHD groups were identified. The frequency of monocytes was found to be significantly lower in ASD compared to ADHD (Figure 4.4A), as well as significantly lower in males than in females independent of diagnosis (Figure 4.4B). The frequency of classical monocytes was also found to be significantly lower in the ASD group than in both the ADHD and TD groups (Figure 4.5). Frequency of B cells was found to be significantly lower in females than in males, independent of diagnosis (Figure 4.6).



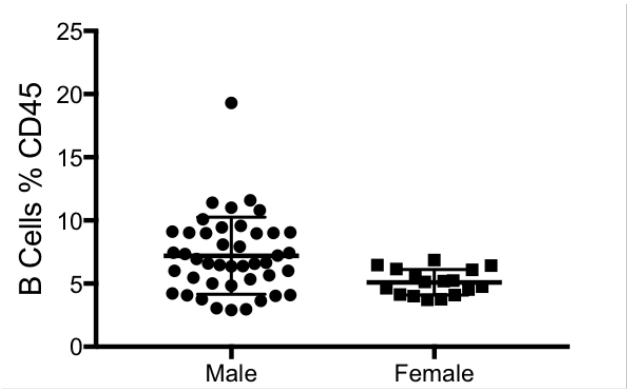
Dependent: Monocytes % CD45				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-0.919	-0.17	0.282
	ADHD	0.95	0.163	0.325
	Sex	1.741	0.298	**0.04***
ADHD	ASD	-1.955	-0.362	**0.02***
	Sex	1.755	0.301	**0.032***

Figure 4.4: *Comparison of monocyte frequency across diagnosis*. Using multiple linear regression, it was found that A) monocyte frequency was significantly lower in ASD than in ADHD and B) monocyte frequency was significantly lower in males than in females.



Dependent: CD14++ CD16- % Monocytes				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-14.177	-0.498	**0.001***
	ADHD	-1.316	-0.043	0.782
	Sex	3.23	0.105	0.434
ADHD	ASD	-13.068	-0.459	**0.002***
	Sex	3.424	0.111	0.392

Figure 4.5: *Comparison of classical monocyte frequency across diagnosis*. Using multiple linear regression, it was found that classical monocyte (CD14++ CD16-) frequency was significantly lower in ASD than in ADHD or TD.

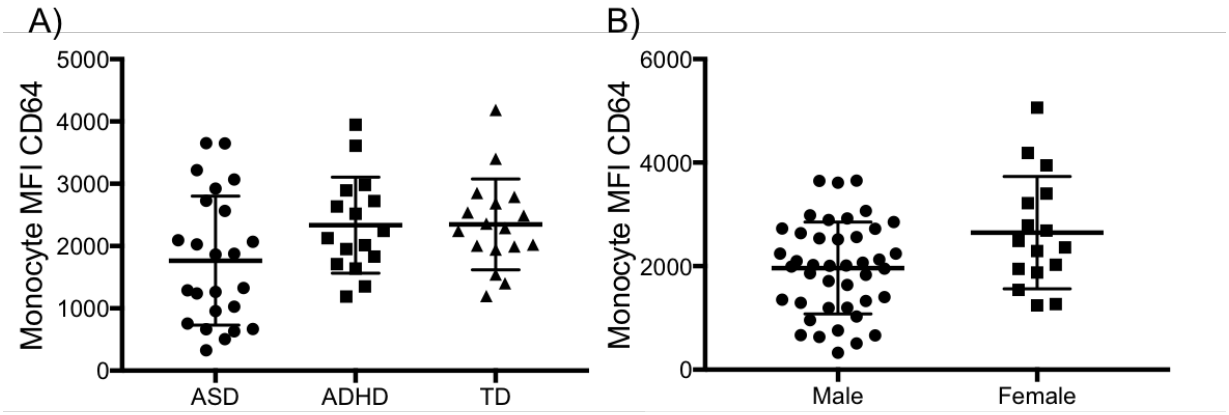


Dependent: B % CD45				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	1.106	0.194	0.218
	ADHD	0.491	0.079	0.619
	Sex	-1.832	-0.296	**0.034***
ADHD	ASD	0.547	0.096	0.516
	Sex	-1.812	-0.293	**0.031***

Figure 4.6: *Sex differences in B cell frequency*. Using multiple linear regression, it was found that B cell frequency was significantly higher in males than females independent of diagnosis.

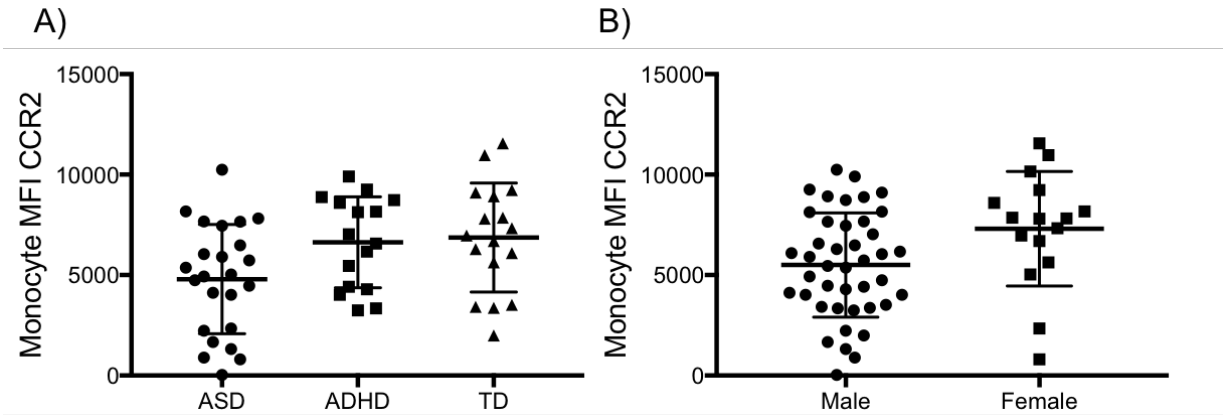
Monocyte surface expression of activation and maturation markers was altered in the ASD group. Monocyte expression of CD64, a high affinity IgG receptor, was significantly lower in ASD than in ADHD and there was a trend of lower expression of this marker in ASD than in TD that did not reach significance (Figure 4.7A). Expression of CD64 on monocytes was also found to be lower in males than in females, independent of diagnosis (Figure 4.7B). Monocyte expression of CCR2, a chemokine receptor associated with monocyte trafficking to tissues, was significantly lower in ASD than in both ADHD and TD (Figure 4.8A). Males also had significantly lower expression of CCR2 on monocytes than females, independent of diagnosis (Figure 4.8B). Intermediate monocyte expression of CCR2 was significantly higher in the ASD group than in the TD group (Figure 4.8C). Monocyte expression of CD16, a marker of monocyte activation, was significantly higher in ASD than in the TD group (Figure 4.9).

Classical monocyte expression of CX_3CR_1, a chemokine receptor associated with monocyte homeostasis, was significantly higher in ASD than both the ADHD and TD groups (Figure 4.10).

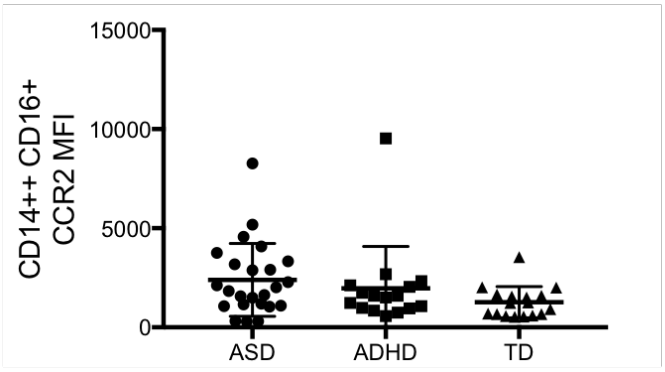


Dependent: Monocyte MFI CD64				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-577.154	-0.294	0.054
	ADHD	183.268	0.085	0.584
	Sex	706.819	0.329	**0.017***
ADHD	ASD	-889.65	-0.452	**0.002***
	Sex	783.94	0.365	**0.006***

Figure 4.7: *Comparison of CD64 expression across diagnosis*. Using multiple linear regression, it was found that A) monocyte expression of CD64 was significantly lower in ASD than in ADHD and B) monocyte expression of CD64 was significantly lower in males than in females independent of diagnosis.

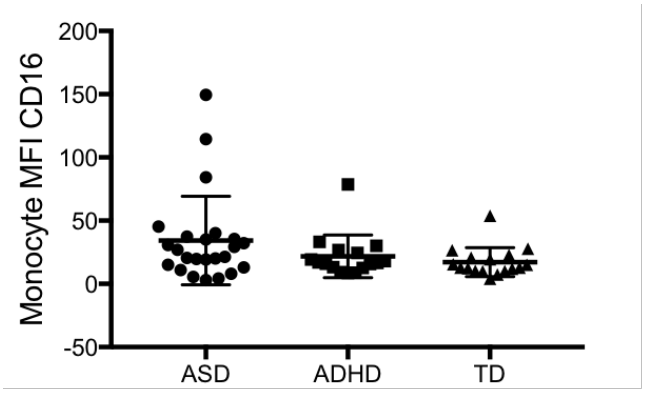


Dependent: Monocytes MFI CCR2				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-1872.402	-0.34	**0.025***
	ADHD	487.328	0.081	0.599
	Sex	1830.92	0.304	**0.025***
ADHD	ASD	-2470.884	-0.448	**0.002***
	Sex	1882.634	0.313	**0.018***



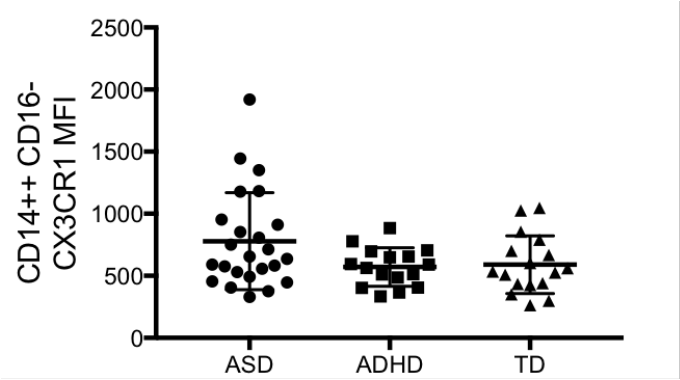
Dependent: CD14++ CD16+ MFI CCR2				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	1220.182	0.352	**0.035***
	ADHD	922.297	0.244	0.156
	Sex	574.718	0.152	0.304
ADHD	ASD	307.965	0.089	0.572
	Sex	527.167	0.139	0.329

Figure 4.8: *Comparison of CCR2 expression across diagnosis*. Using multiple linear regression, it was found that A) monocyte expression of CCR2 was significantly lower in ASD than in ADHD and TD. B) Monocyte expression of CCR2 was significantly lower in males than in females independent of diagnosis. C) Intermediate monocyte expression of CCR2 was significantly higher in ASD than in TD.



Dependent: Monocytes MFI CD16				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	20.588	0.392	**0.017***
	ADHD	9.637	0.168	0.318
	Sex	10.487	0.183	0.209
ADHD	ASD	11.836	0.226	0.15
	Sex	9.476	0.165	0.242

Figure 4.9: *Comparison of CD16 expression across diagnosis*. Using multiple linear regression, it was found that CD16 expression was significantly higher in ASD than in the TD group.



Dependent: CD14++ CD16- MFI CX3CR1				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	230.003	0.37	**0.023***
	ADHD	16.724	0.025	0.882
	Sex	57.723	0.085	0.554
ADHD	ASD	224.361	0.361	**0.022***
	Sex	49.671	0.073	0.598

Figure 4.10: *Comparison of CX_3CR_1 expression across diagnosis*. Using multiple linear regression, it was found that CX_3CR_1 expression was significantly higher in ASD than in both the ADHD and TD groups.

4.24) Soluble and cellular correlations

Since monocyte populations are very sensitive to changes in soluble inflammation, we wanted to investigate the association between markers of soluble and cellular inflammation across disorders. Relationships between concentrations of acute phase proteins and immune cell frequencies or MFIs of maturation and activation markers were determined using Spearman correlations. In the TD group, CRP positively correlated with total monocyte and classical monocyte frequency (Figure 4.11). Also in the TD group, Ferritin positively correlated with expression of CD64 on intermediate and total monocytes, and CRP positively correlated with total monocyte expression of CCR2 and negatively correlated with total monocyte expression of CD16 (Figure 4.12). We hypothesized that the associations identified in the TD group reflect normal adaptations to soluble inflammatory mediators. Interestingly, the observed associations

between markers of soluble inflammation and cellular inflammation in the ASD and ADHD groups did not overlap with those observed in the TD group. In the ASD group, it was found that CRP positively correlated with CD8+ T cell frequency (Figure 4.11). In ASD, CRP also positively correlated with CD13 expression on all subsets of monocytes, and negatively correlated with CD115 expression on total monocytes (Figure 4.12). In the ADHD group, CRP positively correlated with the frequency of intermediate and non-classical monocytes (Figure 4.11). Also in the ADHD group, tPA negatively correlated with CD115 expression on classical and total monocytes, and negatively correlated with CX_3CR_1 expression on total monocytes. Finally, tPA positively correlated with CCR2 expression on intermediate monocytes and CD64 expression on total monocytes in the ADHD group (Figure 4.12). The findings indicate altered monocyte responses to soluble inflammation based on diagnosis.

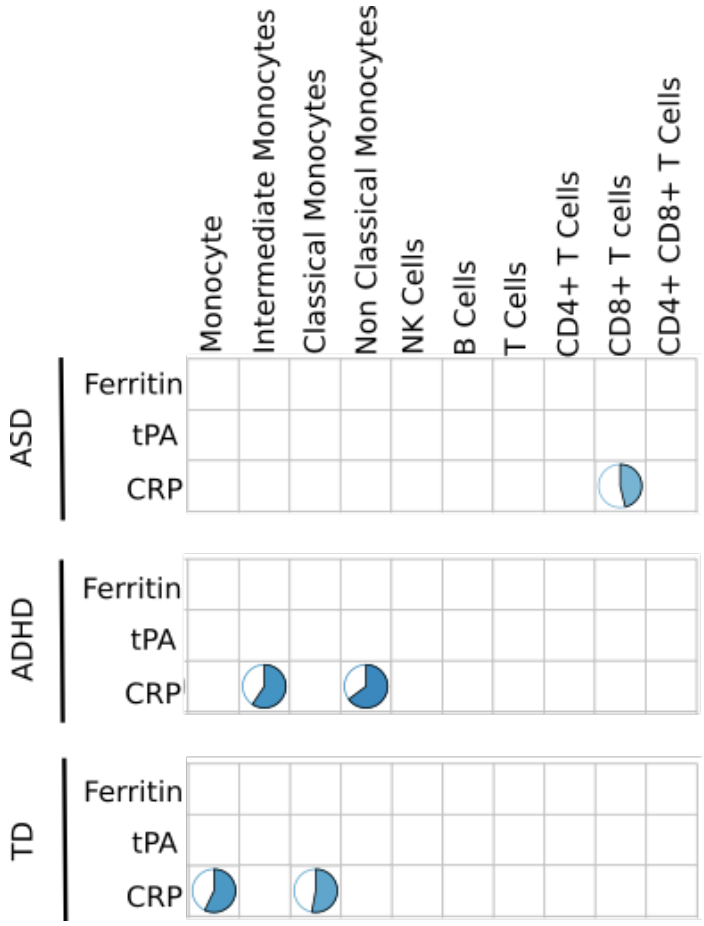


Figure 4.11: *Correlates of acute phase proteins and immune cell frequency.* Spearman correlations were performed to identify associations between APPs and cell frequencies (%CD45+). Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

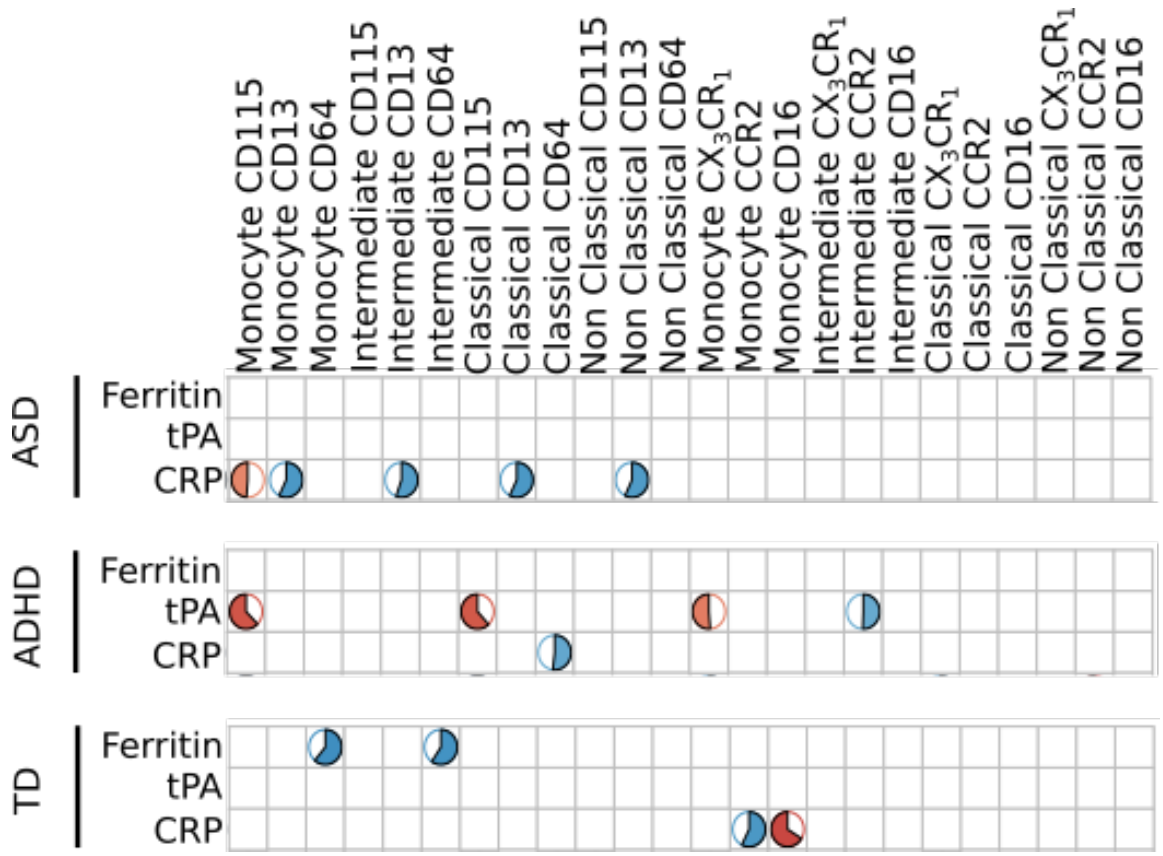


Figure 4.12: *Correlates of acute phase proteins and surface receptor expression.* Spearman correlations were performed to identify associations between APPs and surface receptor expression (MFI). Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

4.25) Inflammation and behaviour

Associations between inflammation and changes in behaviour is well documented[48]. We hypothesized that an increase in soluble and cellular inflammation in neurodevelopmental disorders would be associated with more severe behaviour. To determine if these markers of soluble and cellular inflammation are associated with behavioural outcomes, concentrations of acute phase proteins, frequencies of immune cells and MFIs of maturation and activation markers were compared with behaviour scores. These scores were collected using the ABAS, which measures severity of adaptive behaviours, and the CBCL, which measures the severity of

problem behaviours. More severe adaptive behaviours are given a lower ABAS score, and more severe problem behaviours are given a higher CBCL score.

No significant correlations between acute phase proteins and ABAS scores were identified in any disorder. No significant associations between APPs and CBCL scores in the TD group were identified. In ASD, tPA was found to negatively correlate with CBCL scores (Figure 4.13). Since an increased CBCL score indicates more severe behaviours, this means that increased tPA correlates with less severe internalizing and externalizing problem behaviours. In ADHD, ferritin positively correlated with CBCL scores, indicating that increased ferritin correlates with more severe problem behaviours.

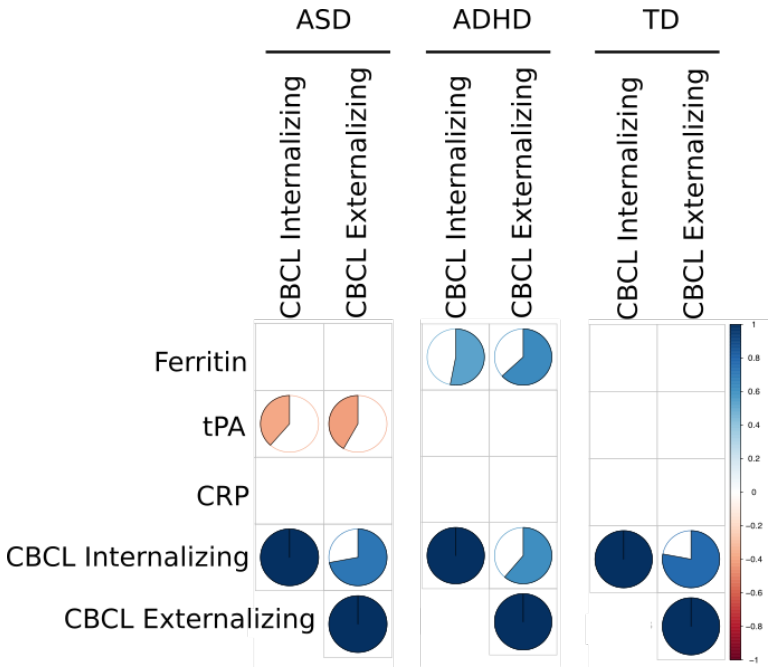


Figure 4.13: *Correlates of acute phase proteins and CBCL scores.* Spearman correlations were performed to identify associations between APPs and CBCL T scores. Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

In the TD group, all significant correlations between surface receptor expression and ABAS score were positive, indicating that an increase in expression of surface receptor expression correlates with less severe adaptive functioning (Figure 4.14). In the ASD and ADHD groups, overall, significant correlations between cell frequencies or surface receptor expression and ABAS score were negative (Figure 4.14). Since a lower ABAS score indicates more severe adaptive behaviours, these correlations mean that an increase in the indicated cell frequencies or surface receptor expression correlates with worse adaptive functioning. The exception to this trend of negative correlations in the neurodevelopmental disorders was seen in NK cell frequency in the ASD group. NK cell frequency positively correlated with ABAS score, indicating that an increase in NK cell frequency correlated with less severe adaptive functioning (Figure 4.14).

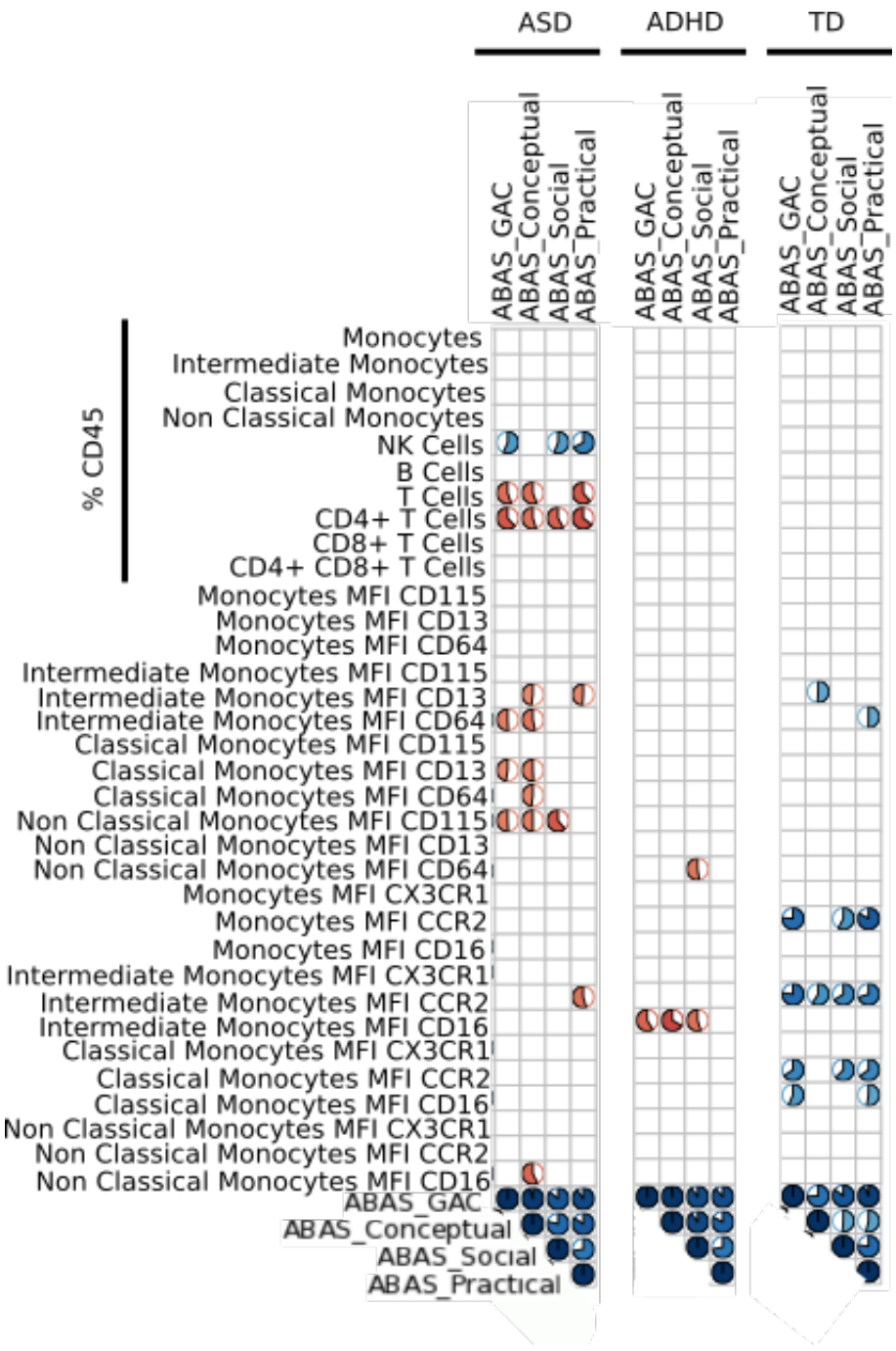


Figure 4.14: *Correlates of cell frequencies, MFIs and ABAS scores.* Spearman correlations were performed to identify associations between cellular factors and ABAS composite scores. Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

In the TD group, all significant correlations of cell frequencies or surface receptor expression with CBCL score were negative (Figure 4.15). This implied that an increase in the indicated cell frequency or MFI correlated with less severe problem behaviours. In the ASD group, most significant correlations between cell frequencies or surface receptor expression and CBCL score were positive (Figure 4.15). Since a higher CBCL score indicates more severe problem behaviours, these correlations mean that an increase in the indicated cell frequency or surface receptor expression correlated with worse problem behaviours. In both ASD and ADHD, NK cell frequency negatively correlated with CBCL internalizing problems score, indicating that an increase in NK cell frequency correlated with less severe internalizing problems.

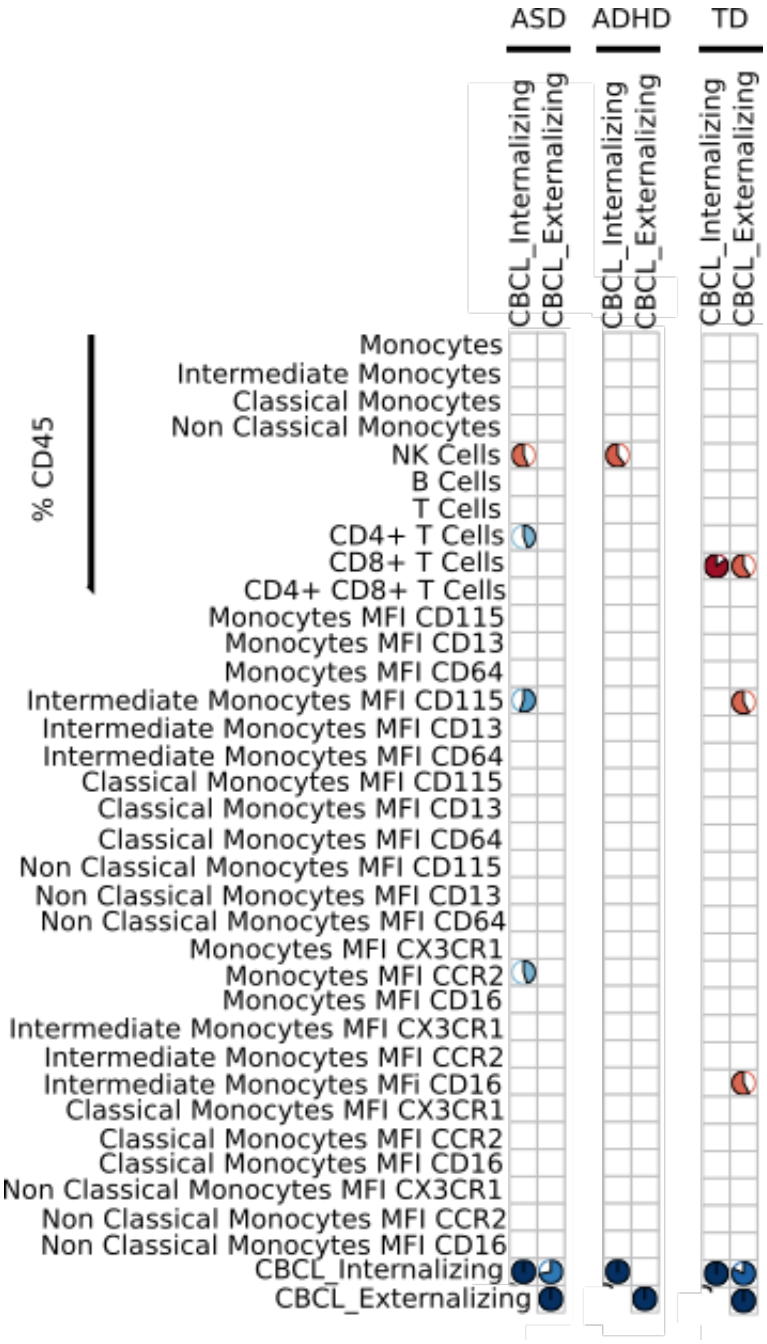


Figure 4.15: *Correlates of cell frequencies, MFIs and CBCL scores.* Spearman correlations were performed to identify associations between cellular factors and CBCL T scores. Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

To replicate and expand upon previous findings with the Omega-3 cohort (Chapter 3), we investigated the associations of acute phase proteins, immune cell frequency and surface receptor expression with ASD-specific behaviour measures. The ADI-R was used to measure ASD like symptoms, with a higher score indicating more severe behaviours. The VABS was used to measure adaptive behaviours, with a lower score indicating more severe adaptive functioning.

We found no significant correlations between levels of circulating acute phase proteins and ADI-R scores. Ferritin was found to negatively correlate with VABS scores of communication, daily living skills and total adaptive composite in males (Figure 4.16). This indicated that increased ferritin correlated with worse adaptive functioning in these categories.

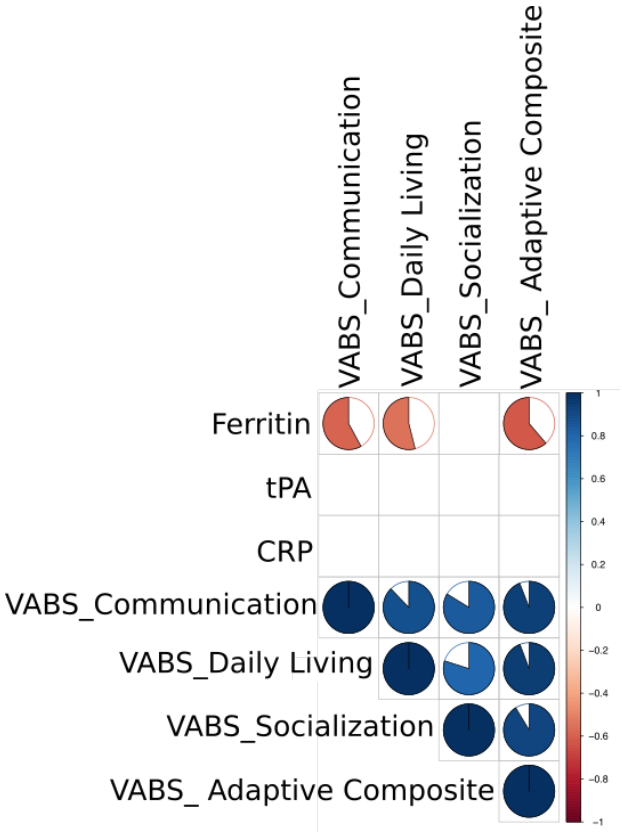


Figure 4.16: *Correlates of acute phase proteins and VABS scores.* Spearman correlations were performed to identify associations between APPs and VABS standard scores in males. Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

To expand upon findings from the Omega-3 cohort in Chapter 3 we investigated associations of immune cell frequencies and monocyte expression of maturation and activation markers with ASD-specific behaviour measures, using the VABS and ADI-R. Frequency of CD4+ T cells and non-classical monocyte expression of CD115 negatively correlated with VABS scores. This means that an increase in cell frequency or surface receptor expression correlated with worse adaptive behaviours (Figure 4.17A). The frequency of NK cells positively

correlated with VABS socialization score, indicating that an increase in NK cell frequency correlated with less severe socialization (Figure 4.17A).

Increased monocyte expression of CD16 and non-classical monocyte expression of CD64 correlated with more severe ADI-R social domain scores. Frequency of CD8+ T cells correlated with a more severe ADI-R repetitive behaviour scores. Non-classical monocyte expression of CD115 correlated with less severe ADI-R communication scores and monocyte expression of CX_3CR_1 correlated with a less severe ADI-R repetitive behaviour score (Figure 4.17B).

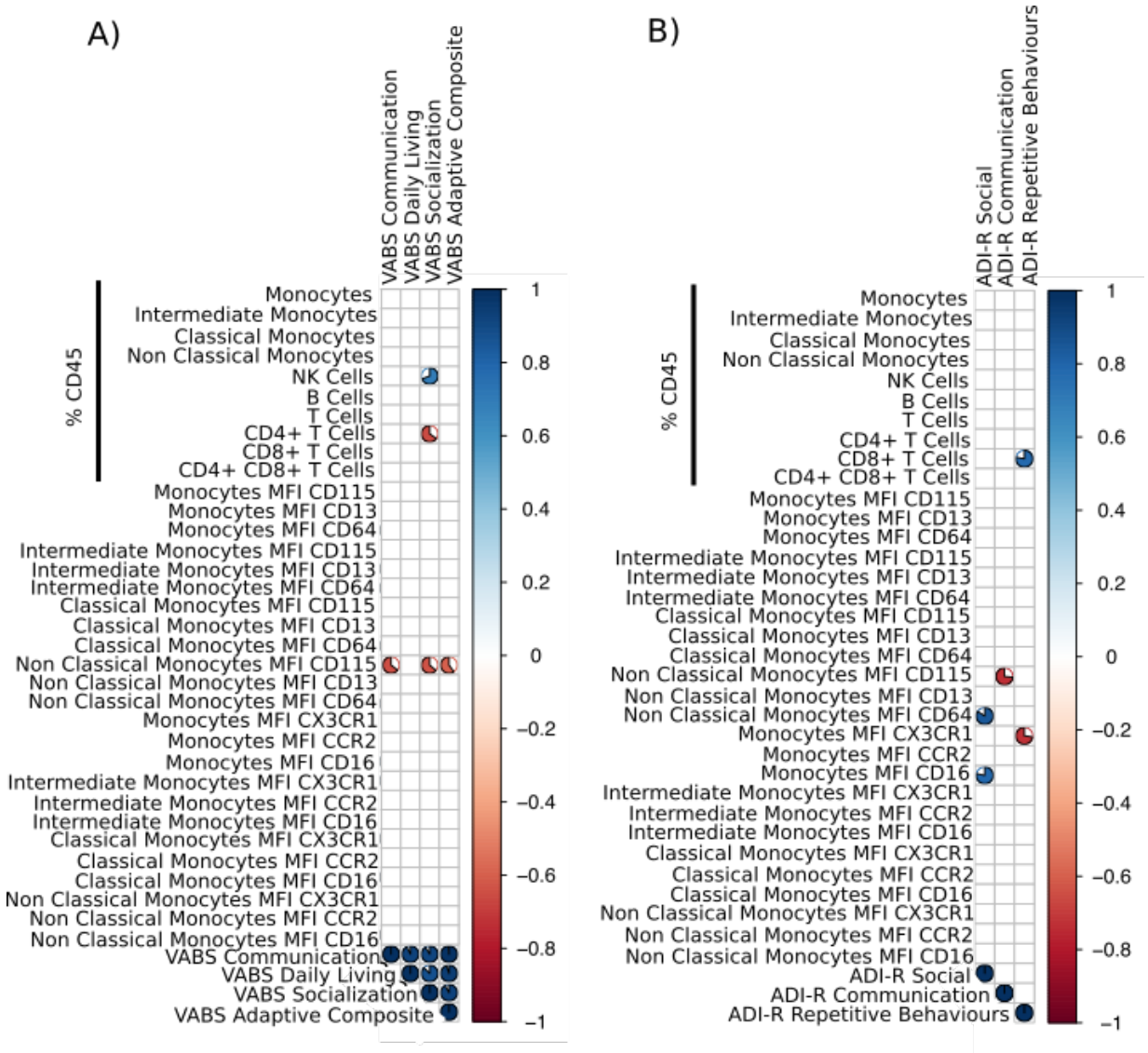


Figure 4.17: *Correlates of immune cell frequencies and surface receptor expression with VABS and ADI-R scores.* Spearman correlations were performed to identify associations of cellular inflammation with VABS standard scores and ADI-R domain totals. Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

4.3) Discussion

To investigate circulating soluble and cellular inflammation in children and young adults with neurodevelopmental disorders, as well as typically developing controls, we measured circulating concentrations of acute phase proteins and analyzed the frequency and phenotype of circulating leukocytes. We found that circulating levels of the acute phase protein CRP was significantly higher in the ASD group compared to the ADHD and TD groups. CRP is a well characterized marker of inflammation that is clinically useful to detect acute or chronic inflammation and is used to assist in the detection or measure progression of inflammatory diseases such as rheumatoid arthritis, osteoarthritis and inflammatory bowel disorders[64,98,99]. One previous study has found CRP to be increased in ASD, and it was found that CRP correlated with more severe ASD-like behaviours, measured using the childhood autism rating scale (CARS)[100]. Otherwise, most studies of soluble inflammation in ASD measure circulating cytokines, however these results have been inconsistent between studies. CRP is a clinically relevant marker of soluble inflammation that is more likely to remain in the circulation in response to inflammatory signals.

In addition to soluble markers of inflammation, we investigated cellular inflammation by quantitating circulating leukocytes. We hypothesized that there would be changes to monocyte frequency or activation and maturation status because these cells are very sensitive to changes in soluble inflammation. We were able to identify alterations in monocyte frequency and phenotype in the ASD group. The frequency of both total monocytes and classical monocytes was lower in the ASD group. Furthermore, monocyte expression of CCR2 was lower in ASD than in both the ADHD group and the TD group. This decrease in monocytes in conjunction with a decreased expression of CCR2 could indicate that the monocytes are being recruited to the tissue in a CCL2

dependent manner. A previous study found CCL2 to be elevated in the circulation of children with ASD and circulating CCL2 correlated with more impaired behavioural outcomes in these children[77]. Furthermore, a study of neuroinflammation in ASD found CCL2 was significantly elevated in the brains of these patients[101].

Classical monocyte expression of CX_3CR_1 was increased in the ASD group compared to both the ADHD and TD groups. Classical monocytes express high levels of CCR2, but some also express low levels of CX_3CR_1 (Appendix Figure 5). If monocytes are being recruited to the tissues in a CCL2 dependent manner, the reduction in CCR2 expression monocytes could lead to an apparent increase in CX_3CR_1 expression on classical monocytes.

We also investigated associations between soluble and cellular inflammation, and found that in all diagnoses, CRP positively correlated with changes in cell frequency. As expected in the TD group, CRP positively correlated with the frequency of total monocytes and classical monocytes. This result is reflective of an adaptive response to soluble inflammation, with monocytes, specifically classical monocytes, increasing in response to CRP. Interestingly, in the ASD group, this did not occur, likely due to the reduction in total and classical monocytes. In ASD, CRP positively correlated with frequency of CD8+ T cells. Whether this apparent relationship is due to an increase in the absolute numbers of CD8+ T cells or is the result of a reduction in circulating monocytes remains to be determined.

Monocytes are known to proliferate in response to soluble inflammation, as well as alter their expression of cell surface markers such as CD16, CD64 and CD115. Thus, we hypothesized that in the TD group, any correlations between monocyte expression of these surface markers with soluble inflammation would be normal adaptations to inflammation. We also hypothesized that if the ADHD and ASD groups did not demonstrate the same associations between soluble

inflammation and monocyte phenotype, these might be maladaptive responses to soluble inflammation. All three groups exhibited completely different correlation patterns. If we assume that the typically developing group represents an adaptive response to soluble changes, then both the ASD and ADHD groups may display maladaptive responses, as none of the correlations found in the TD group were found in the neurodevelopmental disorders. The correlations between ASD group and the ADHD group were also completely different, indicating that there are diagnosis specific immune changes. These findings imply that any underlying immune pathologies that may exist in these two disorders are not congruent.

We found that markers of soluble and cellular inflammation correlated with behaviour. In both ASD and ADHD, increases in immune cell frequency or surface receptor expression generally correlated with more severe behaviour, whereas increases in cell frequency or receptor expression in the TD group consistently correlated with less severe behaviours. This is an interesting finding because it shows that immune changes in neurodevelopmental disorders may negatively impact behavioural outcomes, or represent biomarkers of more severe behaviour in these disorders.

The one exception to this trend was seen in the NK cells. Increases in NK cell frequency correlated with less severe behaviours in both the ASD and ADHD groups. Multiple studies of ASD cohorts have identified low NK cell activity and cytotoxicity in vitro[102–104], but no behaviour analysis was done in these studies. There is also no evidence of NK cells associating with behaviour in mouse models. This may be a novel finding that an increase in NK cell frequency correlates with less severe behavioural outcomes in neurodevelopmental disorders.

Chapter Five

Associations Between Intestinal Permeability and Behaviour in Neurodevelopmental Disorders

5.1) Introduction

Previous studies have shown an increased incidence of gastrointestinal distress (i.e. diarrhea, constipation, bloating and food sensitivities[24–26]) in children with ASD. This may be due to increased intestinal permeability, which has been reported to occur in individuals with ASD[31,105]. Increased intestinal permeability may also contribute to alterations in behaviour, as has been reported in inflammatory bowel disorders[35].

As shown in Chapter 3, we found that I-FABP, a marker of intestinal epithelial damage, positively correlated with circulating levels of the bacterial product lipopolysaccharide (LPS) in children with ASD. Furthermore, both I-FABP and LPS positively correlated with circulating levels of the acute phase protein tissue plasminogen activator (tPA), and correlated with changes in behaviour, as measured by VABS and ADI-R. To replicate these findings in a larger cohort with a broader age range, we accessed cryopreserved plasma, collected as part of the Province of Ontario Neurodevelopmental Disorder (POND) Network's immune platform. To determine whether intestinal permeability and systemic inflammation was associated with behavioural changes across neurodevelopmental disorders, we compared individuals with ASD, ADHD and typically developing controls.

As per our previous study, we measured circulating levels of intestinal fatty acid binding protein (I-FABP) and the bacterial products LPS and MDP to quantitate intestinal epithelial damage and intestinal permeability. Circulating levels of these markers were compared between diagnosis. We measured circulating concentrations of the acute phase proteins ferritin, tPA and

CRP, as described in Chapter 4, to investigate associations between intestinal permeability and soluble inflammation. Using measurements of immune cell frequency and monocyte surface expression of maturation and activation markers outlined in Chapter 4, we will investigate associations between intestinal permeability and cellular inflammation.

In addition to identifying alterations in these markers between diagnosis, we investigated whether intestinal permeability was associated with behaviour using scores of adaptive behaviours, measured with the Adaptive Behaviour Assessment System (ABAS) and scores of problem behaviours, measured using the Child Behaviour Checklist (CBCL). To replicate findings from the Omega-3 cohort, we used ASD-specific behaviour data collected using the Autism Diagnostic Interview-Revised (ADI-R) and the Vineland Adaptive Behaviour Scale (VABS).

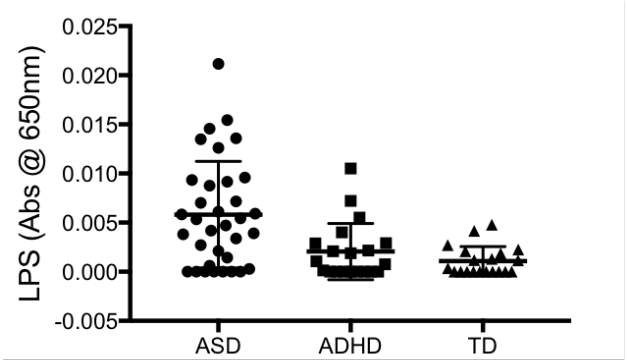
5.2) Results

5.21) Intestinal permeability in neurodevelopmental disorders

To investigate intestinal permeability in neurodevelopmental disorders, three biomarkers were used as indirect measurements. Intestinal fatty acid binding protein (I-FABP) was used as a measure of intestinal epithelial damage and the bacterial products LPS and MDP were measured as indicators of bacterial translocation. These three markers were measured in the plasma of children with neurodevelopmental disorders (Table 5.1). Using multiple linear regression analysis, LPS was found to be significantly higher in the ASD group than in both the ADHD and TD groups (Figure 5.1). No significant changes in the levels of these markers in the TD and ADHD groups were identified.

	ASD			ADHD			TD		
Variable	N	Mean	SD	N	Mean	SD	N	Mean	SD
MDP Relative Abs	34	0.029	0.024	20	0.028	0.014	20	0.036	0.018
LPS Relative Abs	34	0.006	0.005	20	0.002	0.003	20	0.001	0.001
ln (I-FABP) ng/mL	35	0.141	1.117	20	0.515	0.652	20	0.428	0.775

Table 5.1: Descriptive statistics of markers of intestinal permeability.



Dependent: LPS				
Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	0.005	0.507	**<0.001***
	ADHD	0.001	0.054	0.676
	Sex	-0.001	-0.125	0.254
ADHD	ASD	0.004	0.462	**<0.001***
	Sex	-0.001	-0.134	0.219

Figure 5.1: *Comparison of circulating LPS across diagnosis*. Using multiple linear regression, it was found that LPS was significantly higher in the ASD group than in the ADHD or TD groups

Correlations between I-FABP and circulating bacterial products (MDP, LPS) were made within each diagnosis group to indicate intestinal permeability. In the ASD group, a significant positive correlation between MDP and LPS in males was identified (Figure 5.2B). This was not observed in the TD group (Figure 5.2A). In the TD group, a positive trend between LPS and I-FABP in both males and females was identified, however, it did not reach significance (Figure

5.2C). This was not observed in the ASD group (Figure 5.2D). No significant correlations between these markers were identified in the ADHD group.

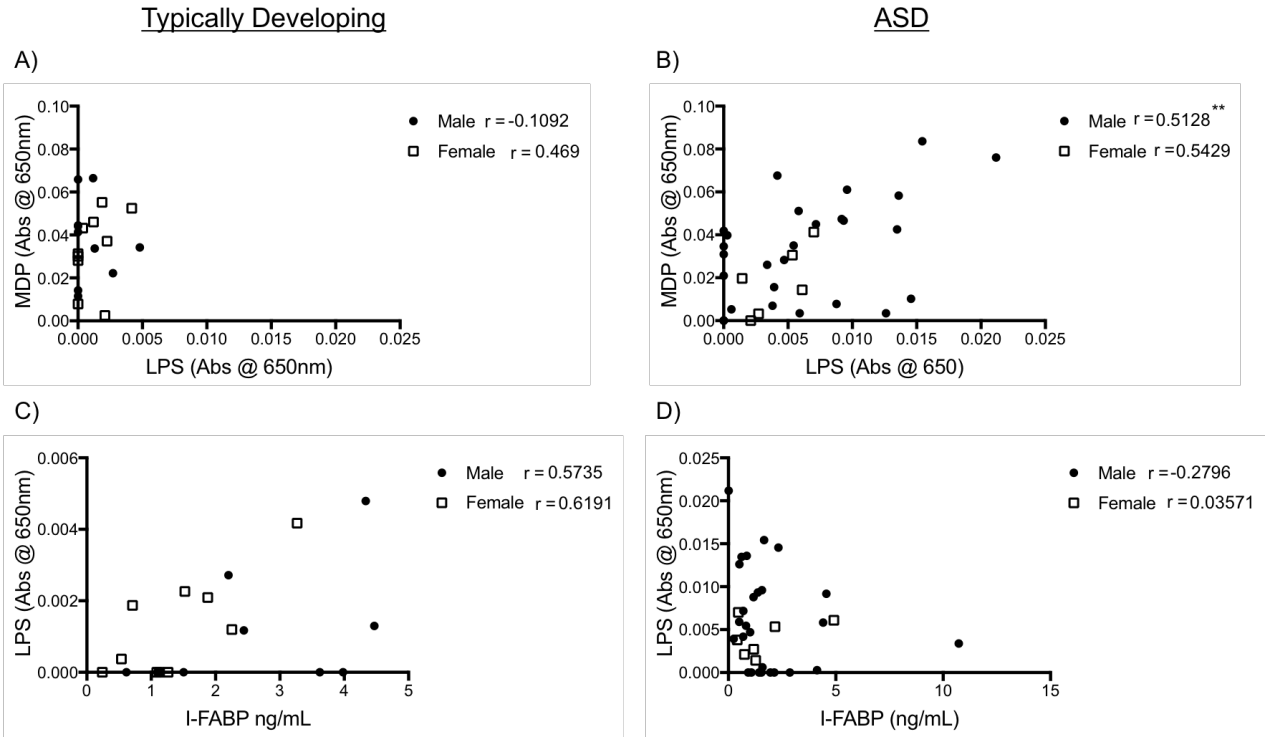


Figure 5.2: *Correlations of markers of intestinal permeability.* Associations were identified using Spearman correlations. B) In the ASD group, MDP and LPS were significantly correlated in males (r=0.5128, p=0.0053). C) In the TD group, there was a positive trend between LPS and I-FABP in both males (r=0.5735, p=0.0881) and females (r=0.6191, p=0.0625) that did not reach significance.

5.22) Associations of intestinal permeability with soluble inflammation

Relationships between markers of intestinal permeability (I-FABP, MDP and LPS) and the acute phase proteins ferritin, tPA and CRP were investigated. No significant correlations were identified in the ADHD or TD groups. In the ASD group, a significant positive correlation between circulating LPS and CRP was identified in males (Figure 5.3).

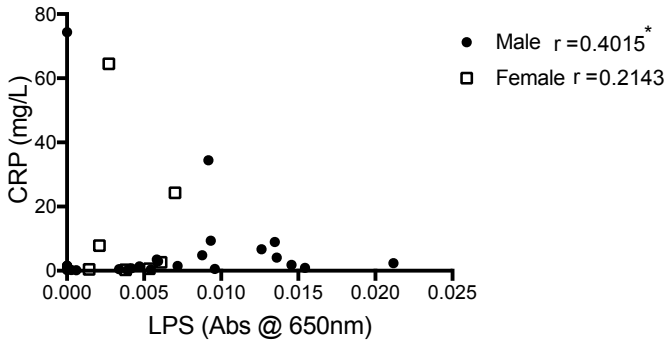


Figure 5.3: In the ASD group, LPS was found to positively correlate with CRP in males using a Spearman correlation (r=0.4015, p=0.0379). No significant correlations were found in the ADHD or TD groups.

5.23) Associations of intestinal permeability and cellular inflammation

To investigate associations between markers of intestinal permeability and cellular inflammation, I-FABP, LPS and MDP were compared with immune cell frequencies and expression of surface receptors (as measured by mean fluorescence intensity or MFI) using Spearman correlations. No associations between circulating bacterial products (MDP and LPS) or I-FABP and circulating leukocytes were identified in the TD group. Although levels of MDP were not significantly different between diagnoses, correlates were identified between MDP and leukocyte frequency. In the ASD group, circulating levels of MDP were found to positively correlate with B cell frequency (Figure 5.4). In the ADHD group, MDP was found to positively correlate with frequency of total monocytes and classical monocytes, and MDP negatively correlated with frequency of NK cells. Also in the ADHD group, LPS positively correlated with frequency of non-classical monocytes (Figure 5.4).

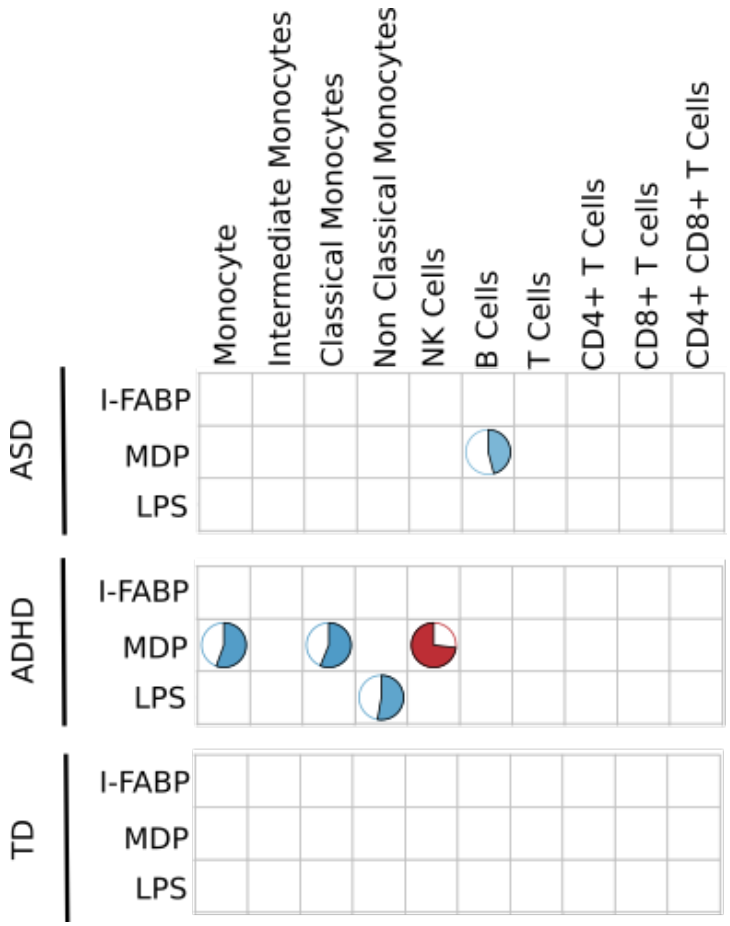


Figure 5.4: *Correlates of markers of intestinal permeability and immune cell frequency.* Spearman correlations were performed to identify associations between markers of intestinal permeability and cell frequencies (% CD45). Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

Monocyte phenotype changes in response to inflammation, thus we predicted that the presence of circulating MDP or LPS would alter monocyte phenotype. We measured the surface expression of activation and maturation markers to investigate alterations in monocyte phenotype. We found associations between circulating bacterial products and monocyte phenotype in the TD group. All significant correlations identified were negative, meaning that as

one factor increases, the other decreases. I-FABP correlated with CD115 expression on intermediate monocytes. MDP correlated with CD115 expression on classical, non-classical and total monocytes, as well as classical and total monocyte expression of CX_3CR_1 Finally, LPS correlated with classical monocyte expression of CX_3CR_1 (Figure 5.5). Because bacterial translocation from the gut is known to occur as a part of normal human digestion[106], we hypothesized that these associations reflected normal adaptations to transient increases in bacterial products. Interestingly, the profile of surface marker expression in ADHD and ASD did not overlap with that of the TD group. In the ASD group, MDP was found to negatively correlate with CD64 expression on all subsets of monocytes, CD16 expression on classical, non-classical and total monocytes, and CX_3CR_1 expression on classical monocytes. LPS was found to positively correlate with expression of CD115 on intermediate monocytes and CCR2 expression on intermediate and non-classical monocytes. LPS also negatively correlated with intermediate monocytes expression of CX_3CR_1 and CD16 (Figure 5.5). In the ADHD group, I-FABP positively correlated with intermediate and non-classical monocyte expression of CD13. MDP positively correlated with total monocyte expression of CCR2 and non-classical monocyte expression of both CX_3CR_1 and CD16 (Figure 5.5).

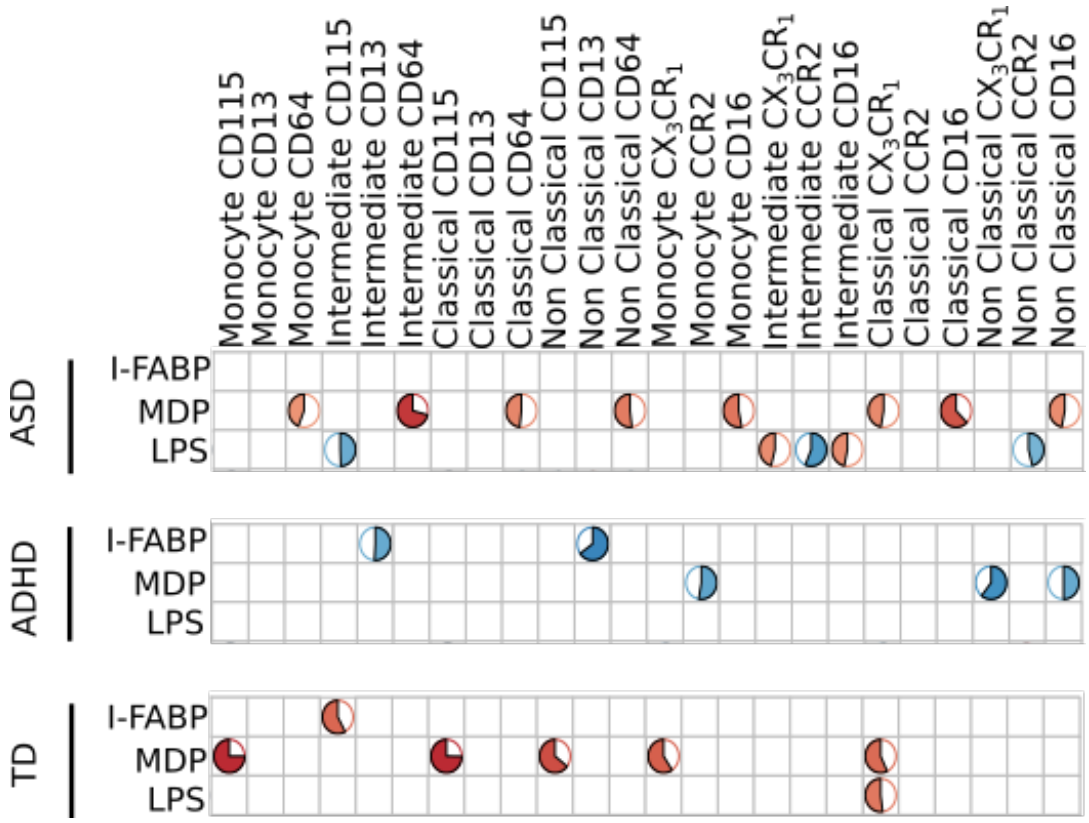


Figure 5.5: *Correlates of markers of intestinal permeability and surface receptor expression.* Spearman correlations were performed to identify associations between markers of intestinal permeability and surface receptor expression. Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

5.24) Intestinal permeability and behaviour

To determine if these alterations in markers of intestinal permeability were associated with behavioural outcomes in neurodevelopmental disorders. Relationships between circulating levels of I-FABP, LPS or MDP and behaviour scores were investigated in each of the disorders, as well as in the TD controls. These scores were collected using the ABAS, which measures adaptive behaviours, and the CBCL, which measures problem behaviours. More severe adaptive behaviours are given a lower ABAS score, and more severe problem behaviours are given a higher CBCL score.

No significant correlations between markers of intestinal permeability and CBCL scores were identified in any of the diagnoses. Significant correlations between markers of intestinal permeability and ABAS score were identified in the TD and ADHD groups, and no significant correlations were identified in the ASD group. In the TD group, there was a significant negative correlation between I-FABP and ABAS socialization score. This means that an increase in I-FABP correlates with worse adaptive functioning (Figure 5.6). In the ADHD group, LPS negatively correlated with ABAS general adaptive composite (GAC) score, and scores of practical behaviour. Since a lower ABAS score is more severe, an increase in LPS correlates with worse adaptive behaviours (Figure 5.6).

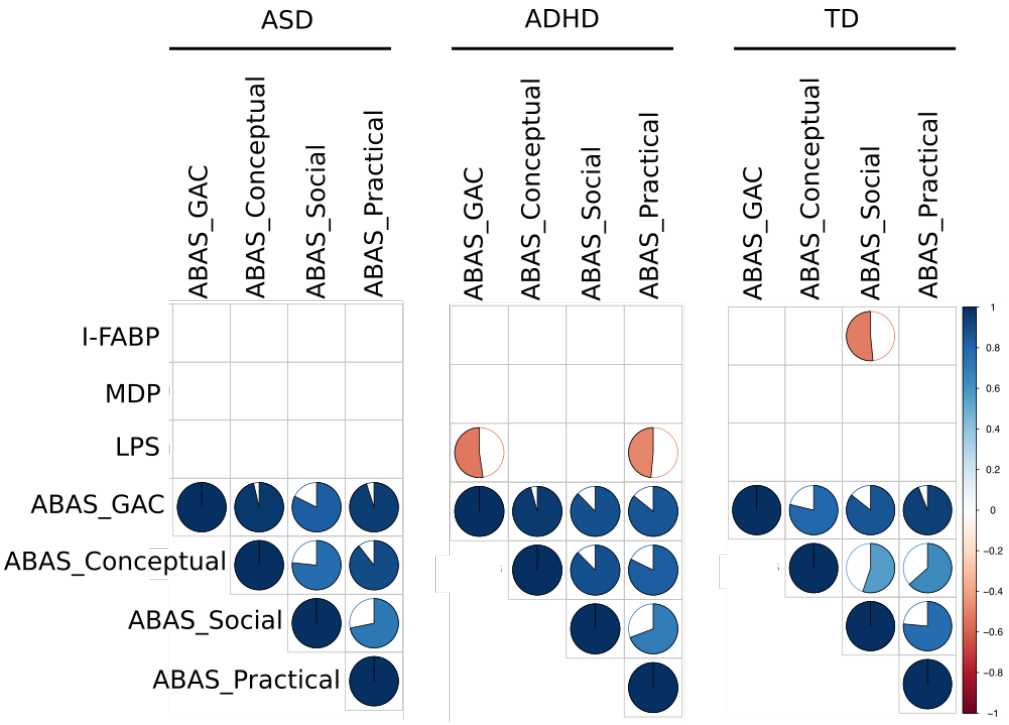


Figure 5.6: *Correlates of markers of intestinal permeability and ABAS scores.* Spearman correlations were performed to identify associations between markers of intestinal permeability and ABAS composite scores. Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation.

To replicate and expand upon previous findings with the Omega-3 cohort, we investigated the association between markers of intestinal permeability and ASD specific behaviour measures. The ADI-R was used to measure ASD like symptoms, with a higher score indicating more severe behaviours. The VABS was used to measure adaptive behaviours, with a lower score indicating more severe adaptive functioning

LPS was found to negatively correlate with VABS adaptive composite score and socialization score in males (Figure 5.7A). Since a lower VABS score indicates more severe adaptive behaviours, this means that an increase in LPS correlated with worse adaptive behaviours. I-FABP was found to negatively correlated with ADI-R communication scores in males (Figure 5.7B). Since a higher ADI-R score indicates more severe behaviours, an increase in I-FABP correlated with less severe communication.

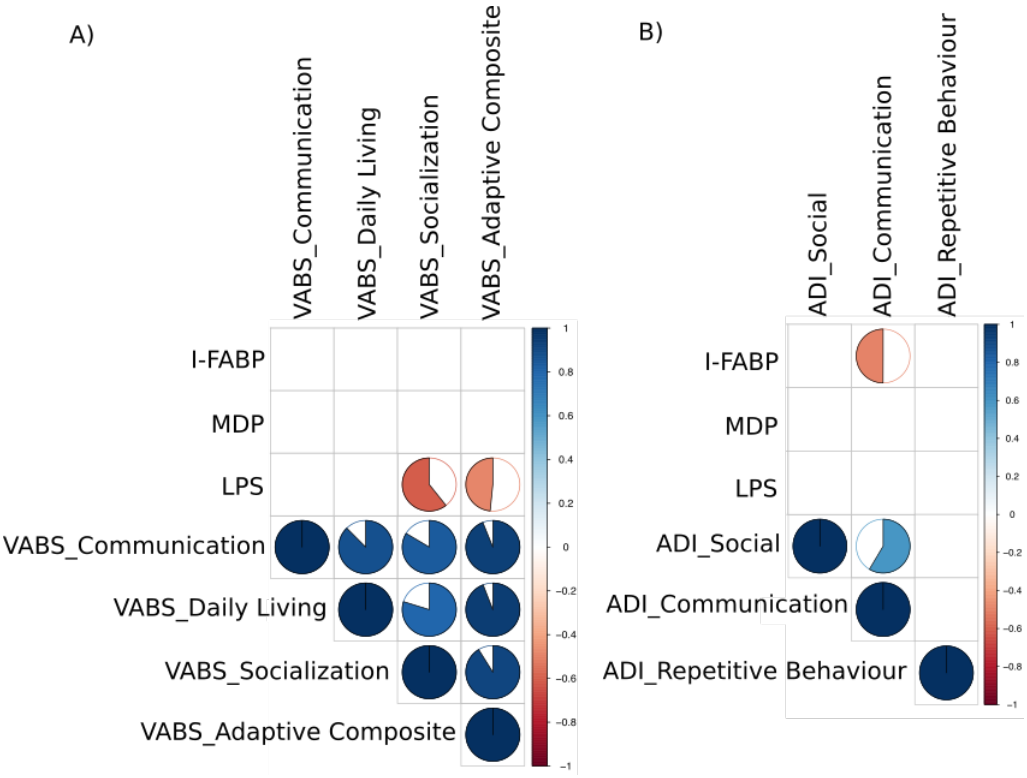


Figure 5.7: *Correlates of markers of intestinal permeability and behaviour scores.* Spearman correlations were performed to identify associations between markers if intestinal permeability, VABS standard scores and ADI-R domain totals. Only significant correlations are shown, indicated by a pie. The amount that the pie is full indicates the strength of the correlation. The colour of the pie indicates the direction of the correlation. Blue is a positive correlation, and orange is a negative correlation. A) Correlations of markers of intestinal permeability and VABS scores. B) Correlations of markers of intestinal permeability and ADI-R scores.

5.3) Discussion

To investigate intestinal permeability in children and young adults with neurodevelopmental disorders as well as TD controls, we measured circulating levels of I-FABP and the bacterial products MDP and LPS. We found that the bacterial product LPS was significantly increased in the ASD group compared to the ADHD and TD groups. LPS is a component of Gram negative bacterial cell walls and is a common pathogen associated molecular pattern (PAMP) recognized by the pattern recognition receptor (PRR) toll-like receptor (TLR)-4. Previous studies have observed that LPS is increased in adults with severe ASD[33].

Although MDP was not found to be significantly increased in the ASD group, LPS positively correlated with the bacterial product MDP in males. This may indicate a source of bacterial translocation in these subjects, and that source may be the gut. Consistent with our hypothesis that increased intestinal permeability and translocation of bacterial products could drive systemic inflammation in ASD, we found that increased LPS positively correlated with increased circulating CRP in males. Furthermore, CRP, was found to be significantly higher in ASD (data in Chapter 4). CRP is increased in the circulation in response to bacterial infection. It is intuitive that an increase in LPS could be associated with an increase in CRP. This indicates that LPS may be leading to increased soluble inflammation in this ASD cohort.

In the TD group, we identified a positive trend between LPS and I-FABP that did not reach significance. This was interesting, as we previously found these two markers positively correlated in females in the Omega-3 cohort (Chapter 3). This shows that in this cohort, intestinal epithelial damage is associated with bacterial translocation in the TD group, but not in the

neurodevelopmental disorders. This also shows that if there is intestinal permeability in the ASD cohort, leading to an increase in LPS, this is not due to intestinal epithelial damage.

We found some surprising associations between markers of intestinal permeability and markers of cellular inflammation. As we expected, there were no significant correlations between cellular inflammation and intestinal permeability in the TD group. Although transient intestinal permeability can occur in the absence of barrier dysfunction, it is quickly resolved and is unlikely to lead to significant cellular changes. In the ADHD group, LPS and MDP positively correlated with monocyte frequencies implying that exposure to bacterial products may be more prolonged. In the ASD group, MDP positively correlated with B cell frequency. This was surprising, as MDP is not known to act directly on B cells. However, we identified significantly lower circulating levels of monocytes in the ASD group in Chapter 4. With this data, we cannot know if this correlation is due to an increase in B cell frequency, or a relative decrease in monocyte frequency, that reflects as an increase in B cell frequency. Furthermore, in the ASD group, cell frequency was not impacted by the increase in LPS seen in this cohort. These findings could indicate a maladaptive immune response to these markers of intestinal permeability in the circulation.

Interestingly, in the ADHD group, MDP negatively correlated with NK cell frequency, meaning that as MDP increases, NK cell frequency decreases, or as NK cells frequency increase, MDP decreases. Previous studies have shown MDP activates NK cells[107]. This negative correlation could indicate a maladaptive NK cell response to MDP.

We also investigated correlations between markers of intestinal permeability and monocyte expression of activation and maturation markers. Similar to the results seen in Chapter 4, there was very little overlap between the correlations in the TD group and those identified in

neurodevelopmental disorders. We hypothesize that this indicates a maladaptive cellular response to intestinal epithelial damage and release of LPS and MDP in the neurodevelopmental disorders. As previously mentioned, classical monocytes are decreased in the ASD group (Chapter 4). In this chapter, we have found that in the intermediate monocytes, LPS correlated with an increase in expression of CCR2 and a decrease in expression of CX_3CR_1 and CD16. Because this more closely resembles the expression profile of classical monocytes, we interpret this as being maladaptive maturation due to chronic exposure to LPS.

There has been discussion of a link between intestinal permeability and behavior in neurodevelopmental disorders, we are the first to extensively characterize this connection. In this cohort, we found that markers of intestinal permeability correlated with behavioural outcomes. The phenomenon of GI distress associated with behavioural repercussions is well documented[35,36]. Interestingly, in the TD group, I-FABP correlated with more severe social adaptive behaviours, even though they did not have an increase in circulating markers of intestinal epithelial damage. We identified correlations between LPS and worse adaptive behaviours in the ADHD group. We did not identify an association between markers of intestinal permeability and ABAS scores in the ASD group. We did, however, identify that increased LPS in the ASD group correlated with more severe VABS adaptive composite and socialization scores, and increased I-FABP correlated with less severe ADI-R communication scores. As LPS was significantly increased in the ASD cohort, we would have expected more associations with behavior. Further investigation into the relationship between bacterial products and behavior needs to be done for a deeper understanding of this connection.

Chapter Six

Discussion

Individuals with ASD more frequently report gastrointestinal distress[24–26]. It was hypothesized that this distress was associated with increased intestinal permeability. Two separate studies found increased intestinal permeability in individuals with ASD using the lactulose/mannitol intestinal permeability test[31,32]. This barrier dysfunction was hypothesized, but not proven, to be a source of circulating bacteria and bacterial products and a trigger of increased inflammation in ASD.

We accessed a cohort of children and young adults (1-21 years, M/F=60/20) with neurodevelopmental disorders (ASD and ADHD) as well as TD controls (which will from here on be referred to as the POND Immune cohort), and found an increase in markers of intestinal permeability that is suggestive of intestinal epithelial damage in individuals with ASD. We found that circulating LPS, a component of the cell wall of Gram negative bacteria was increased in children and young adults with ASD compared to ADHD and TD controls (Figure 5.1) Furthermore, increased LPS was found to positively correlate with circulating MDP, another component of the bacterial cell wall (Figure 5.2B). Since the bacterial load of the gut is higher than any other location in the body, and since we are not experimentally able to determine the source of increased LPS, we hypothesize that these bacterial products originate in the gut. It should be noted that transient, trace amount of LPS can be detected in the circulation in cases with no evidence of barrier dysfunction, such as after a high fat meal or exercise[106,108].

In a different cohort of children with ASD aged 2-5 years old (M/F=25/7) (which will from here on be referred to as the Omega-3 cohort), we identified high levels of circulating intestinal fatty acid binding protein (I-FABP) (Figure 3.1), which positively correlated with

circulating LPS in females (Figure 3.3). This implies that intestinal epithelial damage may be leading to increased bacterial translocation from the gut. We were not able to conclude if these markers were increased in individuals with ASD, because there were no typically developing controls. This is the first study to use bacterial products as a marker of intestinal permeability in ASD.

Evidence of immune abnormalities and increased inflammation in ASD is accumulating, and it is hypothesized that immune abnormalities are present in ADHD. It has been speculated, but have not yet experimentally shown, that increased levels of pro-inflammatory cytokines triggered by an allergic or autoimmune contribute to the development of ADHD[43]. Furthermore, there are higher rates of eczema and asthma, both immune related conditions, in individuals with ADHD[44]. Individuals with ASD have higher levels of circulating pro-inflammatory cytokines, as well as a decrease in regulatory cytokines such as TGF-β compared to typically developing controls[13–15,75,76]. There is also evidence of alterations in circulating immune cell populations and studies show that leukocytes from individuals with ASD respond differently *in vitro* to stimulation than TD controls[3,18,19]. However, due to the behavioural and etiological heterogeneity associated with neurodevelopmental disorders, most notability in ASD, there is no scientific consensus on the existence or extent of immune abnormalities. Since these studies are often performed using small numbers of subjects and intensive behavioural phenotyping is often not performed, consistent results between studies are uncommon.

With this work, we have identified increases in soluble inflammation, as well as alterations in monocyte phenotype and frequency in children and young adults with ASD. In the POND Immune cohort, CRP was significantly increased in the circulation of the ASD group compared to the ADHD and TD groups (Figure 4.3), indicating an increase in soluble

inflammation. These data are consistent with previous studies in which CRP is found to be higher in ASD[100]. This constitutes a clinically relevant marker of soluble inflammation in this cohort. Also in the ASD group, we identified a positive correlation between increased CRP and increased LPS in the circulation (Figure 5.3). This was consistent with our hypothesis that increased bacterial translocation from the gut could drive systemic inflammation in neurodevelopmental disorders.

There was a lower frequency of monocytes, specifically classical monocytes, in the ASD group compared to the ADHD and TD groups in the POND Immune cohort (Figures 4.4 and 4.5). Additionally, these monocytes had altered surface receptor expression, with decreased expression of CCR2 (Figure 4.8), decreased expression of CD64 (Figure 4.7) and increased expression of CD16 compared to the other groups (Figure 4.9). Interestingly, we did not identify correlations of CRP with any alterations in monocyte frequency or in any of the observed phenotypic differences in the ASD cohort. This was unexpected, as increases in CRP are usually reflected in the innate immune response, specifically in monocytes and neutrophils[109]. We hypothesized that the reduction in monocytes and classical monocytes in the circulation is because they are being recruited to the tissue in a CCL2 dependent manner. Consistent with a role for LPS in altering monocyte phenotype and activation, in the ASD group, LPS was found to correlate with increased CCR2 and decreased CX_3CR_1 and CD16 expression on intermediate monocytes (Figure 5.5). This expression profile is more similar to a classical monocyte phenotype, and may reflect an altered developmental process in which intermediate monocytes are filling a void left by classical monocytes migrating to tissues in response to increased LPS.

CRP positively correlated with expression of CD13 on monocytes, as well as on individual monocyte subsets (Figure 4.12). There is no documented evidence that CRP correlates

with CD13 expression. In fact, one study found that CRP and CD13 expression did not correlate in cases of trauma when CRP is significantly increased[73]. Additionally, CD13 expression on monocytes has been shown to increase in response to LPS[73,110], but no such correlation was observed in our ASD cohort, even though LPS was significantly increased in the circulation (Figure 5.5). This indicates to us that circulating monocytes may be responding abnormally to soluble markers of inflammation and intestinal permeability.

One trend seen consistently through this study was the lack of overlap in the correlations observed between the three diagnosis groups in the POND Immune study. We hypothesized that the correlations observed in the TD group represent an adaptive cellular response to intestinal permeability or soluble inflammation. In most cases, there were no similarities in the correlations identified in the TD group with those identified in the neurodevelopmental disorders. This indicated a potential maladaptive cellular response to soluble inflammation in ASD and ADHD. Furthermore, there was often no overlap in the correlations identified in the ADHD and ASD groups. This implied that the underlying causes of these maladaptive responses were not the same between disorders.

The findings in the POND Immune cohort were different than those observed in the Omega-3 cohort. The observation of an increase in circulating CRP could not be experimentally shown, as the Omega-3 cohort only had subjects diagnosed with ASD. Furthermore, we could only investigate markers of soluble inflammation, not cellular inflammation in this cohort. We identified that increases in the acute phase protein tPA correlated with increases in circulating LPS and I-FABP in females (Figure 3.3). This finding was not identified in the POND Immune cohort.

Increased systemic inflammation or immune abnormalities in neurodevelopmental disorders may contribute to worse behavioural outcomes. We know through studies of sickness behaviour that inflammation affects behaviour[48,50], and behaviour alterations in chronic inflammatory conditions such as IBD is well documented[35]. Furthermore, previous studies have found that circulating inflammatory mediators correlated with more severe behaviour in ASD cohorts[76,77]. In both the Omega-3 and POND Immune cohorts, we identified correlations of markers of intestinal permeability, soluble inflammation and cellular changes with behavioural outcomes in all disorders, as well as TD controls. In the Omega-3 cohort, tPA, LPS and I-FABP were found to correlate with changes in ASD-specific behaviours, measured using the ADI-R and ADOS These markers also correlated with more severe adaptive behaviours, measured using the VABS (Figure 3.4 and 3.5). In the POND Immune cohort, ferritin and LPS in ASD correlated with more severe in adaptive behaviours, measured using the ABAS and the VABS. TPA correlated with less severe problem behaviours, measured using the CBCL. No similarities in these behaviour correlates were identified between the two cohorts. Regardless, these findings indicated that markers of soluble inflammation and intestinal permeability in ASD may be contributing to behavioural outcomes.

One of the most interesting findings in the POND Immune cohort was that increases in immune cell frequency and surface receptor expression correlated with more severe adaptive behaviours (measured by the ABAS) and problem behaviours (measured by the CBCL) in neurodevelopmental disorders, while in the TD group, increases in cell frequency and surface receptor expression correlated with less severe adaptive and problem behaviours (Figures 4.14 and 4.15). Furthermore, there was very little overlap in the correlations identified between

diagnosis. This indicates that cellular changes that are associated with more severe behaviours are different than those that are associated with less severe behaviours.

Another interesting finding was that within the neurodevelopmental disorders, NK cell frequency correlated with less severe behavioural outcomes (Figures 4.14 and 4.15). Multiple studies have found that NK cell activity and cytotoxicity is lower in ASD[102–104], and some studies have found higher NK cell numbers in ASD[19]. In our study, we did not find NK cell frequency to be altered in any of the disorders. There are no previous reports of NK cells associating with behaviour in neurodevelopmental disorders. This may be a novel finding that requires further that requires further investigation into the influence of NK cells on behaviour in neurodevelopmental disorders.

One of the main limitations of these studies was the ratio of males to females in the neurodevelopmental disorders. There is a well-documented male bias in both ASD and ADHD[3,42]. In the Omega-3 cohort, we showed that many of the correlations observed were dependent on sex, however, low numbers of females in the ASD and ADHD groups in the POND immune cohort meant we could not investigate sex differences. For example, in the ADHD group, there were only two females, so we could not analyze sex differences within this diagnosis. Furthermore, some subjects were removed from the cellular analysis because the samples were not of sufficiently high quality. This resulted in only four females available for cellular analysis in the ASD group. In the POND Immune study, all experiments were performed with the experimenter blinded to sex and diagnosis. Consequently, we did not produce gender and diagnosis balanced cohorts. For these reasons, analysis of the POND Immune cohort was not separated by sex, unless otherwise indicated.

There were also limitations to the replication of Omega-3 cohort data in the POND Immune cohort. Most of the significant findings in the Omega-3 cohort were in females, so these findings could not be replicated in the POND Immune cohort. Additionally, there were significant demographic differences between the two cohorts. The Omega-3 cohort consisted of children aged 2-5 years. The POND Immune cohort had a mean age of 12 years, the youngest subject was aged 3 and the oldest subject was aged 21 years. It is possible that significant findings identified in the Omega-3 cohort were characteristics of very young children and identified due to the narrow age range. Finally, we could not replicate correlations with ADOS scores that were performed in the Omega-3 cohort. A different version of the ADOS was used to phenotype subjects in the Omega-3 cohort than the version that was used in the POND Immune cohort (ADOS vs ADOS-2). Unfortunately, these values are not comparable and similarities between cohorts could not be drawn.

With this work, we have presented the most robust immunophenotyping performed in ASD and ADHD cohorts. We have identified altered levels of soluble and cellular inflammation in ASD, and we have associated this inflammation with intestinal permeability, indicated by increased circulating LPS. We have identified potential maladaptive monocyte responses to soluble inflammation in both ASD and ADHD. Finally, we have found that changes in monocyte phenotype are associated with more severe behaviours in ASD and ADHD. These findings contribute to the body of knowledge about immune abnormalities in neurodevelopmental disorders, and can inform future studies investigating the relationship between inflammation and behaviour in these disorders.

Chapter Seven

Conclusion

With this work, we have identified alterations in markers of intestinal permeability and soluble and cellular inflammation in children and young adults with autism spectrum disorder. These subjects had increased circulating C-reactive protein which positively correlated with increased circulating lipopolysaccharide. We also identified alterations in monocyte frequency and phenotype in children with ASD, such as a decrease in circulating monocytes and a decrease in monocyte expression of CCR2. These findings indicated increased monocyte recruitment to the tissues. We also identified potential abnormal monocyte responses to increased soluble inflammation in children and young adults with ASD. Finally, we identified that changes in circulating immune cell frequency and phenotype correlated with more severe behavioural outcomes in neurodevelopmental disorders, and less severe behavioural outcomes in typically developing controls.

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Appendix

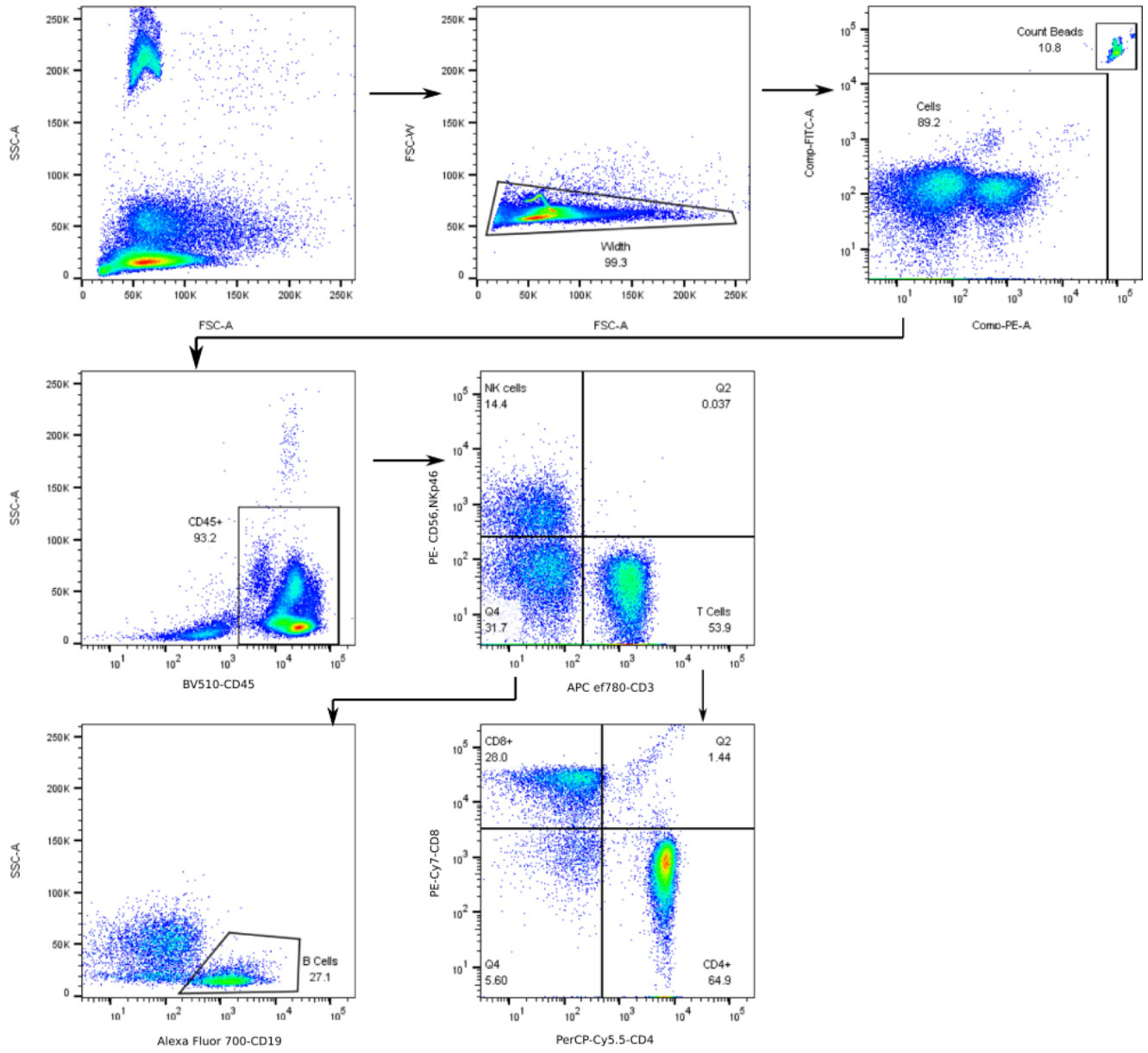

Figure 1) *Lymphocyte gating strategy:* Cell aggregates were excluded based on width and cells were separated from count beads. Count beads are used in future analysis to calculate absolute counts. CD45 was used to isolate all leukocytes. A quadrant gate of CD3 and NKp46.CD56 was used to identify T cells and NK cells. The T cell population was further separated into CD4+ and CD8+ T cells. The cells negative for T cells and NK cell markers were expanded, and CD19 was used to isolate B cells.

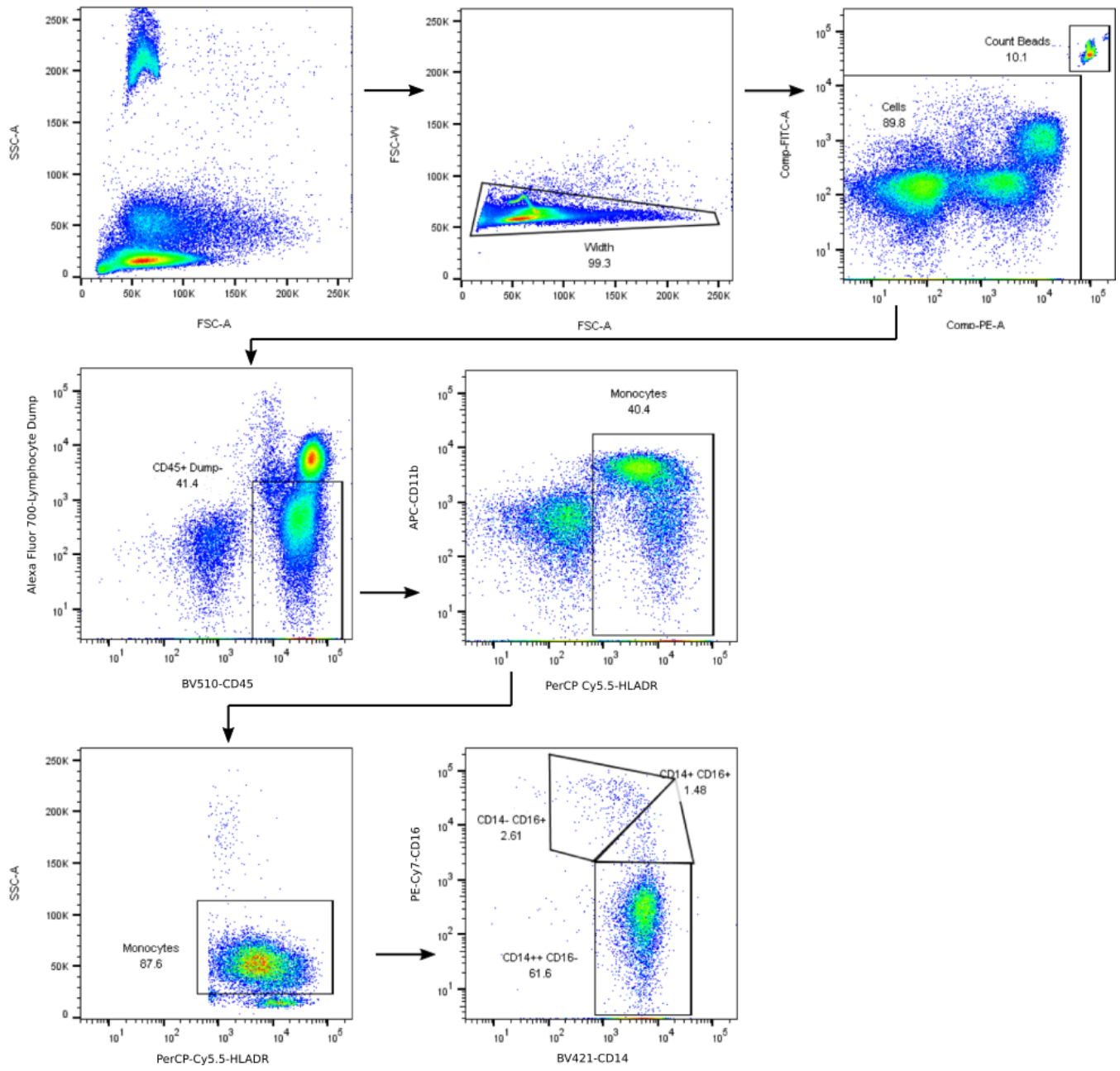

Figure 2) *Monocyte gating strategy.* Cell aggregates were excluded based on width and cells were separated from count beads. Count beads are used in future analysis to calculate absolute counts. CD45 was used to isolate all leukocytes. Lymphocyte populations (CD3+, CD56+ and CD19+) were excluded. HLADR and CD11b were used to identify monocytes. Expression of CD14 and CD16 were used to identify monocyte subsets.

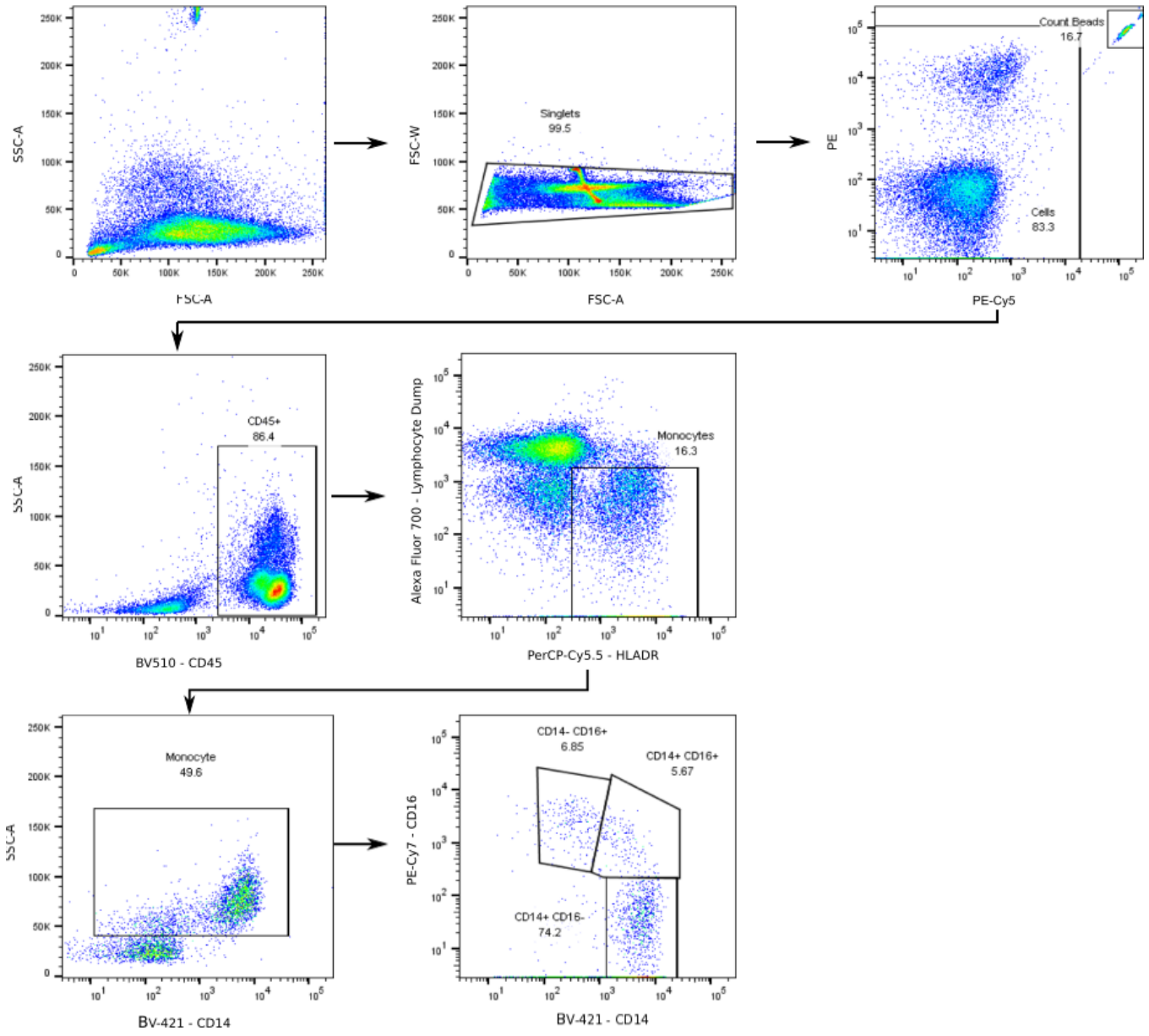

Figure 3) *Monocyte maturity gating strategy.* Cell aggregates were excluded based on width and cells were separated from count beads. Count beads are used in future analysis to calculate absolute counts. CD45 was used to isolate all leukocytes. Lymphocyte populations (CD3+, CD56+ and CD19+) were excluded. HLADR was used to identify monocytes. Expression of CD14 and CD16 were used to identify monocyte subsets.

MONOCYTE STAIN

Marker	Fluorophore	Dilution
CD45	BV510	1/100
CD16	PE-Cy7	1/100
CD14	BV421	1/100
CCR2	PE	1/50
CD11b	APC	1/50
HLA-DR	PerCPCy5.5	1/100
CX3CR1	FITC	1/50
CD19	AF700	1/50
CD3	AF700	1/50
CD56	AF700	1/50

MONOCYTE MATURITY

Marker	Fluorophore	Dilution
CD45	BV510	1/100
CD14	BV421	1/100
CD16	PE-Cy7	1/100
CD115	APC	1/50
HLA-DR	PerCPCy5.5	1/100
CD19	AF700	1/50
CD3	AF700	1/50
CD56	AF700	1/50
CD64	BV605	1/50
CD13	PE	1/100

LYMPHOCYTE STAIN

Marker	Fluorophore	Dilution
CD3	APCef780	1/200
CD4 (OKT4)	PerCPCy5.5	1/100
CD4 (RPA-T4)	PerCPCy5.5	1/100
CD8	PECy7	1/50
CD45	BV510	1/100
CD56	PE	1/50
NKp46	PE	1/50
CD19	AF700	1/50

VIABILITY STAIN

Marker	Fluorophore	Dilution
CD45	BV510	1/100
Zombie		1/100

Figure 4) *Immunophenotyping stains*

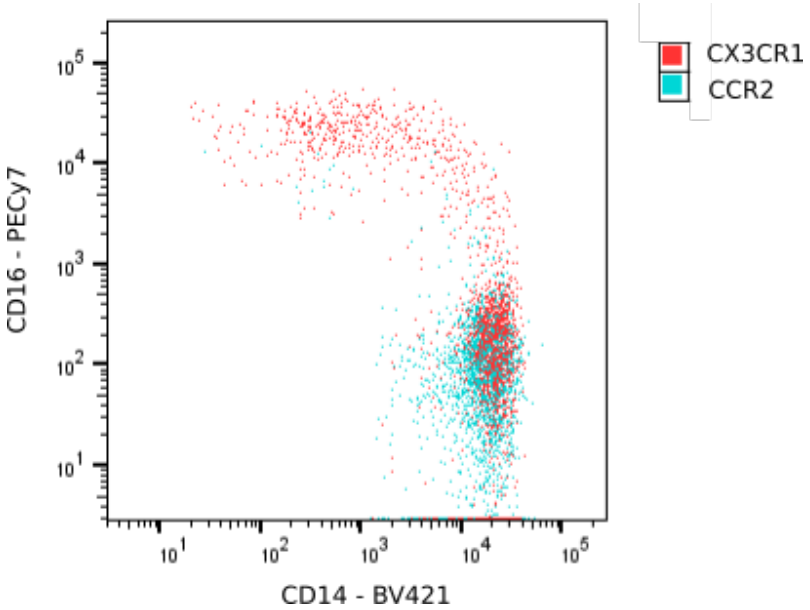

Figure 5) CX_3CR_1 and CCR2 expression on monocyte subsets.

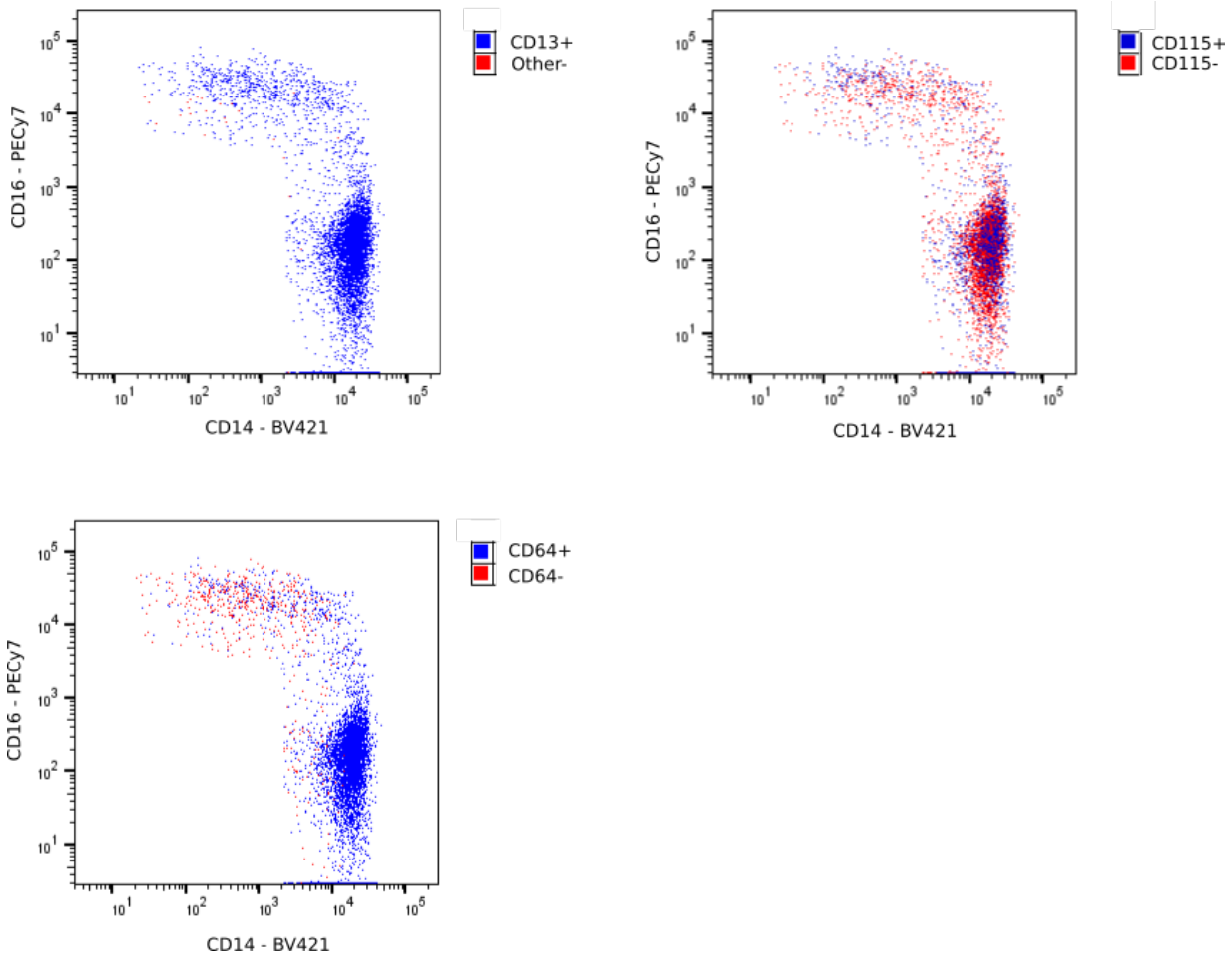

Figure 6) CD13, CD64 and CD115 expression on monocyte subsets

Dependent: ln(Ferritin) ng/mL

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	0.213	0.16	0.278
	ADHD	0.22	0.145	0.328
	Sex	0.177	0.117	0.36
ADHD	ASD	0.01	0.007	0.959
	Sex	0.167	0.11	0.383

Dependent: ln(tPA) ng/mL

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	0.062	0.043	0.771
	ADHD	0.045	0.027	0.855
	Sex	0.117	0.071	0.578
ADHD	ASD	0.001	0.001	0.995
	Sex	0.125	0.075	0.548

Dependent: ln(I-FABP)ng/mL

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-0.437	-0.236	0.098
	ADHD	-0.135	-0.064	0.658
	Sex	-0.43	-0.207	0.095
ADHD	ASD	-0.328	-0.178	0.2
	Sex	-0.416	-0.2	0.103

Dependent: MDP

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-0.009	-0.218	0.129
	ADHD	-0.012	-0.264	0.072
	Sex	-0.009	-0.207	0.096
ADHD	ASD	0.002	0.041	0.765
	Sex	-0.008	-0.188	0.129

Dependent: CD14++CD16+% Monocytes

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	0.134	0.045	0.785
	ADHD	0.467	0.147	0.4
	Sex	0.827	0.26	0.089
ADHD	ASD	-0.307	-0.104	0.517
	Sex	0.789	0.248	0.094

Dependent: CD14+CD16+% Monocytes

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	1.512	0.298	0.075
	ADHD	0.588	0.107	0.535
	Sex	-0.087	-0.016	0.916
ADHD	ASD	0.98	0.193	0.23
	Sex	-0.15	-0.027	0.851

Dependent: NK % CD45

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	1.579	0.135	0.417
	ADHD	-0.639	-0.05	0.766
	Sex	-0.672	-0.053	0.715
ADHD	ASD	2.159	0.185	0.242
	Sex	-0.609	-0.048	0.735

Dependent: T % CD45

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-1.022	-0.065	0.699
	ADHD	-1.178	-0.069	0.687
	Sex	0.53	0.031	0.833
ADHD	ASD	0.393	0.025	0.875
	Sex	0.439	0.026	0.857

Dependent: CD4 % T Cells

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-2.198	-0.102	0.539
	ADHD	0.939	0.04	0.812
	Sex	3.579	0.153	0.294
ADHD	ASD	-3.361	-0.156	0.321
	Sex	3.671	0.157	0.269

Dependent: CD8 % T Cells

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	0.784	0.063	0.705
	ADHD	0.243	0.018	0.916
	Sex	-1.539	-0.114	0.435
ADHD	ASD	1.284	0.104	0.511
	Sex	-1.992	-0.148	0.3

Figure 7.1) Multiple linear regression outputs that did not reach significance

Dependent: CD4+CD8+ % T Cells

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	0.47	0.267	0.102
	ADHD	0.607	0.318	0.057
	Sex	0.475	0.249	0.082
ADHD	ASD	-0.119	-0.067	0.658
	Sex	0.438	0.23	0.099

Dependent: CD14++CD16+ MFI CD64

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	498.418	0.267	0.102
	ADHD	94.289	0.046	0.783
	Sex	523.382	0.257	0.081
ADHD	ASD	329.684	0.177	0.256
	Sex	568.311	0.279	0.05

Dependent: Monocyte MFI CD115

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	16.61	0.042	0.803
	ADHD	13.653	0.032	0.857
	Sex	-48.43	-0.113	0.459
ADHD	ASD	-1.248	-0.003	0.984
	Sex	-46.262	-0.108	0.465

Dependent: CD14++CD16- MFI CD115

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	42.879	0.08	0.634
	ADHD	1.6	0.003	0.988
	Sex	-71.826	-0.123	0.418
ADHD	ASD	35.811	0.067	0.679
	Sex	-68.29	-0.117	0.426

Dependent: Monocyte MFI CD13

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-1492.426	-0.238	0.146
	ADHD	-620.84	-0.091	0.592
	Sex	1261.602	0.184	0.211
ADHD	ASD	-569.447	-0.091	0.558
	Sex	1089.821	0.159	0.259

Dependent: CD14++CD16- MFI CD13

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-541.938	-0.052	0.754
	ADHD	-1384.588	-0.122	0.483
	Sex	1644.958	0.145	0.336
ADHD	ASD	1329.664	0.128	0.421
	Sex	1385.089	0.122	0.397

Dependent: CD14++CD16+ MFI CD115

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-126.634	-0.162	0.312
	ADHD	-170.11	-0.2	0.234
	Sex	196.828	0.231	0.113
ADHD	ASD	51.565	0.066	0.666
	Sex	199.036	0.234	0.096

Dependent: CD14++CD16- MFI CD64

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	282.868	0.103	0.534
	ADHD	248.491	0.083	0.631
	Sex	825.834	0.275	0.069
ADHD	ASD	-134.717	-0.049	0.755
	Sex	926.363	0.308	**0.034***

Dependent: CD14++CD16+ MFI CD13

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-970.332	-0.065	0.698
	ADHD	-2318.523	-0.142	0.417
	Sex	1663.288	0.102	0.5
ADHD	ASD	2001.041	0.134	0.402
	Sex	1335.439	0.082	0.571

Dependent: CD14+CD16+ MFI CD115

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	141.55	0.261	0.113
	ADHD	18.744	0.032	0.852
	Sex	-79.879	-0.135	0.359
ADHD	ASD	110.325	0.203	0.197
	Sex	-72.477	-0.122	0.389

Figure 7.2) Multiple linear regression outputs that did not reach significance (continued from page 90).

Dependent: CD14+CD16+MFI CD13

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-325.202	-0.037	0.825
	ADHD	-147.454	-0.015	0.93
	Sex	1709.499	0.178	0.241
ADHD	ASD	235.832	0.027	0.868
	Sex	1443.21	0.15	0.304

Dependent: CD14++CD16-MFI CCR2

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	2042.706	0.304	0.066
	ADHD	430.003	0.059	0.73
	Sex	1163.994	0.159	0.282
ADHD	ASD	1531.632	0.228	0.15
	Sex	1198.405	0.164	0.252

Dependent: CD14+CD16+MFI CD64

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	17.527	0.046	0.78
	ADHD	99.925	0.241	0.166
	Sex	94.716	0.228	0.129
ADHD	ASD	-107.258	-0.282	0.071
	Sex	106.684	0.257	0.069

Dependent: CD14++CD16-MFI CD16

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	4.366	0.322	0.054
	ADHD	3.564	0.241	0.164
	Sex	2.008	0.136	0.362
ADHD	ASD	0.725	0.053	0.735
	Sex	1.902	0.128	0.37

Dependent: Monocytes MFI CX3CR1

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-20.203	-0.04	0.808
	ADHD	50.928	0.093	0.591
	Sex	123.995	0.226	0.134
ADHD	ASD	-58.667	-0.117	0.466
	Sex	113.515	0.207	0.157

Dependent: CD14+CD16+MFI CX3CR1

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-52.391	-0.043	0.794
	ADHD	278.792	0.211	0.225
	Sex	25.256	0.019	0.898
ADHD	ASD	-286.386	-0.237	0.145
	Sex	-16.66	-0.013	0.931

Dependent: CD14++CD16+MFI CX3CR1

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-244.633	-0.156	0.359
	ADHD	-135.267	-0.079	0.655
	Sex	-41.722	-0.024	0.873
ADHD	ASD	-80.64	-0.051	0.752
	Sex	-54.673	-0.032	0.828

Dependent: CD14+CD16+MFI CCR2

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	18.308	0.074	0.661
	ADHD	-16.528	-0.061	0.728
	Sex	-22.097	-0.082	0.59
ADHD	ASD	28.583	0.116	0.478
	Sex	-17.239	-0.064	0.665

Dependent: CD14++CD16+MFI CD16

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-707.835	-0.293	*0.08*
	ADHD	-516.289	-0.196	0.258
	Sex	-412.778	-0.157	0.295
ADHD	ASD	-189.114	-0.078	0.622
	Sex	-391.486	-0.149	0.304

Dependent: CD14+CD16+MFI CD16

Reference	Test	Unstandardized B	Standardized β	Sig
TD	ASD	-1645.07	-0.212	0.206
	ADHD	-41.311	-0.005	0.978
	Sex	487.812	0.058	0.701
ADHD	ASD	-1850.93	-0.238	0.14
	Sex	652.697	0.077	0.595

Figure 7.3) Multiple linear regression outputs that did not reach significance (continued from Page 91

		Monocyte	Int Monocyte	Classical Monocyte	Non-Classical Monocyte	NK Cells	B Cells	T Cells	CD4+ T Cells	CD8+ T Cells	CD4+ CD8+ T Cells
ASD	Ferritin										
	tPA										
	CRP									r= 0.548 p=0.008 n=22	
ADHD	Ferritin										
	tPA										
	CRP		r=0.593 p=0.015 n=16		r=0.648 p=0.007 n=16						
TD	Ferritin										
	tPA										
	CRP	r=0.507 p=0.038 n=17		r=0.473 p=0.049 n=17							

Figure 8) Statistics for correlates of APPs and immune cell frequency. Corresponds to Figure 4.11

		Monocyte			Intermediate		Classical			Non-Classical		
		CD115	CD13	CD64	CD115	CD13	CD64	CD115	CD13	CD64	CD115	CD13	CD64
ASD	Ferritin												
	tPA												
	CRP	r=-0.435 p=0.049 n=21	r=0.578 p=0.006 n=21			r=0.531 p=0.013 n=21			r=0.544 p=0.011 n=21			r=0.588 p=0.005 n=21	
ADHD	Ferritin												
	tPA	r=-0.624 p=0.010 n=16						r=-0.618 p=0.011 n=16					
	CRP									r=0.518 p=0.040 n=16			
TD	Ferritin			r=0.571 p=0.017 n=17			r=0.520 p=0.033 n=17						
	tPA												
	CRP												

Figure 9.1) Statistics for correlations of APPS with MFIs. Corresponds to Figure 4.12

		Monocyte			Intermediate		
		CX_3CR_1	CCR2	CD16	CX_3CR_1	CCR2	CD16
ASD	Ferritin						
	tPA						
	CRP						
ADHD	Ferritin						
	tPA	r=-0.512 p=0.043 n=16				r=0.506 p=0.046 n=16	
	CRP						
TD	Ferritin						
	tPA						
	CRP		r=-0.549 p=0.022 n=17	r=-0.694 p=0.002 n=17			

Figure 9.2) Statistics for correlations of APPS with MFIs (continued from page 94). Corresponds to Figure 4.12

		Monocyte	Int Monocyte	Classical Monocyte	Non-Classical Monocyte	NK Cells	B Cells	T Cells	CD4+ T Cells	CD8+ T Cells	CD4+ CD8+ T Cells
ASD	I-FABP										
	MDP						r= 0.487 p=0.021 n=22				
	LPS										
ADHD	I-FABP										
	MDP	r= 0.556 p=0.025 n=16		r=0.562 p=0.024 n=16		r=-0.706 p=0.002 n=17					
	LPS										
TD	I-FABP										
	MDP										
	LPS										

Figure 10) Statistics for correlates of intestinal permeability with immune cell frequency. Corresponds to Figure 5.4

		Monocyte			Intermediate			Classical			Non-Classical		
		CD115	CD13	CD64	CD115	CD13	CD64	CD115	CD13	CD64	CD115	CD13	CD64
ASD	I-FABP												
	MDP			r=-0.440 p=0.046 n=21			r=-0.709 P<0.001 n=21			r=-0.483 p=0.027 n=21			r=-0.519 p=0.016 n=21
	LPS				r=0.494 p=0.023 n=21								
ADHD	I-FABP					r=0.509 p=0.044 n=16						r=0.650 p=0.006 n=16	
	MDP												
	LPS												
TD	I-FABP				r=-0.578 p=0.015 n=17			r=-0.748 p=0.001 n=17					
	MDP	r=-0.750 p=0.001 n=17											
	LPS												

Figure 11.1) Statistics for correlations of intestinal permeability with MFIs. Corresponds to Figure 5.5

		Monocyte			Intermediate			Classical			Non-Classical		
		CX_3CR_1	CCR2	CD16	CX_3CR_1	CCR2	CD16	CX_3CR_1	CCR2	CD16	CX_3CR_1	CCR2	CD16
ASD	I-FABP												
	MDP			r=-0.531 p=0.013 n=21						r=-0.624 p=0.003 n=21			r=-0.469 p=0.032 n=21
	LPS				r=-0.460 p=0.036 n=21	r=0.562 p=0.008 n=21	r=-0.468 p=0.032 n=21	r=-0.472 p=0.031 n=21					
ADHD	I-FABP												
	MDP		r=0.526 p=0.036 n=16								r=0.606 p=0.013 n=16		r=0.500 p=0.049 n=16
	LPS												
TD	I-FABP												
	MDP	r=-0.593 p=0.012 n=17						r=-0.569 p=0.017 n=17					
	LPS							r=-0.520 p=0.032 n=17					

Figure 11.2) Statistics for correlations of intestinal permeability with MFIs (continued from page 97). Corresponds to Figure 5.5

	ASD		ADHD	
	CBCL Internalizing	CBCL Externalizing	CBCL Internalizing	CBCL Externalizing
Ferritin			r=0.531 p=0.034	r=0.632 p=0.009
tPA	r=-0.386 p=0.043	r=-0.418 p=0.028		
CRP				

Figure 12) Statistics for correlates of APPS and CBCL scores. Corresponds to Figure 4.13.

	ADHD				TD			
	ABAS GAC	ABAS Conceptual	ABAS Social	ABAS Practical	ABAS GAC	ABAS Conceptual	ABAS Social	ABAS Practical
I-FABP							r=-0.516 p=0.029	
MDP								
LPS	r=-0.523 p=0.026			r=-0.485 p=0.041				

Figure 13) Statistics for correlates of intestinal permeability and ABAs scores. Corresponds to Figure 5.6.

	ASD									
	R values				P Values					
	ABAS GAC	ABAS Conceptual	ABAS Social	ABAS Practical	ABAS GAC	ABAS Conceptual	ABAS Social	ABAS Practical		
NK Cell %CD45	0.565360302		0.559222397	0.67530221	0.014480166		0.015828919	0.002102678		
T Cells % CD45	-0.552912002	-0.557684225		-0.612122096	0.017317451	0.016182037		0.006932522		
CD4 %CD45	-0.619302936	-0.549406908	-0.580167056	-0.646301502	0.006129607	0.018190778	0.011600108	0.003755231		
Intermediate MFI CD13		-0.479049714		-0.488869085		0.044281821		0.039516127		
Intermediate MFI CD64	-0.508305592	-0.533886939			0.03124894	0.022483657				
Classical MFI CD13	-0.474072767	-0.508020323			0.046858282	0.031359694				
Classical MFI CD64		-0.480084379				0.043760008				
Non-Classical MFI CD115	-0.508305592	-0.500777671	-0.611584045		0.03124894	0.034274689	0.006995952			
Intermediate MFI CCR2				-0.546870502				0.018844137		
Non-classical MFI CD16		-0.5701002				0.013502455				
	ADHD									
	R values				P Values					
	ABAS GAC	ABAS Conceptual	ABAS Social	ABAS Practical	ABAS GAC	ABAS Conceptual	ABAS Social	ABAS Practical		
Non-Classical MFI CD64			-0.52451107				0.044891933			
Intermediate MFI CD16	-0.586763014	-0.666666933	-0.5491958	7	0.021484517	0.006641412	0.033969338			
	TD									
	R values				P Values					
	ABAS GAC	ABAS Conceptual	ABAS Social	ABAS Practical	ABAS GAC	ABAS Conceptual	ABAS Social	ABAS Practical		
IntMFICD13		0.510363964				0.04339152				
IntMFICD64				0.501477108				0.047813868		
MonoMFICCR2	0.762887425		0.576698674	0.831862026	0.000587562		0.019355949	6.45E-05		
IntMFICCR2	0.77081819	0.591421706	0.659780215	0.701108729	0.000473801	0.015821156	0.005419843	0.002478401		
ClassMFICCR2	0.656849019		0.62389652	0.648970375	0.005704343		0.009799646	0.006528495		
ClassMFICD16	0.565954465			0.525462542	0.022299903			0.036590022		

Figure 14) Statistics for correlates of cellular markers and ABAS scores. Corresponds to Figure 4.14.

	ASD					
	R Values			P Values		
	CBCL Internalizing	CBCL Externalizing		CBCL Internalizing	CBCL Externalizing	
NK Cells % CD45	-0.565939653	-0.300565505		0.009294011	0.197871242	
CD4 % CD45	0.445366624	0.375141908		0.049080595	0.103138522	
Intermediate MFI CD115	0.552375187	0.416573243		0.011552167	0.067684826	
Mono MFI CCR2	0.452148857	-0.009039564		0.045333725	0.969828257	
	ADHD					
	R Values			P Values		
	CBCL Internalizing	CBCL Externalizing		CBCL Internalizing	CBCL Externalizing	
NK.CD45	-0.604143103			0.028757251		
	TD					
	R Values			P Values		
	CBCL Internalizing	CBCL Externalizing		CBCL Internalizing	CBCL Externalizing	
CD8 % CD45	-0.852636828	-0.590139166		0.000425046	0.043386689	
Intermediate MFI CD115		-0.579537864			0.04827682	
Intermediate MFI CD16		-0.579537864			0.04827682	

Figure 15) Statistics for correlates of cellular markers and CBCL scores. Corresponds to Figure 4.15.