IMMUNOLOGICAL BIOMARKERS IN AUTISM: A REVIEW

By

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A thesis submitted to the Department of Pharmacy
in partial fulfilment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

Department of Pharmacy
Brac University
December 2020

Declaration

It is hereby declared that

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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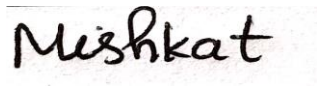

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Approval

The thesis titled “Immunological Biomarkers in Autism: A Review” submitted by Mishkat Ahmed Chowdhury (16346048) of Summer, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on 14/12/2020.

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Ethics Statement

No living organism were harmed during this project.

Abstract

Autism spectrum disorder (ASD) is a specific heterogenous neurodevelopmental disorder focused on the occurrence and intensity of core anomalies in social contact and repetitive behaviour, with complex pathogenesis and diagnosis. However, investigations have linked immune dysregulation as a hallmark of ASD. The prime justification for this claim lies in the numerous studies in the last four decades which found a frequent impaired immune response in individual with ASD. The aim of this review is to identify some immunological biomarker to investigate immune dysfunction in the diagnosis and treatment of ASD. Many studies have documented several immune cell abnormalities in ASD subjects, namely T-cells, B-cells, natural killer cells, and monocytes. Additionally, impairment in immunoglobulin regulation, chemokine activities and elevated autoantibodies production have been reported in many analyses. On top of that elevated peripheral cytokines, and chemokines and related neuroinflammation have been observed in a substantial chunk of individual diagnosed with ASD.

Key Words: Autism, immune dysregulation, cytokine, immunoglobulin, chemokine.

Dedication:

Dedicated to my parents

Acknowledgements

Firstly, I sincerely want to thank Almighty Allah for making me enable to come to this field and study Pharmacy. Without his blessings, I would not be able to continue this project paper and submit it for passing my bachelor's degree in pharmacy. Foremost, I would like to express my deepest and sincere gratitude to my project supervisor Dr. Sharmind Neelotpol, Associate Professor, Department of Pharmacy, Brac University. Without her helpful guidance, sincere encouragement, motivational directions, and mentoring capacity I would not be able to carry out my project work. Lastly and most importantly, I sincerely put forward my regards and gratitude to Dr. Eva Rahman Kabir, Professor and Chairperson, Department of Pharmacy, BRAC University for her contribution and support to the students and department. Moreover, I give my gratitude to the Department of Pharmacy which has enriched me with all the knowledge and technical support. It has also given me the opportunity to run the research in partial fulfilment of the requirement for the degree of Bachelor of Pharmacy. Last but not the least, I am also thankful to my family as well as my fellow mates and friends for their cooperation and mental support which I needed to complete my project works.

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Lists of Acronyms

ASD: Autism Spectrum Disorder

ID: Intellectual Disabilities

CNV: Copy Number Variants

DSM: Diagnostic and Statistical Manual of Mental Disorders

CARS: Childhood Autism Rating Scale

ID: Intellectual Disabilities

MIA: Maternal Immune Activation

PDD-NOS: Pervasive Developmental Disorder – Not Otherwise Specified

MIF: Migration Inhibitory Factor

NK: Natural killer

PTEN: Phosphatase and tensin homolog

GABA: Gamma aminobutyric acid

GI: Gastrointestinal

WHO: World Health Organization

CSF: Cerebrospinal Fluid

CNS: Central Nervous System

Ig: Immunoglobulin

ABC: Autism Behaviour Checklist

GFAP: Glial fibrillary acidic protein

TD: Typically Developing

DD: Developmental Disability.

IQR: Interquartile range

TNF: Tumour Necrosis Factor

TGF: Transforming Growth Factor

PBMC: Peripheral blood mononuclear cell

IL: Interleukin

TLR: Toll like receptor

BDNF: Brain-derived neurotrophic factor

MIP: macrophage inflammatory protein

MCP-1: Monocyte chemoattractant protein-1

VABS: Vineland Adaptive Behaviour Scales

PHA: polyhydroxyalkanoates

Chapter 1

Introduction

Autism can be identified by a very wide range of conditions, basically a neurodevelopmental disorder that manifest during early childhood. Individual with impairment in social communication and interaction, repetitive behaviour patterns, cognitive comorbidities form the basis of autism (Masi et al., 2017). These symptoms start to appear in early childhood which results in the clinically remarkable developmental impairment. Autistic disorder, pervasive (not otherwise specified) developmental disorder (PDDNOS), As perger syndrome and childhood disintegrative disorder have all been integrated into one ASD diagnosis. Additionally, inflammation and neuro-immune impairments are very frequently occurred and common features of autism spectrum disorder (Siniscalco et al., 2018). In the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III) in 1980, the definition 'Autism' was first used. Being a developmental disorder, ASD varies in the severity from person to person and males are documented to be more affected than females accounting a ratio of 5:1 approximately (Gottfried et al., 2015). Children with autism struggles to communicate with other, unable to interpret anger or sorrow and more often they are unable to talk properly rather try to exhibit gesture (Ratajczak, 2011). Moreover, it accompanies the symptoms of social anxiety disorder, hyperactivity disorder and intellectual disability (Masi et al. 2017). Autism spectrum disorder (ASD) is not a single entity rather characterised by a broad range of abnormalities. As per the 5th edition of the Psychiatric Disorders Diagnostic and Statistical Manual (DSM), ASD is identified in subjects exhibiting three social communication or interaction deficits and at least two symptoms manifestation of restricted or repetitive behaviours (Hsiao, 2013).

The pathogenesis of autism is controversial and complex. There is no common or single cause known for autism spectrum disorder. However, it might occur due to any abnormalities in brain structure or function and alliance of impaired immune response. Etiology refers to the genetical predisposition along with environmental influence affecting the developing brain. Nearly 1000 genes are believed to be responsible for autism (Masi et al., 2017). These genes can play a role in the function of neurotransmitter and neuronal excitability (Hodges et al., 2018). Moreover, evidence shows that autism can be a result of cerebral dysconnectivity of axons in the central nervous system (Stienman and mankuta, 2019). Genetic alterations in the adaptive and innate immune regulation have an involvement with autism. To be specific, the MET proto-oncogene tyrosine kinase C gene PRKCB1 serine, have been shown to lead to autism spectrum disorder, a mediator of activation of B cells and neuronal activity. Other susceptibility genes have been shown to be aligned with ASD, including those correlate d with NK cells, MIF, and Reelin (Gesundheit et al., 2013). Any dysfunction in neurotransmitter system might leads to autism spectrum disorder. Neurotransmitters are the endogenous substances that enable neurotransmission by connecting one neuron to another. They play a critical role in cognitive development, motor activity and behaviour regulations. In the pathogenesis of ASD, GABAergic, glutamatergic, and serotonergic systems are most frequently associated. Additionally, any dysfunction in glutamate system might cause ASD as it is accountable for several neurological functions such as cognition, memory, behaviour, sensation, and movement (Otaish et al., 2018), although there is a conflict whether inhibition or hyperactivity of glutamatergic system is involved in ASD (Zieminska et al., 2018).

When a condition develops such as ASD due to environmental factors or genetical predisposition, there are some characteristic changes observed in many naturally occurring molecules (Jepson, 2007a). There lies the concept of biomarker which is specific to any condition/ disease (Uddin et al., 2017). According to WHO biomarker is defined as 'any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease.
It reveals or confirms the existence of a disease or condition of concern or identifies a person with a disorder subtype and is also important for the rational development of drugs and medic al devices (Califf, 2018). A biomarker nominee can be characterized when there is a biological variable involves with a disease condition and it can be measured at a significant level in the patient or biomaterials through some reliable and sensitive qualitative and quantitative measurement procedure (Klin,2018). However, autism cannot be diagnosed accurately with single biomarker array. These normal variants can include biomarker array which can be diagnosed when properly developed and understood. In addition, observations in biomarker profiles may provide evidence for anticipating treatment regimens (Ratajczak, 2011).

In the recent studies, scientists found a strong relationship between immunological system and autism. Impairment in immunologic system have been reported in ASD individual which increases the possibilities of finding peripheral biomarker for the subsets of ASD. Maternal immune activation in the first or second trimester of pregnancy accounts one of the predominant nongenetic causes for ASD.

Autism in offspring has been found to be correlated with viral or bacterial infection and antibi otic treatment during the first trimester of pregnancy. Increased psoriasis, asthma and allergies during pregnancy have also been mentioned as a threat for the origination of ASD. Besides, studies show that exposure to thalidomide and valproic acid during pregnancy might be a great threat of autism in child. Moreover, maternal diabetes, thyroid disease, or psoriasis has an

increased risk to have a child with ASD (Hodges et al., 2019). Studies on animal models revealed mechanisms by which MIA can lead to autism, suggesting that maternal responses such as inflammatory cytokines, immunoglobulins, growth factors etc can swiftly move to the developing embryo that might result in autism and behavioural abnormalities (Hsiao, 2013).

Plasma, CSF, brain, and peripheral immune cells of autistic subjects have been analysed and production of pro-inflammatory signalling proteins reported to be altered. Moreover, there is an elevated level of autoantibodies, substantially to CNS protein present in children with ASD (Siniscalco et al., 2018). This study focuses on immunological biomarkers, particularly cytokine, chemokine, and immunoglobulin alterations in subjects with ASD. These biomarkers are crucial in inflammation and any relevant dysfunction might cause impairments in fatal brain development.

Biomarkers could be established as candidates for the therapeutic approaches and used in the ASD population to achieve the efficacy of such therapies. A subgroup of ASD individuals could be therefore from immune based drug intervention. Additionally, this study clarifies few roles of immune system in the manifestation of autism spectrum disorder which involves accumulation of recent research and expert's opinion. As immune dysfunction is one of the major hallmarks of ASD, this study focuses on the exact linking between them additionally with behavioural abnormalities.

1.1 Aim

Therefore, the aim of the study is to evaluate the changes in biomarkers involved in the immunologic systems of autism.

1.2 Objectives

The objectives of this study are to

- Find out any alteration occurs in the biomarker of immunologic system due to autism.
- Evaluate the mechanism of action of these alterations take place in autistic individual.

Chapter 2

Methodology

A review paper focuses on the existing state of understanding of a subject or concept. It typically summarizes published research or paper on a specific subject in academic publishing. In this study, established study on predefined topic were searched by key words, selected, and reviewed to obtain the information. In order to evaluate biomarker studies for ASD, search on common medical literature databases such as NCBI resources, PubMed, Mendeley, science direct, and ResearchGate have been conducted. Abstract were screened, and only relevant article were analysed.

Assessing the potential biomarkers associated with autism, papers documenting immunological alterations involved in ASD were reviewed. If they met specific requirements such as: comparison between autistic and control group in terms of immunological and neurological biomarker, neuro-immune dysregulation due to ASD, studies were included. However, more recent studies were prioritized to be included. Additionally, this review compared the production of pro-inflammatory and anti-inflammatory cytokines between autistic group and control group and their association with typical autistic behaviour. The key words that we have used to search the relevant articles are: Immunological biomarker, cytokine, chemokine, autism, immunoglobulin, interleukin, inflammation, immunological alteration.

Chapter 3

Discussion

3.1 Immunoglobulin

Many studies have demonstrated alterations in immunoglobulins level in subjects diagnosed with autism spectrum disorder. However, these studies are associated with a little controversy having no clear conclusion. More recent studies have found the association of immunoglobulin changes with severity of autism spectrum disorder and its characteristic behaviour (Gottfried et al., 2015; Heuer et al. 2008; Hughes et al., 2018).

IgG and IgM have been reported to be either elevated (Croonenberghs et al. 2002) or having a downfall in plasma concentration (Gupta et al. 1996; Heuer et al. 2008) in patient with ASD compared to healthy controls. On the other hand, Heuer and colleagues (2008) have found that autistic children have a lower level of immunoglobulin G and M (IgG and IgM).

Previous studies have shown that in comparison to controls, it is more likely that children with ASD will have a higher synthesis of immunoglobulin E generating CD4+ T cells. Additionally, it has been specified that about 5% individual diagnosed with ASD have IgA inadequacy and 30-40% patients have a decreased serum IgA level (Gupta et al. 1996, Wasilewska et al. 2012). With an alteration in specific Ig sub-type, a downfall in the levels of total Ig have been demonstrated in individual with ASD while there were no differences mentioned in the functionality of immunoglobulin producing B cells (Heuer et al., 2012).

Warren et al., (1997) conducted a study in order to identify any immunoglobulin abnormalities involved with autism. The authors analysed serum of 40 individuals with an average age of 11.6 years with ASD and compared the result with healthy controls. They found a significant

decrease ($p = 0.006$) in the serum immunoglobulin A (IgA) in subjects with autism spectrum disorder. Additionally, eight of 40 analysed participants had a below normal serum IgA level whereas no anomalies were reported. Mean serum immunoglobulin A (IgA) level of individual with ASD were remarkably reduced compared to healthy controls group (105 mg/100 ml vs. 143 mg/100 ml).

Along with that, Enstrom and colleagues (2008) performed a study with over 100 people with autism spectrum disorder and analysed the amount of immunoglobulins in plasma concentration. A decreased level of immunoglobulin G (IgG) and immunoglobulin M (IgM) has been identified, which is inversely associated with Aberrant Behaviour Checklist (ABC) ratings. The Authors also observed a substantial rise in subject with ASD in IgG4 concentration, along with an uplifting of IgG2 subtype. IgG4 is a blocking antibody that is developed under chronic antigen exposure (Hughes et al., 2018).

In spite of the fact that individuals with ASD present a diminished concentration of IgM and IgG immunoglobulins, they exhibit an elevated level of antibodies against diverse proteins expressed in the nervous tissue, e.g., myelin basic protein, serotonin receptors, glial fibrillary acidic protein (GFAP) and heat shock protein (Gottfried et al., 2015). According to Croonenberghs et al., (2002) there is an increased level of total serum protein observed in patients with autism along with an up regulation of gamma-globulins and albumin concentration.

However, further extensive investigation has revealed that the discovered abnormalities are not due to B-cell dysfunction. Rather, the authors hypothesized that the mentioned shortcomings might take place by either having a defect in during immune system development or any abnormalities in any other type of immune cell that is associated with immunoglobulin

production (Gładysz et al., 2018). In addition, the authors proposed that a cytokine-related effect on autoimmune B cells could be involved in the changes of IgG subclasses.

Decreased Levels of IgG and IgM in Children with Autism

A study of Heuer et al., (2008) focuses on the humoral immune responses, investigated over 250 children with autism and the result are shown in Figure 1.

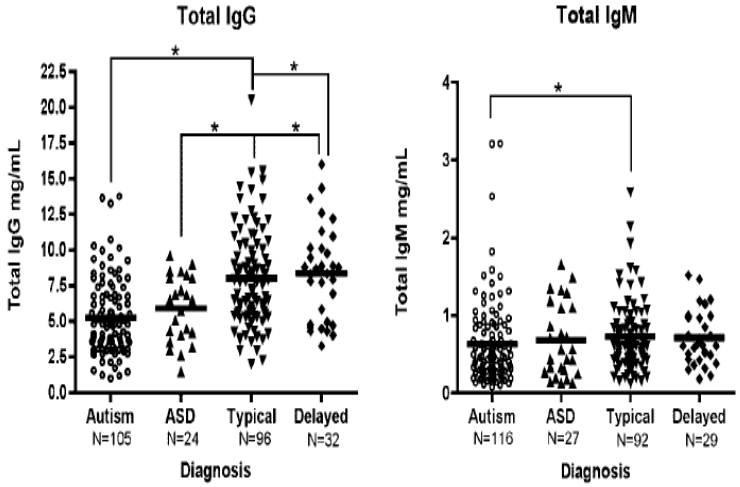

Figure 1 : Median plasma immunoglobulin levels as determined by ELISA. Plasma from children diagnosed with AU, ASD, DD, or TD controls was assayed for levels of total immunoglobulin by ELISA. Children with AU displayed a significantly reduced level of total IgG were also significantly reduced in children with AU compared to TD controls (B) (Heuer et al., 2008).

They found a diminished plasma IgM and IgG level in children with ASD, especially IgG which is found to correlate negatively with Aberrant Checklist Behaviour ratings (ABC). Moreover. Children with autism shows a remarkable difference in plasma concentration of

immunoglobulin G (IgG) compared to typically developing (TD) groups. Evidence provided that autistic children demonstrated a considerable lessen median level of total IgG (4.77 mg/ mL, interquartile range (IQR) 3.27–6.77) compared with both TD controls (7.76 mg/mL, IQR 5.52–10.20) and children with DD (8.54 mg/mL, IQR 4.85–10.14).

On top of that, autistic children present a significantly decreased plasma level of immunoglobulin M (IgM) (0.48 mg/ mL, IQR 0.29–0.85) than TD controls (0.65 mg/mL, IQR 0.45–0.89, P50.01). On the other hand, neither the ASD population (0.57 mg/mL, IQR 0.27–1.11) nor the DD subjects (0.62 mg/mL, IQR 0.43–0.97) display a substantial difference relative to either of the plasma IgM group (Heuer et al., 2008).

3.2 Cytokines

Cytokines are tiny proteins (8–25 kDa), essentially polypeptides include interleukins, interferons, growth factors, tumor necrosis factors (TNFs), and chemokines, which are used by the immune system to establish a cell liaise and control immune responses, additionally known as the shared language between the immune and nervous system. It is very well known that the nervous system and the immune system has an extensive intercommunication which therefore is not unanticipated that immune system dysfunction is frequently observed to be associated with neurological disorder (Goines and Ashwood, 2012. Xu et al., 2015). According to immunological perspective, CD4+ lymphocyte helper-cell functions can be categorised into cell mediated immunity or T-helper 1 (Th1) and humoral immunity or T-helper 2 (Th2). Th1 basically acts as a first line defence against any antigen such as virus, bacteria, protozoa, fungi etc and Th2 assists B-cells with producing antibody. Impairments in Th1 and Th2 activities has been observed in patients with autism spectrum disorder which ultimately results in the alterations in cytokine production (Hsiao and Malkova, 2016).

Cytokines jointly aid in the growth of the brain, cognitive function and collaborate to restore the central nervous system (CNS) following an injury or trauma, along with contributing to the

immune response (Rose and Ashwood, 2014). According to Gottfried et al., (2015) cytokines such as TNF-α, IL-1β, IL-10, IL-6, and TGF-β family can regulate neuronal functions and oligodendrocyte survival has known to be assisted by IL-6. Additionally, cytokines regulate systemic and central nervous system responses towards injury, inflammation, or infection. In their study, the authors mentioned a research with 100 autistic children ages ranging from 4-11 years where their cytokine profile were analysed and compared with 100 healthy controls. Meta-analysis of data investigated in subjects with ASD revealed impairment in numerous cytokines level both in brain and in plasma, as tabulated in Table 1.

Table 1: various Cytokines level in ASD children in compared with healthy controls.

Cytokines	Level compared to control group	Source	Evaluated subjects
		INTERLEUKINS	
IL-1β	↑	Plasma	Children with ASD
	↑	Plasma	Children with ASD
	↑	Plasma	Adults with severe ASD
	↑	Blood cells	Children with ASD
	↑ (TLR2 or TLR4 stimulation)	Blood cells	Children with ASD
	↓ (TLR-9 stimulation)	Blood cells	Children with ASD
IL-6	↑	Plasma	Children with ASD
	↑	Plasma	Adults with severe autism
	↑	Blood cells	Children with ASD
	↑ (TLR2 or TLR4 stimulation)	Blood cells	Children with ASD
	↓ (TLR-9 stimulation)	Blood cells	Children with ASD
	↑	Lymphoblasts	Children with ASD
	↑	Cerebellum (postmortem)	Children with ASD
	↑	Brain (postmortem)	ASD subjects (children and adults)
	↑	Brain (postmortem)	ASD subjects (children and adults)
IL-12 P40	↑	Plasma	Children with ASD
		CHEMOKINES	
CCL2	↑	Brain (postmortem)	ASD subjects (children and adults)
		Plasma	Children with ASD
		TUMOR NECROSIS FACTOR	
TNF-α	↑	CSF	Children with ASD
		Brain (postmortem)	Children with ASD
		INTERFERON	
IFN-γ		Serum (mid-gestational)	Mothers giving birth to child with ASD
	↑	Whole blood and serum	Children with ASD
		Brain (postmortem)	ASD subjects (children and adults)
		GROWTH FACTORS	
TGF-β1	↓	Plasma	Children with ASD (Lower levels correlated with more severe behavioral scores)
	↓	Serum	Adults with ASD
BDNF	↑	Brain (postmortem)	ASD subjects (children and adults)
	↑	Plasma	Children with ASD

IK, interleukin; IFN, interferon; TGF, transforming growth factor; TLR, toll-like receptors.

(Gottfried et al., 2015).

Individuals with autism have been shown to be in a chronic condition of cytokine induction and to display a deviation from the usual cytokine profile when put together with healthy control (Bjorklund et al., 2016). It has recently been established that an autistic individual develops cytokine functional impairment, and the inflammatory cytokine concentration differs in various body fluids and cells including serum, plasma, blood mononuclear cells, cerebrospinal fluid and brain tissue of autistic subjects in compare with healthy individuals (Xu et al., 2015). Furthermore, the altered cytokines level has been found to be involved with impaired aberrant behaviour and the severity of autism (Qasem et al., 2017).

Cytokines can be differentiated in many non-identical families based on their function or structure. However, it is frequently classified by their capability to regulate or induce inflammation and often named as pro-inflammatory and anti-inflammatory (Rose and Ashwood, 2014).

Therefore, it can be anticipated that any alterations in cytokine profile can trigger neurodevelopmental defects. Disruption of normal cytokine production has been shown to play a significant role as a risk factor for a number of neurodevelopmental conditions, including autism and schizophrenia (Meltzer and Water, 2016).

3.3 Pro-inflammatory cytokines

In the recent studies, evidence and facts progressively points out a strong inflammatory state involved with autism spectrum disorder. This inflammatory state is recurrently associated with immune system dysfunction. Jyonouchi and colleagues (2001) performed a research to observe the adaptive and innate immune activities in ASD children. They reported that autistic children's peripheral blood mononuclear cells (PBMCs) generate relatively high levels of pro-inflammatory cytokines compared to control group.

An up-regulated activity of inflammatory state has been documented in patients with ASD by means of pro-inflammatory biomarker study (Siniscalco et al., 2018). Involvement of pro-inflammatory cytokines during neurodevelopment have been analysed extensively in subjects with ASD and authors reported that interleukin 1 (IL-1), IL-6, IL-12, tumor necrosis factor α (TNF-α), and interferon-γ (IFN-γ), are associated with CNS pleiotropic effects (Gottfried et al., 2015). Additionally, studies have shown that pro-inflammatory cytokines are significantly generated by triggering cultured peripheral mononuclear blood cells (PBMCs) in vitro in

children with ASD (Enstrom et al., 2009). In compare to healthy control, autistic individual also shows an increase amount of pro-inflammatory cytokines in cerebrospinal fluid (Hsiao, 2013). Another study observed that elevated levels of Th1 cytokines in other words pro-inflammatory cytokines are thought to be associated with ASD due to the association of Th1 cytokines in the development of brain and involved in core characteristics of autism (Ashwood et al., 2001).

3.3.1 Interleukin-1 beta (IL-1β)

At the very initial phase of immune responses, IL-1β acts as an inflammatory cytokine. Along with its inflammatory action, additionally it triggers the production of another cytokine, IL-5 and in the end, an acute phase response in the liver. Moreover, systemic IL-1β has the potential to overcome blood brain barrier where it can stimulate its own expression in the hypothalamus resulting in the neuroendocrine alterations engaged with sickness behaviour and fever (Goines and Ashwood, 2012).

Many studies reported an elevated level of IL-1β in subjects with ASD (children and adult) and claimed that the alteration is associated with behavioural severity. According to Suzuki and colleagues (2011), individual with ASD, adult or children, shows a higher level of plasma IL-1β and distorted cellular IL-1β responses following stimulation, such as viral or bacterial infection, immunogens provoking humoral or cell-mediated immune responses.

Ricci and colleagues (2013) graphically illustrated the difference in the amount of IL-1β present in healthy control group (n=29) and autistic group (n=29) of children.

IL-1beta: t (df: 1, 28) = 12.75, $p<0.0001$: autism>control.

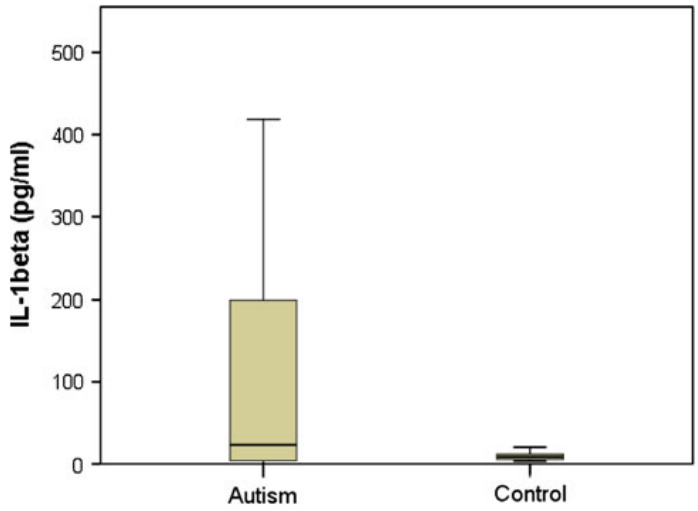

Figure 2: IL-1β concentrations (pg/ml) in control versus autism group. (values expressed as mean ± SD), autism>control) (Ricci et al., 2013).

Supporting this fact, immunological alterations in innate immunity has been observed in patient with ASD. Inflammatory cytokines including IL-1β, TNF-α, and IL-6 has been found to be released at an elevated level from the peripheral blood monocyte of autistic individual (Bjorklund et al., 2016). Moreover, in vitro, Jyonouchi and colleagues (2001) noticed that children with ASD develop significantly higher level of soluble IL-1β relative to healthy controls when their peripheral blood mononuclear cells (PBMCs) is being stimulated. Rose and Ashwood (2014) also demonstrated an elevated amount of IL-1β detected in the cerebral spinal fluid (CSF) and brain tissue. Another study of Ashwood et al., (2010) revealed that IL-1β is prominently up regulated in plasma samples from children with autism (median 110.6; interquartile range 25.7–245.8 pg/mL) making an analogy with TD (Typically developing) controls (62.8; 15–148.8 pg/mL; $p = 0.04$).

However, differences did not achieve statistical significance when compared with DD (Developmental delay) controls (46.1; 15–153.8 pg/mL; $p = 0.1$), after rectification for

miscellaneous comparisons and may be because of having comparatively a small quantity of DD subjects in their study. In Addition, they distinguished that the elevation of IL-1β is more prominent to the children having a more regressive form of ASD presenting a higher level of cytokine expression than the ASD children.

An early study examining mediated immune cells responses in children with ASD exhibit improved production of inherent cytokines, including IL-1β following stimulation of peripheral blood mononuclear cells (PBMC) with TLR-4 (Toll like receptor 4) ligand lipopolysaccharide (LPS), contrasted with typically developing children (Jyonouchi et al., 2001). Investigators evaluated innate responses to many environmentally important pathogen-associated molecular patterns (PAMPs) to enhance understanding of differential innate responses to various TLR stimuli in ASD subjects. After exposure to several innate immune ligands, the result of this study showed elevated cytokine production. Manipulation of isolated monocytes with TLR2 (Toll like receptor 2) Ligand lipoteichoic acid (LTA) have shown a substantial increase in the production of TNF-alpha, IL-1β and IL-6 in children with ASD against the standard development of controls, supporting earlier work. Increased IL-1β was also provided by TLR4 stimulation with LPS. In addition, increased IL-1β output following stimulation of LPS was found to be correlated with deteriorating behaviour (Hughes et al., 2018).

Inflammatory cytokines in the post-mortem brains with autism spectrum disorder (ASD) has contributed to the belief that chronic neuroinflammation plays a role in the pathogenesis of ASD. However, only a handful of pro-inflammatory usually interleukin-1β (IL-1β), IL-6, tumour necrosis factor (TNF) and interferon γ have been examined by various studies cited to support this theory without reporting any other hallmarks of neuroinflammation (Estes and

McAllister, 2015). Additionally, they assumed that it might be accountable for an impairment in immune response.

3.3.2 Interleukin (IL)-6

Originally, interleukin (IL)-6 was considered to be a powerful immune and inflammatory response inducer. Latest research indicates that IL-6 has a key role on the central nervous system (CNS). IL-6 can induce cellular responses in the CNS that mediate inflammation, neurogenesis, gliogenesis, cell formation, cell survival, myelination, and demyelination (Wagoner and Benveniste., 1999).

Autism is associated with an elevated cytokine response and IL-6 has been consistently shown to be increased in the autistic brain. Changes in pro-inflammatory cytokine levels such as IL-12, IL-1, IL-6, TNF-alpha, IL-23 and brain-derived neurotrophic factor (BDNF) have recently documented in ASD by Ricci et al., 2013. In addition, the discrepancy in the amount of IL-6 in plasma present in the healthy control group (n=29) and the autistic group of children (n=29) was graphically demonstrated.

IL-6: $t(1, 29) = 5.10$, $p<0.0001$: autism>control (see Fig. 3).

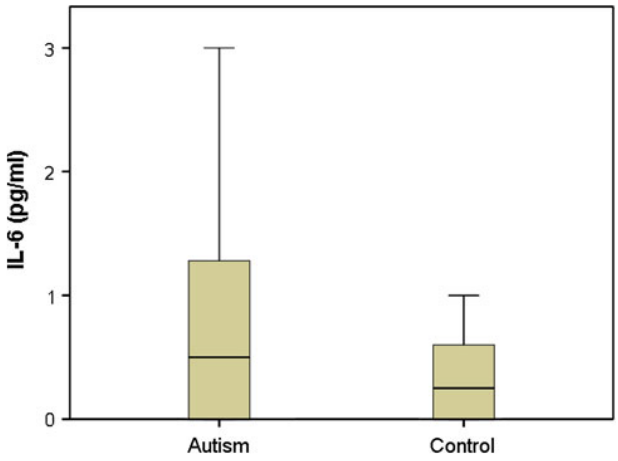

Figure 3: IL-6 concentrations (pg/ml) in control versus autism group. (values expressed as mean ± SD), autism>control (Ricci et al., 2013).

In addition, lipopolysaccharide-stimulated TNF-α and IL-6 development has been shown to be higher in peripheral blood mononuclear cells from ASD subjects than those from controls (Suzuki et al., 2011).

In a study of Wei et al (2011), Immunohistochemistry experiments were carried out to investigate the expression of IL-6 in the cerebellum of six autistic subjects and six age-matched controls using IL-6 antibodies. The results are shown in Figure 4.

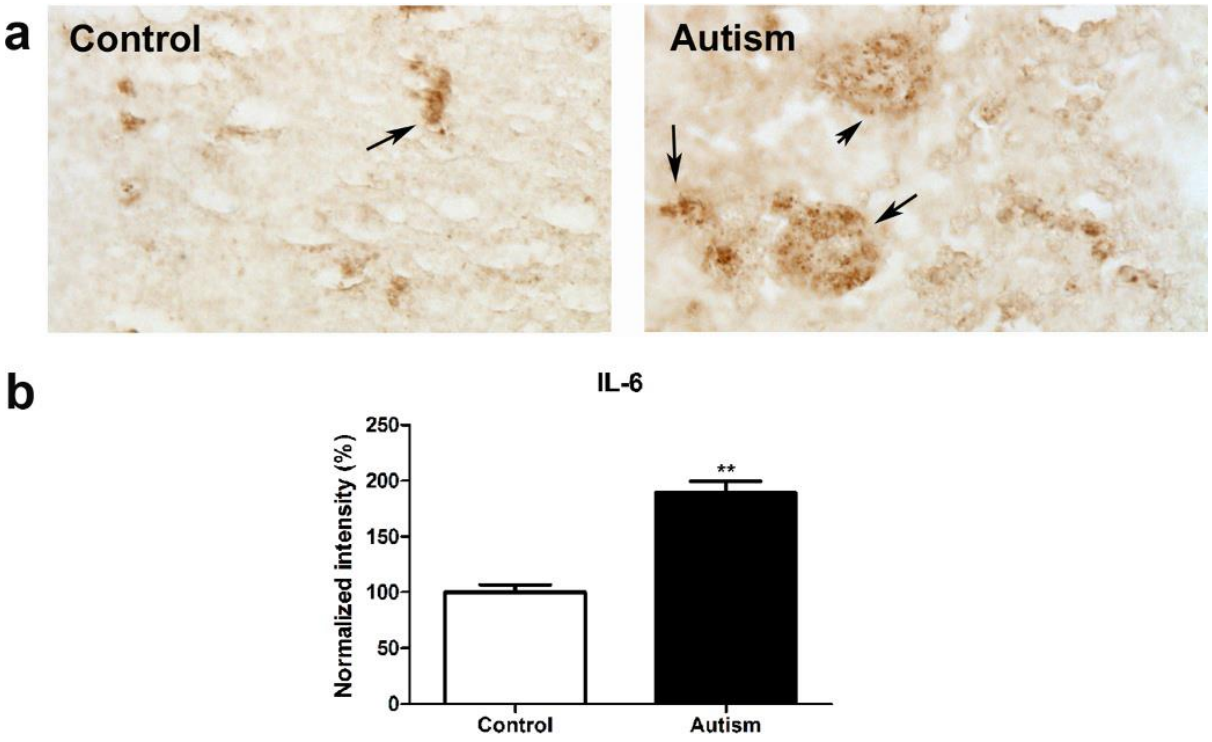

Figure 4: IL-6 expression increased in the cerebellum of autistic subjects. Immunohistochemistry studies were carried out on cerebellar homogenates from 6 autistic subjects and 6 age-matched controls using an IL-6 antibody (dilution 1:100). Stronger immunostaining.

They found that relative to age-matched controls, positive stain of IL-6 is clearly greater in the cerebellum of six autistic subjects. Quantitative analysis using Image the authors showed that in autistic subjects, IL-6 increased significantly by 89% ASD sample ($p < 0.01$).

Increase in the amount of plasma IL-6 level in young children have been demonstrated compared to controls with having no family history of autism spectrum disorder (Manzardo et al., 2012). In Addition, Kalkbrenner et al., (2010) reported an increased level of interlukin-6 (IL-6) in the brain of autistic individual compared to control subjects.

Excessive IL-6 and and IL-1β development was associated with increased social behavioural dysfunction diagnosed with ASD (Enstrom et al., 2009).

3.3.3 Tumor necrosis factor- alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-alpha) seems to be a type of cytokine that induces systemic inflammation and an acute-phase reaction. TNF-alpha is is synthesized mainly by activated macrophages (M1), despite the fact that some other type of cells, such as CD4+ lymphocytes and Natural Killer cells, can produce it (Xu et al., 2015).

Evidence showed that there is a rising number of circulating monocytes, a precursor of TNF-α production, have been observed to be present in the blood and post-mortem of brain tissue immune alterations including an elevated production of TNF- α in the cerebrospinal fluid of ASD children which is also responsible for stereotypic behaviours matched with those found in ASD individuals (Gottfried et al., 2015; Gent et al., 1997).

Additionally, analysis of mid pregnancy serum samples collected from mothers giving birth to autistic child revealed an increased amount of TNF-α (Goines and Ashwood, 2013). Another analysis on cerebrospinal fluid and brain tissue studies exposed an increased amount of tumour necrosis factor-alpha (TNF-α) in autistic individuals. Moreover, with regards to food hypersensitivity and dietary proteins, peripheral blood mononuclear cells (PBMCs) from children with ASD produce more elevated levels of TNF-α (Meltzer and Water, 2016).

Statistical analysis showed a meaningful difference in the amount of serum TNF- α levels between autistic and control group (figure-5) (Ghaffari et al., 2016).

Table 2: Mean values of serum TNF-α level in children autism and healthy children.

Adipokine serum levels	Autistic children $n = 30$ Boys/girls (22/8)	Health children $n = 30$ Boys/girls (22/8)	t-value	p value
TNF-α pg/mL				
All subjects	6.7 ± 1.43	5.38 ± 1.45	3.5	0.001
Boys	6.53 ± 1.43	5.4 ± 1.53	3.5	0.002
Girls	7.16 ± 1.44	5.23 ± 1.25	1.77	0.12

(Ghaffari et al., 2016).

They analysed the plasma of 30 autistic and 30 healthy children and made a comparison which shows a remarkable statistical difference in plasma TNF-α levels.

Jyonouchi and colleagues (2001) performed a study with 71 ASD children aged 2-14 years and put together with healthy control groups. They found an increased amount of TNF- α in the autistic subjects. Moreover, the authors reported that in most autistic subject (83.1%), activated peripheral blood mononuclear cells (PBMCs) produce more TNF- α compared with healthy controls and it exhibit an extravagant or poorly regulated innate immune response. Furthermore, TNF- α production was found remarkably elevated in autistic individuals in compared with controls when both of their PBMCs were being stimulated by polyhydroxyalkanoates (PHA) and tetanus and it is found to be associated with stereotypic behaviour (Ashwood et al., 2011). Moreover, a study with 32 autistic children found an increased level of TNF- α in sera and reported to be associated with ASD severity (Siniscalco et al., 2018). Guloksuz et al. (2017) also reported an increased plasma TNF- α level in ASD children compared to healthy control group. A graphical elastration (figure 5) might make it easy to understand the difference between these two groups.

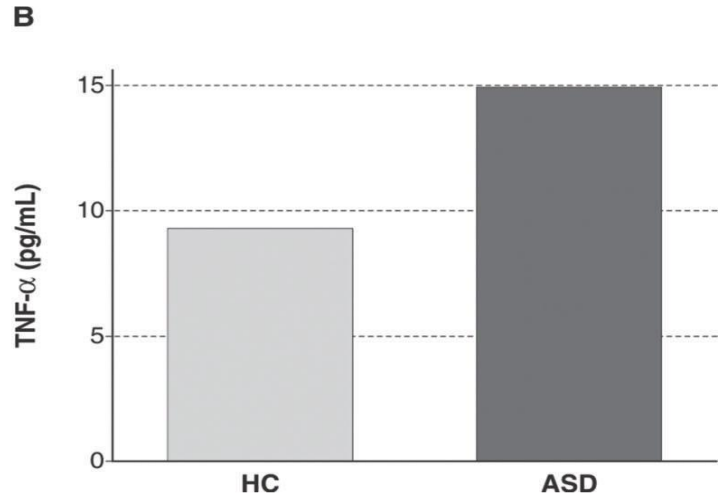

Figure 5: Plasma concentration level of TNF-α in typically developing healthy children and in children with ASD (Guloksuz et al. (2017).

Another study of ricci et al., (2013) showed that the blood serum level of autistic individual contains an elevated level of TNF- α compared to healthy control (Figure 6). In their study, they analysed the data with 29 ASD patient and contrasted with the control group considering the gender and age as well. They hypothesized that the alterations in terms of TNF- α production might be due to the hyperactivity of immune system or cytokine dysregulation caused by neuro-immune alterations.

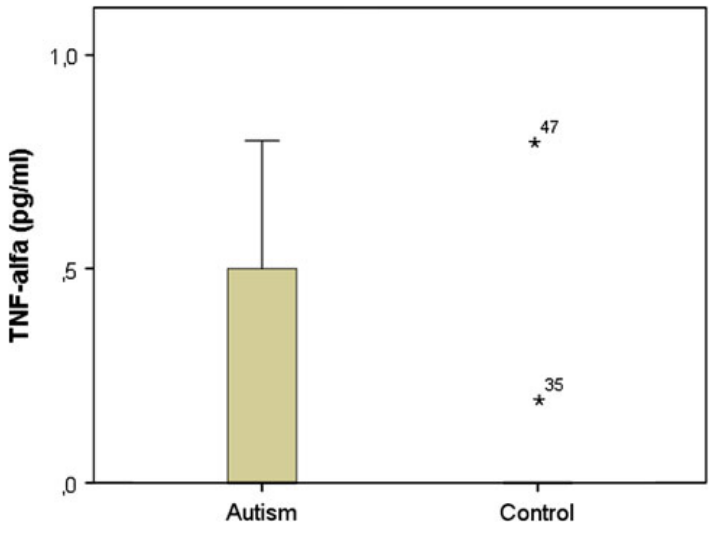

*Figure 6: TNF-a concentrations (pg/ml) in control and autistic group (mean values ± SD), autism>control. In the control group, individual values: *47 and *35 gave values deviating markedly from the mean value (Ricci et al., 2013)*

3.4 Anti- Inflammatory cytokines

As previously discussed, dysregulations in the cytokine system accounts a key mediator of inflammation in the pathophysiology of neuropsychiatric disorder. Numerous study approaches to find out any imbalance in the pro-inflammatory and anti-inflammatory cytokines regulation. Anti- inflammatory cytokines are those basically acts as immunosuppressive or immunoregulatory which diminish immune reactions and predominantly include IL-10 and TGF-β. They both are well recognised by the fact of their inhibitory effect to the immune system (Rose and Ashwood, 2014). The fact is correlated with probable hyperimmune state in individual with autism spectrum disorder possibly due to hyperactivation of NK cells, T-cell responses, and monocyte and associated with behavioural impairment (Gesundheit et al., 2013).

3.4.1 Interleukin- 10 (IL-10)

Saghazadeh and colleagues (2019) collected data from 14 studies and reported that ASD subjects (n= 682) shows a remarkable decreased concentration of blood IL-10 compared with healthy controls (n= 487). Ashwood et al., (2011) also outlined a reduced level of IL-10 in autistic individual.

In a study of Molloy et al., (2006), the authors specified some parameters such as mean age 6.9 years having a scale of 3.5- 10 years, 85% boys in each group, no remarkable differences in immune responses between the groups, and performed an analysis with cytokines such as IL-4, IL-5, IL-10 and IL-13. Primary series of DPT immunizations were given to all the subjects. The authors observed the production of cytokines from PBMC and significant differences were noticed. All other cytokines were produced at a remarkable higher level compared to control group except IL-10. They reported a decreased amount of IL-10 produced from PBMC of cases than control PBMC.

Moreover, impairment in adaptive immune responses with a decreased level of IL-10 was reported by Gesundheit et al., (2013). Several studies confirm that children with ASD having GI abnormalities are more likely to have e decrease amount of IL-10 in blood or plasma compared to controls. This fact has been correlated with immune hyperactivity and linked with aberrant behaviour of subjects with ASD (Gładysz et al., 2017).

However, in a study, Siniscalco et al., (2018) reported no significant changes of IL-10 production between the control and autistic group. They analysed the brain tissue and PBMC of each group in order to demonstrate the differences. The fact is also supported by Croonenberghs et al. (2002) where they specified no remarkable differences in the IL-10 production in subjects with ASD compared to controls. On the other hand, a study of Gladysz et al., (2017) revealed that there is an elevated production of IL-10 by PBMCs in pregnant women who later gave birth to autistic child.

Another study of Hsiao (2013) reported a decreased level of anti-inflammatory cytokines where he analysed over 300 ASD cases and concluded with a remarkable decrease IL-10 in the neonatal blood.

In addition, a study reported a decrease in the blood IL-10 level in dried neonatal blood spots of 359 neonates who were later diagnosed with ASD, compared to typically developed controls (Rose and Ashwood, 2014). Furthermore, in ASD children, decreased frequencies of IL-10 or T-cell production were documented due to immune alterations when plotted against healthy controls (Gesundheit et al., 2013).

Jyonouchi et al., (2001) performed a study in order to determine T-cell cytokines production. The cells were cultured with recall antigen for 4 days (dust mite extract and tetanus toxoid), concanavalin A; Con A (1 micro g/ml), mitogens [phytohemagglutinin; PHA (10 microgm/ml)]. They identified IL-10 as type two T (T2) cytokines representative and cytokine

level were quantified by using enzyme-linked immunosorbent essay (ELISA). They reported a decreased amount of IL-10 and other anti-inflammatory cytokine production in subjects with autism compared with healthy controls.

Numerous studies reported immune system dysfunction and dysregulation in the activity of monocytes, regulatory T cells, NK cells etc. In essence, Hsiao and Malkova (2016) reported approximately 40% higher NK cells and a decreased percentage of T-helper cells in autistic children compared to control, which ultimately leads a path towards hyperimmune states. The authors also linked the immunological profile of autistic individual with their innate immune responses which involves an increased amount of pro-inflammatory cytokine production and reduced anti-inflammatory cytokines including IL-10. Hsiao (2013) suggested that it might be due to an early life suppression of Th-2, T helper cell-1 responses and alterations in peripheral immune activities in ASD individual.

3.4.2 Transforming growth factor beta1 (TGF- β1)

Transforming growth factor beta1 (TGF- β1), an immunosuppressive regulatory cytokine which is involved in the immunosuppressive activity by deactivating or inducing unresponsiveness in functioning T-cells, is crucial for maintaining immune homeostasis. It occupies a great importance among immune regulators that can impressively restrict the manifold aspects of immune activity (Ashwood et al., 2008).

Two studies demonstrated that the amount of serum or plasma TGF-β1 level were found remarkably lower in subjects with autism spectrum disorder when assimilated with standard developing controls. A relatively greater study with 36 TD children, 32 DD children and 75 children with autism ages ranging from 2-5 years investigated the plasma level of TGF-β1 and reported that there is a notable reduction in the plasma level of TGF-β1 in ASD children in compare with TD children and DD children (Molloy et al., 2006).

A study of Hsiao (2013) reported a rise in pro-inflammatory cytokine level and a decline with anti-inflammatory cytokine level such as TGF- β1. In addition, a downfall in the level of plasma TGF- β1 is also reported by Manzardo et al. (2012).

A study was performed by Ashwood et al., (2008) in order to demonstrate the TGF- β1 level in ASD children. Additionally, they compared the findings to the children having developmental disabilities and healthy control group. In their study, they investigated 143 children with having some specific criteria such as ASD confirmation, confirmed developmental disability without ASD, healthy controls, and age matching. For each subject, their plasma was collected and the level of TGF- β1 concentration were determined by enzyme linked immunosorbent assay (ELISA). Figure 7 demonstrates the differences.

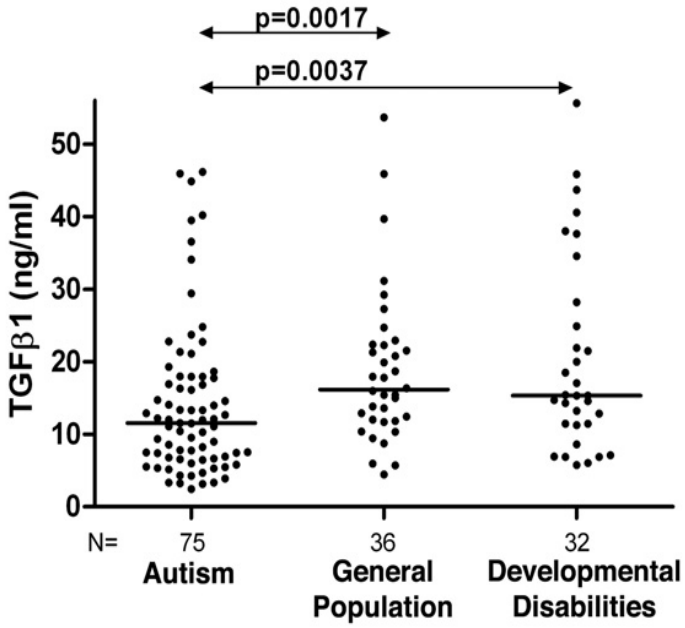

Figure 7: Circulating TGFβ1 levels (ng/ml) in autism, general population, and developmental disabilities. TGFβ1 levels were remarkably reduced in ASD children compared to healthy control group (p=0.0017) and developmental disabilities group. The bar signifies the differences (Ashwood et al., (2008).

The researchers found that, there was a noticeable decline in plasma TGF- β1 concentration in autistic children compare to healthy control group (General population), (median=11.54,

interquartile range (6.73–17.84) ng/ml versus 16.16 (11.97–22.32) ng/ml, p=0.0017) or to the children having developmental disabilities (median=15.34, interquartile range (11.41–25.71) ng/ml, p=0.0037).

Furthermore, A cytokine/chemokine profile have been found to be impaired in subjects with ASD and a down regulation of TGF- β1 was reported additionally (Hughes et al., 2018). The decreased level of TGF-β1 in subjects with ASD is inversely associated with typical autistic behaviour as determined by Aberrant Behaviour Checklist (ABC) and Vineland Adaptive Behaviour Scales (VABS) (Molloy et al., 2006). Down regulation of TGF- β1 in relation to social impairment was also described in a study of Gładysz et al., (2017). The authors reported that stereotypic behaviour seemed to be associated with low TGF- β1 and also correlated with ASD severity. Gesundheit et al., (2013) have described that a dysregulation in immune mediators such as cytokine profile, serum antibodies, brain antibodies are involved in autism spectrum disorder and its severity. They reported that a reduction in the anti-inflammatory cytokines such as IL-10 and TGF- β1 can lead to a hyperimmune state and is observed in subjects with ASD. The decrease in the TGF- β1 level is associated with lower adaptive and cognitive behaviour and additionally with ASD severity. On top of that, microRNAs (miRNAs) responsible for regulating TGF- β1 signalling pathway have been found to be altered in the cortex and serum of ASD subjects (Hughes et al., 2018).

Several studies have reported diminished plasma levels of transforming growth factor (TGF-β) associated with cell migration, apoptosis and immune system and CNS regulation; these findings indicate that TGF-β malfunction can play a role in ASD by not being capable of regulating inflammation, resulting in a chronic inflammation state that has disastrous consequences on CNS (Okada et al., 2006).

3.5 Chemokine

A group of cytokines known as chemokines having a closely related molecular structure that are known to influence peripheral immune cell proliferation and are labelled for their chemo-attractive attributes. They are assisted in neurodevelopment and synaptic transmission in the Central Nervous System (CNS), in addition to chemotaxis (Rostene et al., 2011).

By regulating CNS inflammatory states or neurogenesis, chemokines may contribute to temporary or permanent alterations in cognitive function (Stuart et al., 2015). According to some researchers, changes in levels and responses of chemokine may play a role in the pathophysiology of ASD (Ashwood et al., 2011).

Compared with typically developing (TD) children, one of the most critical steps for validating this hypothesis is to analyse chemokine levels in ASD patients. In addition to an increased neuroinflammatory response in the autistic brain, changes in circulating chemokine levels were also documented (Shen et al., 2016). The study recruited a total of 42 autistic children and 35 age-matched TD children.

In the case group, 38 subjects were male and 4 were female; their mean age was 4.29±0.97 years, ranging from 3.8 to 6.0 years old. In the control group, 19 subjects were male and 16 were female; their mean age was 4.37±1.11 years, ranging from 3.7 to 6.1 years old. The level of the seven chemokines (CCL5, Eotaxin, MIP-1 α, MIP-1 β, MCP-1, IP-10, and MIG) in the plasma samples were measured by using a commercial kit.

The current study demonstrated that some plasma levels of chemokine were altered in autistic patients as opposed to children with TD. In the autism community, the authors reported higher plasma levels of CCL5, MCP-1, MIP-1a, and MIP-1β. It is compatible with some previous research (Ashwood et al., 2011). Heuer et al., (2019) have also demonstrated a significant

plasma MCP-1 level. In addition, they observed reduced IP-10 and MIG plasma levels in autistic children.

In a study by Han et al. (2017), in plasma and cerebrospinal fluid (CSF) specimens of people with ASD, elevated levels of macrophage migration factor (MIF) CXCL8 were found relative to those from normally developing (TD) controls and participants with other developmental disorders. In addition, elevated CCL5 and CCL2 plasma levels in ASD were observed (Noriega and Savelkoul, 2014).

A research was carried out by Ashwood and colleagues (2011) to differentiate the degree of plasma chemokine in autistic people from the stable control group and children with developmental disabilities (Table-3).

Table 3: Comparison of plasma chemokine levels in children with autism spectrum disorders (n=80), typically developing controls (n=58) and children with developmental disabilities other than autism (n=37). Data are presented as median and interquartile ranges. $p<0.05$ compared with typically developing controls; $p<0.05$ compared with developmentally delayed controls.

	Autism spectrum disorder	Typically developing	Developmental delays
MCP-1	139.7 (86–254.7)	90.5 (62–246.6)	84 (56.7–119.1)
MIP-1α	123.7 (72.9–321.8)	125.3 (49.8–406.7)	96.5 (56.9–136.6)
MIP-1β	81.4 (46.8–196.6)	85.1 (12.7–192.3)	35.6 (25.4–86.2)
CCL5	7967 (4294–19,100)	4040 (2341–9008)	4170 (2430–5271)
Eotaxin	42.8 (30.9–56.6)	30.1 (18.1–42.6)	30 (19.7–44.1)
IP-10	8.5 (5–14.1)	7.5 (5–13.9)	7.7 (6.0–13.5)

(Ashwood et al., 2011).

They analysed the plasma of 175 children which includes 80 ASD, 58 TD and 37 DD children. they observed extraordinary elevations in plasma MCP-1, CCL5 and eotaxin levels in children with ASD relative to TD and DD controls in their study (Table-1).

Compared with TD (median 90.5, IQR 62–246.6 pg/ml; p=0.027) and DD controls (median 84, IQR 56.7–119.1 pg/ml; p=0.011), MCP-1 levels in children with ASD (median 139.7, IQR 86-254.7 pg/ml) were dramatically improved.

In children with ASD (median 7967, IQR 4295– 19,100 pg/ml), plasma levels of CCL5 were nearly two-fold higher compared with TD ((median 4040, IQR 2341– 9008 pg/ml; ; p=0.001) and DD controls (median 4170, IQR 2431–5271 pg/ml; p=0.005).

Compared to TD (median 30.1, IQR 18.1– 42.6 pg/ml; p=0.013) and DD (median 30, IQR 19,7- 44.1 pg/ml; p=0.022), the amounts of eotoxin found in children with ASD were substantially higher (median 42.8, IQR 30.9–56.6).

Furthermore, in children with ASD, MIP-1β levels were substantially elevated (median 81.4, IQR 46.8–196.6 pg/ml) relative to DD (median 35.6, IQR 25.4–86.2 pg/ml; p=0.01).

Compared to DD (median 96.5, IQR 25.4–86.2, p=0.059), there was also a tendency for elevated MIP-1 alpha levels in children with ASD (median 123.7, IQR 72.9–321.8).

However, in autistic children, the levels of MIP-1 alpha and MIP-1β were found to be significantly lower than in TD children. in addition, the authors identified substantial correlation between elevated levels of MCP-1, CCL5 and exotoxin and more aberrant cognitive and adaptive function habits or impairments, as measured by ABC, MSEL and VABS, accordingly. In addition, Manzardo et al. (2012) recorded a marked decline ($p<0.05$) in the level of MIP-1 alpha and MIP 1β in autistic individuals which serve as attractants in humoral immune response and development of antibodies, in support with the prior findings.

Chapter 4

Conclusion

ASD accompanies a great margin of heterogenous neurodevelopmental disorder which includes a wide variety and level of inflammatory biomarkers. With the heterogeneity of ASD, researchers are more oriented on recognizing possible biological markers as a way of defining ASD subjects. Researchers identified the immune impairment and inflammation as a very effective component in the diagnosis and treatment option for the disorder. Some risk factors including familial autoimmunity, immune stimulation such as inflammation during pregnancy increases the possibilities of having an offspring with developmental disabilities.

Although, the influence of immune impairment in aberrant behaviour is not clearly understood yet, however many studies included in this review demonstrated an association of immune dysfunction with worsening behaviour. Immune cells interaction resulting in inflammation and a dysregulation in cytokine production in subjects with ASD has been found to be responsible for abnormal brain development and act as a biomarker in the identification of ASD.

In addition, signs of inflammation have been identified in all post-mortem brain tissues, CSF, plasma/sera, and peripheral immune cell activation responses in people with ASD. To be specific, altered T cell activation, increased monocyte cell activation, skewed immunological profile, and changes in complement component levels have also been defined in ASD and can include pathogenic indications. In addition, cytokine inequalities in autism are well known and have many interesting consequences.

Many researchers using a large number of participants from a population-based case-control sample have found that there are substantial rises in IL-1β, IL-6, TNF-alpha plasma level as well as dramatic drop in IL-10 and TGF- β1 levels in young children who have reported ASD relative to confirmed and age matched children who develop typically.

Increases in cytokines in children who suffered lack of language or social skills were the key drivers of these levels. In addition, altitudes and declines in these cytokine levels are linked to increased frequency in the central domains of ASD and greater deterioration in aberrant behaviour. The modified levels of cytokines can make it easier to distinguish subtypes of ASD that shares common values and profiles, and even provide biological markers.

These results indicate that persistent inflammatory responses can be contributed to behavioural disruptions. The malfunctioning immunophenotype seen in ASD specimens may play a crucial role in the pathogenesis of ASD and therefore would be of potential therapeutic importance. Taken together, these results indicate that immune factors, including cytokines, can serve as helpful biomarkers to classify certain persons with more extreme behavioural consequences and can contribute to potential new approaches to therapy.

4.1 Limitation of the study

Since the search for the etiology of autism is still an undergoing research, few information included in this study are not supported universally. More investigations are needed to establish these hypotheses.

4.2 Future research plan

Future research could be done on targeting immune-based therapy and its application. Additionally, future research could be conducted to link the immunological biomarker with behavioural aberrant in patients with ASD.

Chapter 5

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