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A Systematic Review of Gut-Immune-Brain Mechanisms in Autism Spectrum Disorder

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Abstract: Despite decades of research, the etiological origins of Autism Spectrum Disorder (ASD) remains elusive. Recently, the mechanisms of ASD have encompassed emerging theories involving the gastrointestinal, immune and nervous systems. While each of these perspectives presents its own set of supporting evidence, the field requires an integration of these modular concepts and an overarching view of how these subsystems intersect. In this systematic review, we have synthesized relevant evidences from the existing literature, evaluating them in an interdependent manner and in doing so, outlining their possible connections. Specifically, we first discussed gastrointestinal and immuno-inflammation pathways in-depth, exploring the relationships between microbial composition, bacterial metabolites, gut mucosa and immune system constituents. Accounting for temporal differences in the mechanisms involved in neurodevelopment, prenatal and postnatal phases were further elucidated, where the former focused on maternal immune activation (MIA) and fetal development, while the latter addressed the role of immune dysregulation in contributing to atypical neurodevelopment. As autism remains, foremost, a neurodevelopmental disorder, this review presents an integration of disparate modules into a “Gut-Immune-Brain” paradigm. Existing gaps in the literature have been highlighted and possible avenues for future research with an integrated physiological perspective underlying ASD have also been suggested.

Key words: *Autism spectrum disorder, gut-brain axis, immune system, physiological mechanisms*

1. Introduction

1.1 Autism Spectrum Disorder

Deficits in social communication and restricted, repetitive patterns of behaviors and interests have been identified as the two overarching branches of symptoms in Autism Spectrum Disorder (ASD) (American Psychiatric Association, 2013). However, the unique constellation of symptoms that differs strikingly across individuals hints at more complex underlying mechanisms. The past decades have since witnessed a paradigm shift in ASD research, veering from its once predominantly genetic focus to encompass more systemic approaches (Frye & Rossignol, 2011; Herbert, 2010; Rossignol & Frye, 2012a; Rossignol & Frye, 2012b; Arndt, Stodgell, & Rodier, 2005; Harumi Jyonouchi, Sun, & Itokazu, 2002). Emerging evidence shows that ASD is associated with extensive dysregulation across numerous biological modules, the most prominent of which implicate the gastrointestinal environment (e.g. McElhanon, McCracken, Karpen, & Sharp, 2014; Adams, Johansen, Powell, Quig, & Rubin, 2011; Arentsen, Raith, Qian, Forssberg, & Heijtz, 2015; Clarke et al., 2012; Desbonnet, Clarke, Shanahan, Dinan, & Cryan, 2014; Heijtz et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011; Sudo et al., 2004), immuno-inflammation pathways (e.g. Rossignol & Frye, 2012a; El-Ansary & Al-Ayadhi, 2014; Pardo, Vargas, & Zimmerman, 2005; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2004) and nervous system (e.g. Catani et al., 2016; Ha, Sohn, Kim, Sim, & Cheon, 2015; Herrington, Miller, Pandey, & Schultz, 2016; Yang, Beam, Pelphrey, Abdullahi, & Jou, 2016).

1.2 The Gut-Brain Axis

Notable features of gastrointestinal abnormalities associated with autism include alterations of microbial composition (Kinross, Darzi, & Nicholson, 2011; Adams, Johansen, et al., 2011; Finegold et al., 2002; Kang et al., 2013; Song, Liu, & Finegold, 2004; Williams et al., 2011; de Theije, Koelink, et al., 2014; de Theije,

Wopereis, et al., 2014; Finegold et al., 2010; Parracho, Bingham, Gibson, & McCartney, 2005; Song et al., 2004), overproduction of bacterial metabolites (Macfabe, 2013; Calabrese & Rizza, 1999; Wajner, Latini, Wyse, & Dutra-Filho, 2004; Wang et al., 2012) and increase in gastrointestinal mucosa permeability (Hsiao et al., 2013; de Magistris et al., 2010; D'Eufemia et al., 1996; Adams, Audhya, et al., 2011; Cade et al., 2000; D'Eufemia et al., 1996). Dysbiosis of the gut is hypothesized to influence mammalian brain development via communication between gastrointestinal microbiota and the central nervous system (CNS) (Sarkar et al., 2016; McVey Neufeld, Mao, Bienenstock, Foster, & Kunze, 2013; Bercik et al., 2011), which is usually modulated by immune responses (Carabotti, Scirocco, Maselli, & Severi, 2015; Erny et al., 2015; Heijtz et al., 2011; Midtvedt, 2012; Nicholson et al., 2012; Alkanani et al., 2015; Breban et al., 2017; Köhling, Plummer, Marchesi, Davidge, & Ludgate, 2017; Ma, Shi, Li, Chen, & Niu, 2015; Miyake et al., 2015).

1.3 The Immuno-Inflammation Pathways

Accumulating evidence drives at the indubitable link between ASD and generalized immune dysfunction. A subset of children with ASD has been shown to exhibit upregulated quantities of natural killer (NK) cells, interferon gamma (INF-γ) (Gregg et al., 2008; Enstrom, Lit, et al., 2009; Vargas et al., 2004), tumour necrosis factor-alpha (TNF-α) (Chez, Dowling, Patel, Khanna, & Kominsky, 2007), TNF-receptor II (Zimmerman et al., 2005), interleukin-6 (IL-6)(Li et al., 2009; Wei et al., 2011), IL-8 (Li et al., 2009), IL-1β (Ashwood, Schauer, Pessah, & Van de Water, 2009) and autoantibodies (Rossignol & Frye, 2012a; Frye, Sequeira, Quadros, James, & Rossignol, 2013; Rossignol & Frye, 2012a; Gesundheit et al., 2013), with concomitant reduction in concentration of plasma transforming growth factor-beta (TGF-β1) (Ashwood et al., 2008). Contradicting results have surrounded findings on immunoglobulin atypicalities in ASD (Croonenberghs et al., 2002; Stern et al., 2005; Gupta, 2000). Neuroinflammation in microglial activation has also been reported in various brain regions (Vargas et al., 2004; Morgan et al., 2010; Tetreault et al., 2012; Suzuki et al., 2013). Finally, the presence of immuno-inflammatory biomarkers have been shown to correlate with acuteness of ASD symptoms (Khakzad et al., 2012; Al-

ayadhi & Mostafa, 2011; Al-Ayadhi & Mostafa, 2012a, 2013; Mostafa & Kitchener, 2009).

The current review will begin by unravelling the mechanistic connections between the gut and immune system, followed by the immune system and brain development. This discussion will coalesce on the multiple routes that these networks can possibly take to influence brain maturity, linking etiological mechanisms of autism across the 'gut-immune-brain' axis.

2. Methods

--- FIGURE 1 – About here ---

Pubmed and PsycInfo databases were utilized to browse for articles, from the year 2000 to 2018, relating to the gut-brain axis and immune/inflammation system in Autism Spectrum Disorder (ASD). Firstly, five database searches were made using the Boolean operator "AND": 1) Gut, Brain and Autism; 2) Immune, Brain and Autism; 3) Microbiome and Autism; 4) Gut, Brain, Immune and Autism; 5) Gut, Brain, Inflammation and Autism. These searches generated a long list of records (n=931), which were subsequently organized into a database and screened according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines as illustrated in Figure 1. A total of 358 records were eventually included for qualitative analysis.

3. The Gut and Immuno-Inflammation

3.1 Microbiota, T-cells and Cytokines

The human gastrointestinal (GI) tract is home to 100 trillion microorganisms, collectively known as the microbiota (Weinstock, 2012). The medley of gut microbiota has astounding implications on the profile of circulating cytokines (e.g. IL-12p40, IFN-γ, TGFβ, IL-β, IL-6, TNF-α, MCP-1) (El-Ansary & Al-Ayadhi, 2014; Jyonouchi, Sun, & Le, 2001; Suzuki et al., 2011; Xu, Li, & Zhong, 2015; Harumi Jyonouchi, Geng, Ruby, & Zimmerman-Bier, 2005), and lymphocyte development,

with some species stimulating the differentiation of specific T-cell subtypes (Estes & McAllister, 2015; Hsiao, 2013; Morgan et al., 2010; Vargas et al., 2004). In ASD, a dysbiosis in microbiota, featuring an overrepresentation of *Clostridial*, *Bacteriodetes*, *Firmicutes* and *Lactobacilli* (Finegold, Downes, & Summanen, 2012; Tomova et al., 2015; Wang, Conlon, Christophersen, Sorich, & Angley, 2014) has been observed. Synthesis of T regulatory (T-reg) cells producing anti-inflammatory IL-10 is only enhanced by a strain-specific cluster of *Clostridial* species and *Bacteroides fragilis* (Ochoa-Reparaz et al., 2010; Round et al., 2011), whereas accretion of Th17 cells producing pro-inflammatory IL-17 is stimulated by segmented filamentous bacteria (SFB) (Lee, Menezes, Umesaki, & Mazmanian, 2011; Wu et al., 2010). The balance between Th1/Th2 cytokine response has also been shown to be modulated by specific bacterial species (Mulle, Sharp, & Cubells, 2013), although conflicting findings of Th1 and Th2 dominance in ASD have been reported (Anthony et al., 1998; Furlano et al., 2001). Recently, Rose et al. (2018) stratified sub-groups of children with (ASD) and without ASD (TD), who either displayed irregular (GI) or normal GI symptoms (NoGI). Upon endotoxin stimulation, the ASD-GI group was found to display higher levels of IL-5, IL-15 and IL-17 than ASD-noGI. This group also exhibited lower concentrations of anti-inflammatory TGF-β1 than ASD-noGI and TD-noGI, and their bacterial composition was distinctly different from other groups. In a similar study, Luna et al. (2017) observed an increased abundance in *Clostridiales* genera and reduced levels of *Sutterella*, *Dorea* and *Blautia*, with parallel elevations in IL-6 and tryptophan concentrations amongst ASD-GI, as compared to other groups. Gene sequencing analyses by Foley and colleagues (2015) revealed that infants with ASD have a higher abundance of bacterial genera *Faecalibacterium*, and lower levels of *Blautia*, in fecal and blood samples. Recently, a study by Coretti et al. (2017) on mice models have elucidated markedly unique microbial and immune profiles in male and female autistic mice, suggesting the possible role of the microbiota in influencing sex-specific susceptibility to autism.

3.2 Microbiota and Antibodies

An altered gut microbiome may be sufficient to stimulate the production of an army of antibodies (Gutzeit, Magri, & Cerutti, 2014). Indeed, Immunoglobulin A (IgA), the main antibody secreted in the gut, has been shown to be significantly higher in ASD

populations as compared to controls (Zhou et al., 2017). IgA is responsible for maintaining mucosal immunity (Lutgendorff, Akkermans, & Soderholm, 2008), the overstimulation of which suggests a consistent assault to the gut. Several species of microbiota have been postulated to be associated with ASD. In particular, the presence of *Micobacterium paratuberclosis* may evoke antibodies that interact with the myelin basic protein in the CNS (Dow, 2011), while *Clostridium bolteae* and *Sutterella* species have been proposed to elicit antibody production that aggravates gastrointestinal co-morbidities (Pequegnat et al., 2013; Williams, Hornig, Parekh, & Lipkin, 2012). Re-establishing microbial balance through Fecal Microbiota Transplant (FMT) has been shown to alleviate gastrointestinal and core autistic symptoms (Kang et al., 2017; Moon et al., 2015; Palm et al., 2014).

3.3 Microbiota and Interferon (IFN) Signalling

In autism, mechanisms of interferon (IFN), a signalling protein released in response to pathogens (De Andrea, Ravera, Gioia, Gariglio, & Landolfo, 2002), are altered. Genes associated with type-I IFN signalling have been shown to be substantially reduced in germ-free (GF) mice devoid of gut microbiota (Erny et al., 2015). Inoue et al. (2016) recently discovered that, as compared to healthy controls, autistic infants exhibited significantly different expression patterns of genes implicated in IFN-γ and type-I IFN signalling. Elevated quantities of IFN-α or IFN-γ, with (Jyonouchi et al., 2002) and without endotoxin induction, have also been reported in the cerebrospinal fluid (CSF) and peripheral mononuclear blood cells (PBMC) of autistic children (Singh, 1996; Stubbs, 1995; Vargas et al., 2004). Interestingly, correlational analysis generated significant association between IFN-related genes and quantity of *Faecalibacterium* (Ashwood et al., 2011; Suzuki et al., 2011).

3.4 Microbiota and Maternal Immune Activation (MIA)

Whilst earlier reports have associated specific infectious agents (e.g. rubella, influenza), during the prenatal phase, to an increased incidence of ASD, it was later discovered that it was the inflammatory response towards an infection, known as maternal immune activation (MIA) (Wong & Hoeffer, 2018), rather than a specific causative agent, that presents a risk (Atladóttir et al., 2010; Wilkerson, Volpe, Dean,

& Titus, 2002; Libbey, Sweeten, McMahon, & Fujinami, 2005; Lintas, Altieri, Lombardi, Sacco, & Persico, 2010; Atladottir, Henriksen, Schendel, & Parner, 2012; Zhang et al., 2010; Meltzer & Van de Water, 2016). In addition to MIA, evidence points to the involvement of functional Toll-like receptor 3 (TLR3) and commensal microflora in inducing autistic phenotypes (Atarashi et al., 2015). In a study by Kim et al. (2017) on mice, administration of the antibiotic vancomycin prior to inducing MIA by poly (I:C) treatment did not lead to observation of abnormal behavioral phenotypes. Moreover, vancomycin treatment substantially diminished Th17 levels in the offspring's small intestine. The study also revealed that certain maternal intestinal bacteria modulate Th17 abundance and the emergence of autistic behaviors. Specifically, offspring which came from mothers with human commensal bacteria or mouse commensal SFB showed significantly more Th17 cell differentiation (Kim et al., 2017), which is potentially linked to aberrant behaviours mediated by an increase in expression of IL-17a receptor in the offspring's frontal cortex (Choi et al., 2016a).

3.5 Gastrointestinal Mucosa Permeability

The "leaky gut theory" postulates that an exceptionally increased permeability of the intestinal mucosa barrier represents a fundamental mechanism underlying autistic pathology (de Magistris et al., 2010; D'Eufemia et al., 1996; Horvath & Perman, 2002; Panksepp, 1979; Wakefield et al., 1998). Studies on subgroups of ASD subjects have revealed that this deficiency is partly contributed by (1) alterations in microbial composition, with an overrepresentation of bacterium *Akkermansia muciniphila*, known to degrade the mucus lining (Wang et al., 2011), (2) prevalence of *Clostridia* and reduced abundance of *Bifidobacteria*, increasing pro-inflammatory cytokines production, which aggravates mucosa permeability (Heberling, Dhurjati, & Sasser, 2013), (3) elevated plasma levels of zonulin, a protein that regulates permeability (Esnafoglu et al., 2017), (4) decreased amounts of tight junction proteins at the intestinal barrier (Fiorentino et al., 2016), potentially due to increased toxins from *Clostridia* (Hecht, Pothoulakis, LaMont, & Madara, 1988), (5) infection by *Escherichia coli*, which leads to transformation of actin and tight junction structures (Long, Nisa, Donnenberg, & Hassel, 2014), (6) abundance of *Candida* fungi, which expresses root-like formations that invade the intestinal wall (de Magistris et al.,

2010) and (7) increased levels of claudin, a molecule that contributes to pores formation (Fiorentino et al., 2016). Individuals with both autistic and gastrointestinal disorders exhibit a distinct cytokine (Ashwood & Wakefield, 2006; Torrente et al., 2002) and regulatory (Ashwood, Anthony, Torrente, & Wakefield, 2004) profile. However, while Ashwood et al. (2003) reported a link between ASD with GI symptoms and intestinal permeability, de Magistris et al. (2010) found no association. Animal model studies suggest that the composition of microbiota modulates the integrity of both the intestinal barrier (Jakobsson et al., 2014), and the blood-brain barrier (BBB) (Braniste et al., 2014). Permeability of the brain and the GI appears to be developmentally-sensitive. Stolp and colleagues (2005) revealed that BBB permeability in rats is propagated by inflammation in early life, while Braniste et al. (2014) and Cani et al. (2009) showed that the gut microbiota determines permeability of the gut mucosa lining in later adulthood.

An impaired intestinal barrier leads to resoundingly detrimental outcomes. Firstly, intestinal permeability upsets normative levels of major histocompatibility complex (MHC)-presenting epithelial cells, which affects activation of T-regs (Rabinowitz & Mayer, 2012) and exacerbates inflammation (Pastorelli, De Salvo, Mercado, Vecchi, & Pizarro, 2013). Secondly, neuroactive opioids from digested products, such as amyloid beta peptides, have been shown to leak into the bloodstream and permeate the BBB, influencing only neural but not glial cells (Clifford et al., 2007), to elicit ASD symptoms (Panksepp, 1979; Shattock & Whiteley, 2002). However, no significant difference in opioid levels has been found between those with and without ASD (Cass et al., 2008). Lastly, toxins from the gut that pass through the intestinal barrier have been shown to induce antibody production (de Magistris et al., 2014) that exacerbates chronic inflammation (Visser, Rozing, Sapone, Lammers, & Fasano, 2009). Indeed, deposition of IgG and complement C1q at the epithelium supports the presence of an autoimmune reaction (Torrente et al., 2002). Additionally, bacterial lipopolysaccharides (LPS) from the gut stimulate liver cells to secrete TNF-α, which modulates BBB permeability (Kim, Wass, Cross, & Opal, 1992). Studies on ASD patients revealed that TNF-α cascades to produce pro-inflammatory cytokines, which leads to peripheral inflammation (Breese et al., 1994), followed by activation of microglia in the brain (Qin et al., 2007), indicating neuroinflammation (Derecki et al., 2012; Laurence & Fatemi, 2005; Morgan et al., 2010; Tetreault et al., 2012).

Moreover, TNF-α indirectly inhibits interferon-beta (INFβ), a molecule which defends the mucosa lining (Long et al., 2014). Most interestingly, studies comparing ASD and typical populations with GI symptoms found that increased permeability of the intestinal barrier allows for an almost imperceptible infiltration of immune cells into the GI (Ashwood et al., 2003, 2004; Ashwood & Wakefield, 2006; Furlano et al., 2001; Torrente et al., 2002; Torrente et al., 2004), including lymphocytes bearing pro-inflammatory characteristics (i.e. CD3+ staining revealed increased TNF-α+, IFN-γ+ and reduced IL-10+), although Fernell and colleagues (2007) did not find any association between gastrointestinal inflammation and autism.

3.6 Bacterial Metabolites

The gut microbiome produces three classes of short-chain fatty acid (SCFA): propionic acid (PPA), acetic acid and butyric acid (Stilling, Dinan, & Cryan, 2014), which were all shown to be overexpressed in ASD populations (Wang et al., 2012), though contradictory findings remain prevalent (Cryan & Dinan, 2012; Louis, 2012; Mangiola et al., 2016; Vuong & Hsiao, 2017). Animal model studies that replicate autistic-like behaviours have elucidated an overgrowth in *Firmicutes* bacteria, a reduction in *Bacteroidetes*, along with increased levels of SCFA, especially butyric acid, in male mice (de Theije, Koelink, et al., 2014; de Theije, Wopereis, et al., 2014). Although Hsiao et al. (2013) did not generate similar findings using MIA mice models, they demonstrated an increase in diversity of *Clostridium* and *Bacteroides*, in line with predominant findings in ASD human subjects (Parracho et al., 2005; Tomova et al., 2015). Several studies have illustrated that SCFAs are capable of permeating the BBB (Karuri, Dobrowsky, & Tannock, 1993) to modulate neural characteristics of brain cells (El-Ansary, Ben Bacha, & Kotb, 2012; Kratsman, Getselter, & Elliott, 2016; Macfabe, 2012; Erny et al., 2015). Indeed, administration of PPA in rats, prenatally through a pregnant mother (Foley, Ossenkopp, Kavaliers, & Macfabe, 2014) and in early life (Foley et al., 2015; MacFabe, 2015; Thomas et al., 2012; Wikoff et al., 2009), as well as increasing PPA diet in children (Mellon, Deshpande, Mathers, & Bartlett, 2000), facilitated the emergence of autistic-like behaviors across animal and human studies. Intriguingly, administration of sodium butyrate alleviated autistic symptoms (Kratsman et al., 2016; Takuma et al., 2014), either by reducing concentrations of IL-17 and IL-23 while simultaneously increasing

T-regs, IL-10 and IL-12 production (Zhang, Liao, Sparks, & Luo, 2014) or through different metabolic processes (Ríos-Covián et al., 2016; Jung, Park, Jeon, & Han, 2015; Peng, Li, Green, Holzman, & Lin, 2009).

SCFAs from bacterial species act upon specific GI and immune pathways. A consortium of *Clostridia* species is capable of inducing the production of tryptophan catabolites (Maes & Rief, 2012), such as propionic acid, that interact with aryl hydrocarbon receptor on the surface of differentiating T-cells to stimulate production of IL-22 (Fallarino, Grohmann, & Puccetti, 2012; Mezrich et al., 2010; Opitz et al., 2011; Qiu et al., 2013), which promotes T-cell differentiation (Cavaglieri et al., 2003; Qiu et al., 2012; Veldhoen et al., 2008). This process regulates T-reg/Th17 balance (Zhang et al., 2014) and intestinal barrier integrity (Mjösberg, Bernink, Peters, & Spits, 2012; Qiu et al., 2012). Previous animal studies have revealed that mice with microbial dysbiosis, lacking Trp-metabolising bacteria, or gene (e.g. ACE2, Card9 knockout mice), subsequently synthesised low amounts of IL-22, creating pro-inflammatory conditions (Hashimoto et al., 2012; Lamas et al., 2016) and disrupting the intestinal barrier (Mjösberg et al., 2012; Qiu et al., 2012). Table 1 summarizes central gastrointestinal-immune mechanisms in ASD.

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4. Prenatal Phase: Maternal Immune System and Fetal Development

4.1 Maternal Immune Activation (MIA) and Neurodevelopment

Fetal development of organs tethers on a delicate course that, when interrupted, leads to deleterious consequences. Extensive retrospective studies have suggested a link between prenatal maternal infection and altered neurodevelopment that ultimately elevates the risk of ASD in infants (Lee et al., 2015; Meltzer & Van de Water, 2016). Moreover, maternal immune activation (MI) during the prenatal phase has long been established to play a crucial role in inducing inflammatory states (Lintas et al., 2010; Atladottir et al., 2012) and behavioral dysregulation (Bauman et al., 2014a; Machado, Whitaker, Smith, Patterson, & Bauman, 2015; Malkova, Yu, Hsiao, Moore, & Patterson, 2012). The immune system comprises of a myriad of

cells which are capable of producing pro-inflammatory cytokines responsible for the inflammatory state observed in MIA (Meltzer & Van de Water, 2016). Animal studies do not only support this observation, but also further suggest that the mere activation of immune response in the mother, devoid of any infection, is sufficient enough to propagate changes in the offspring (Shi et al., 2003).

Regardless of whether immune activation was triggered by direct infection (i.e. Influenza), dsRNA mimic poly (I:C), or bacterial LPS, MIA models have replicated significant autistic traits in their offspring, further buffeting the role of a generalized inflammatory state in ASD (Gilmore, Jarskog, & Vadlamudi, 2005; Meyer, Feldon, Schedlowski, & Yee, 2005; Urakubo, Jarskog, Lieberman, & Gilmore, 2001; Zuckerman & Weiner, 2005; Meltzer & Van de Water, 2016; de Cossío, Guzmán, van der Veldt, & Luheshi, 2017). In one study, the authors utilized a direct infection of human influenza and poly (I:C) to trigger immune activation in two separate groups of pregnant mice and found that both experiments yielded similar autism-relevant traits in offspring (Shi et al., 2003). These findings suggest that MIA was responsible for the offspring's aberrant phenotypes (Shi et al., 2003). Interestingly, altered cerebellar development, also observed in autistic individuals, was demonstrated in a follow up study (Shi et al., 2009). Poly(I:C) injection in pregnant rhesus macaques at either the late-first or late-second trimester presented atypical social interactions and vocal communications along with repetitive behaviors, all of which align with ASD traits (Bauman et al., 2014b). Another study injected bacterial LPS in pregnant mice, producing offspring where only the males exhibited autistic-relevant behavioral changes, with an associated reduction of CX3CR1 expression - a receptor involved in neuronal pruning (de Cossío et al., 2017). MIA models have thus proven to be successful in establishing and replicating autism-relevant socio-behaviours. However, much of the mechanistic approach remains unresolved (Gilmore et al., 2005; Meyer et al., 2005; Urakubo et al., 2001; Zuckerman & Weiner, 2005).

4.2 Maternal Pro-inflammatory Cytokine Profile

Atypical cytokine profiles in pregnant mothers and their offspring have become a ubiquitous observation associated with autism. A study done in 2007 revealed that upon injection of pregnant mice with IL-6, one of the main pro-inflammatory

cytokines, offspring with autism-relevant behaviors were observed (Smith, Li, Garbett, Mirnics, & Patterson, 2007). Interestingly, these behaviors were not seen in offspring of pregnant mice that were injected with other relevant pro-inflammatory cytokines such as IL-1a, TNF-α or IFN-γ (Smith et al., 2007). The authors went on to prove IL-6's key role in ASD when autistic-related behaviors were rescued upon the co-injection of anti-IL-6 antibody onto pregnant dams (Smith et al., 2007). Rescued phenotype was not apparent upon co-injection with anti-IFN-γ or anti-IL-1β. An extended study demonstrated no significant difference in social behavior between poly(I:C) treated IL-6 knockout (KO) mice and untreated IL-6 KO mice, thus further supporting the integral role of IL-6 in the etiology of ASD (Smith et al., 2007). Besides IL-6, Choi et al. (2016b) recently discovered that atypical socio-behaviors were observed in the offspring of IL-17 injected dams. Likewise, pre-treatment of dams with anti-IL-17 produced offspring with 'rescued' phenotypes (Choi et al., 2016b). Indeed, Th17 cells may be another critical player in MIA-induced ASD that is acting downstream of elevated IL-6 levels (Choi et al., 2016b). The authors showed that other pro-inflammatory cytokines such as TNF-α, IFN-β and IL-1β were increased in MIA induced models (Choi et al., 2016b). This was also reflected in a recent human study that saw elevated pro-inflammatory cytokines, at mid-gestational stage, in mothers of children with ASD (Jones et al., 2017). More importantly, some of these cytokines are capable of crossing the placenta and fetal BBB via active transport mechanisms. These cytokines potentially activate fetal microglia and mast cells, exacerbating the neuro-environment which could contribute to autism (Abdallah, Larsen, Grove, Nørgaard-Pedersen, et al., 2013; Zaretsky, Alexander, Byrd, & Bawdon, 2004; Ferretti & Hollander, 2015a).

4.3 Autoantibodies Against Fetal Brain Protein

Aside from MIA, emerging findings of maternal autoantibodies against fetal proteins strongly support the correlation between maternal immune dysregulation and ASD (Dalton et al., 2003a; Croen et al., 2008; Zimmerman et al., 2007; Singer et al., 2008; Braunschweig et al., 2013a; Enstrom, Van de Water, & Ashwood, 2009). During pregnancy, protective maternal IgG antibodies cross the placenta to transfer immunity to the developing fetus. Consequently, maternal autoantibodies against fetal brain can cross the placenta and through the BBB, which, although is actively forming, remains permissive and as such results in the disruption of

neurodevelopment (Bake, Friedman, & Sohrabji, 2009). Injection of IgG from mothers of children with autism into pregnant macaque monkeys produced offspring of enlarged brain volume as well as aberrant social behavior (Dalton et al., 2003b; Bauman et al., 2013). Interestingly, a specific set of maternal antibodies, found in 12% of mothers with autistic children, were shown to be reactive towards fetal brain proteins of sizes 37 kDA, 73 kDa (Braunschweig & Van de Water, 2012; Fox, Amaral, & Van de Water, 2012; Daniel Braunschweig et al., 2008) and around 39 kDa (Piras et al., 2014). Subsequently, seven developmentally regulated proteins were identified from the bands: Lactate Dehydrogenase A and B (LDH-A and LDH-B), stress-induced phosphoprotein 1 (STIP1), collapsin response mediator proteins 1 and 2 (CRMP1 and CRMP2), cypin and Y-box binding protein 1 (YBX1) (Braunschweig et al., 2013b). These findings not only provide insights into the mechanisms of autoantibodies, but also highlight the potential predictive markers for autism risk (Meltzer & Van de Water, 2016). Animal model studies that injected maternal anti-brain antibodies from mothers of children with autism into pregnant mice and monkeys, at varying gestational stages, yielded offspring with autism-relevant traits, supporting the promising clinical utility of antibodies (Dalton et al., 2003b; Singer et al., 2009; Braunschweig et al., 2012; Martin et al., 2008).

5. Postnatal Phase: Immune System and Neuroinflammation

5.1 Immune Dysregulation

Once thought to be immune-privileged, the CNS has been shown to be in constant communication-relay with the immune system (Skaper, Facci, Zusso, & Giusti, 2018). Immune cell derived mediators influence the CNS environment through interaction with key players such as microglia and mast cells (Garden & Möller, 2006; Hanisch & Kettenmann, 2007a). Post-mortem of brain specimens in ASD individuals ages four to forty-five suggests chronic neuroinflammation as well as increased microglial abundance and activation, with greatest activity found in the cerebella and anterior cingulate gyrus (Li et al., 2009; Morgan et al., 2010; Vargas et al., 2004). Interestingly, growing evidences have suggested that neuroinflammation is a double-edged sword that provides both neuroprotection and deleterious consequences (Tilleux & Hermans, 2007; Griffiths, Neal, & Gasque, 2007; Skaper, 2007). Persistent activation of microglia, mast cells and astroglia contribute heavily

to elevation in pro-inflammatory cytokine levels that shift the exquisite balance between pro- and anti-inflammatory states to favor a perilous pro-inflammatory condition, hindering neurodevelopmental processes (Abdallah et al., 2012; Abdallah, Larsen, Grove, Bonefeld-Jørgensen, et al., 2013; Zerbo et al., 2014). While extensive post-natal studies saw elevated levels of pro-inflammatory cytokines, including IL-6, IL-8, IL-12, IFN-γ, TNF-α and TH1, in both the brain and CSF, there were also contradictory findings which reported that, as compared to controls, ASD individuals exhibited lesser IL-6, one of the prominent pro-inflammatory cytokines (Pardo et al., 2005; Abdallah et al., 2011; Al-Ayadhi & Mostafa, 2012b; Masi et al., 2014; Croonenberghs et al., 2002; Croonenberghs, Bosmans, Deboutte, Kenis, & Maes, 2002; Li et al., 2009; Ricci et al., 2013; Chez & Guido-Estrada, 2010; Chez et al., 2007; Garbett et al., 2008; Goines & Ashwood, 2013; Falcone & Franco, 2015).

5.2 Microglial Cell Activation

Microglia, a resident mononuclear phagocytic cell, contributes greatly towards neurodevelopment by modulating synaptic pruning, maturation of brain circuitry, and immunosurveillance (Bessis, Béchade, Bernard, & Roumier, 2007; Hanisch & Kettenmann, 2007b; Di Marco, Bonaccorso, Aloisi, D'Antoni, & Catania, 2016; Kettenmann, Kirchhoff, & Verkhratsky, 2013; Blinzinger & Kreutzberg, 1968; Tremblay et al., 2011; Paolicelli et al., 2011; Schafer et al., 2012). Both animal studies and post-mortem on brains of ASD individuals showed salient pathology differences as compared to controls, with an abundance of activated microglial cells in various brain regions (Morgan et al., 2010; Tetreault et al., 2012; Vargas et al., 2004; Careaga, Schwartzer, & Ashwood, 2015). Activated microglial cells undergo two major changes that begins with a drastic cell morphology transformation from a highly branched order to an amoeboid form (M1) before a final transformation into active phagocytes (M2) (Hanisch & Kettenmann, 2007a). The former is responsible for neuronal homeostasis while the latter results in neuroinflammation (Kalkman & Feuerbach, 2016). Morphological assessments of ASD brains have shown greater proportions of microglia in M1 activated state, which further supports its role in neuroinflammation (Morgan et al., 2010; Tetreault et al., 2012). Influenced greatly by external immune signals, activated microglia secrete numerous pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α, and generate reactive oxygen species that alter the CNS environment (Garden & Möller, 2006; Hanisch & Kettenmann, 2007a).

Phagocytic events during neuronal debris clearance may occur without the induction of pro-inflammatory cytokines (Ransohoff & Perry, 2009; Kettenmann et al., 2013). As such, an upregulation of cytokines associated with the activation of microglia presents evidence of chronic neuroinflammation (Ferretti & Hollander, 2015b). Consequently, these findings espouse the critical role of abnormal microglial activation in neurodevelopment (Fernández de Cossío et al., 2017), and in creating a neuro-inflammatory state which may underlie the pathogenesis of ASD (Di Marco et al., 2016),

5.3 Mast Cell Activation

Alongside microglia, mast cells, which belong to the innate arm of the immune system, were also reported to be activated in autism (Theoharides, Doyle, Francis, Conti, & Kalogeromitros, 2008; Theoharides et al., 2012; Theoharides, Stewart, Panagiotidou, & Melamed, 2016). Indeed, the incidence of ASD increases by ten-folds in children with mastocytosis, a condition defined as the accumulation of functionally defective mast cells that contribute greatly to pro-inflammatory secretions (Kempuraj et al., 2010). Aside from immune cells-derived mediators, other substances, such as neurotensin, microbial products and immunoglobulin-free light chains from the gut can also trigger mast cell activation (Theoharides & Kalogeromitros, 2006). In turn, mast cells secrete numerous pro-inflammatory cytokines, as well as vasoactive molecules such as histamines (Kim et al., 1992; de Boer & Breimer, 1998; Abbott, 2000). Recent findings have shown that neurotensin is present at elevated levels in both the brain and gut of ASD patients (Angelidou et al., 2010). Aside from stimulating lymphocyte proliferation and activating T cells and mast cells, neurotensin is also capable of stimulating mast cells to secrete mitochondrial DNA extracellularly, a phenomenon that has inflammatory consequences (Zhang, Asadi, Weng, Sismanopoulos, & Theoharides, 2012), and which is also observed in ASD patients (Evers et al., 1994; Ramez et al., 2001; Lemaire, 1988; Carraway et al., 1982; Zhang et al., 2010). More importantly, these mast cells-derived inflammatory and vasoactive mediators increase BBB permeability, which further exacerbates the neuro-inflammatory state (Theoharides, Tsilioni, Patel, & Doyle, 2016). Table 2 summarizes fundamental immune-brain mechanisms in ASD.

--- TABLE 2 – About here ---

6. Perspective

This section integrates the main etiological pathways of ASD in Figure 2 and Figure 3. Future research in this field may opt to adopt three key suggestions. Firstly, the interconnected nature of these processes emphasize the need for extensive systemic approaches with an eye for detailed mechanistic investigation. Researchers should direct their focus on reinforcing or extending existing theories, allowing for sequential processes to be elucidated. Secondly, the literature is rife with glaring missing connections. For instance, the dearth of longitudinal studies that continuously investigate pre- and post-natal processes is concerning, given that autism is a temporally-sensitive neurodevelopmental disorder with processes in both stages of development being implicated. Another unaddressed matter is how little in the way of testing has been conducted to investigate how neuroinflammation leads to autistic behavioral phenotypes, although numerous studies have cited this phenomenon to be a central etiological factor. The disjoint between systemic pathways and the manifestation of abnormal behaviors should be bridged with rigorous testing for causality. Indeed, the final suggestion reiterates the need for the field to unearth causal mechanisms. With studies on the association of systems dysfunction and ASD sketching the scaffold of autistic etiology, it is time for causal mechanisms to be brought to the fore, allowing for more definitive etiological pathways to be uncovered.

--- FIGURE 2 – About here ---

--- FIGURE 3 – About here ---

7. Conclusion

Autism remains one of the most perplexing unsolved mysteries of the human condition. Taking the reader through the gastrointestinal, immune and nervous systems, this review has holistically elucidated the mechanisms of the “gut-immune-brain” pathway in ASD. Drawing from studies that have investigated these modular

connections, potential gaps in the literature were deciphered. Future studies in this field should adopt a more systemic and causal approach, explicating missing links and conflicting evidences in a systematic manner.

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Table and Figure Captions

Table 1. Table depicting fundamental mechanisms in the gut-immune connection implicated in autism spectrum disorder (ASD).

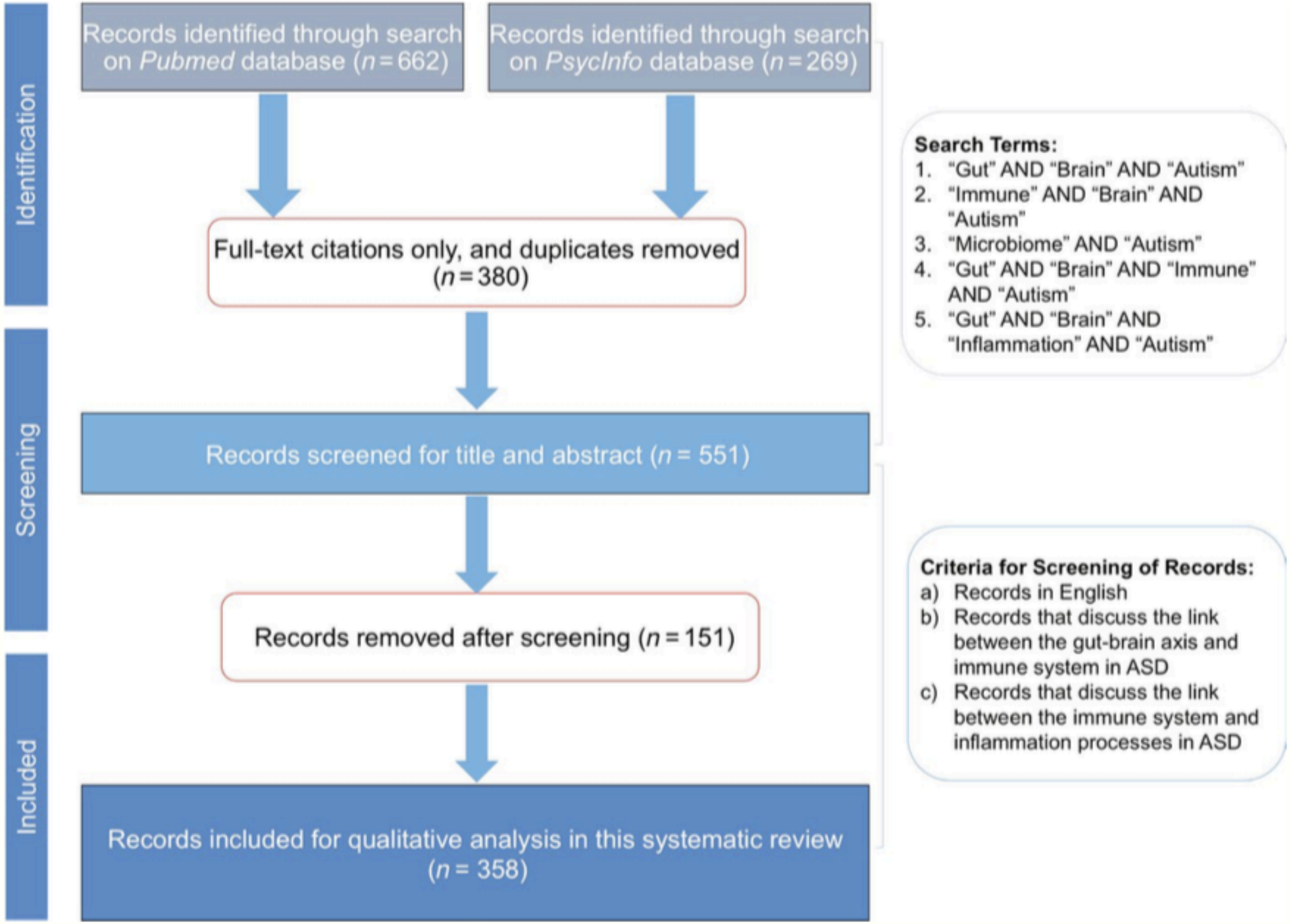
Table 2. Table depicting fundamental mechanisms in the immune-brain connection implicated in autism spectrum disorder (ASD).

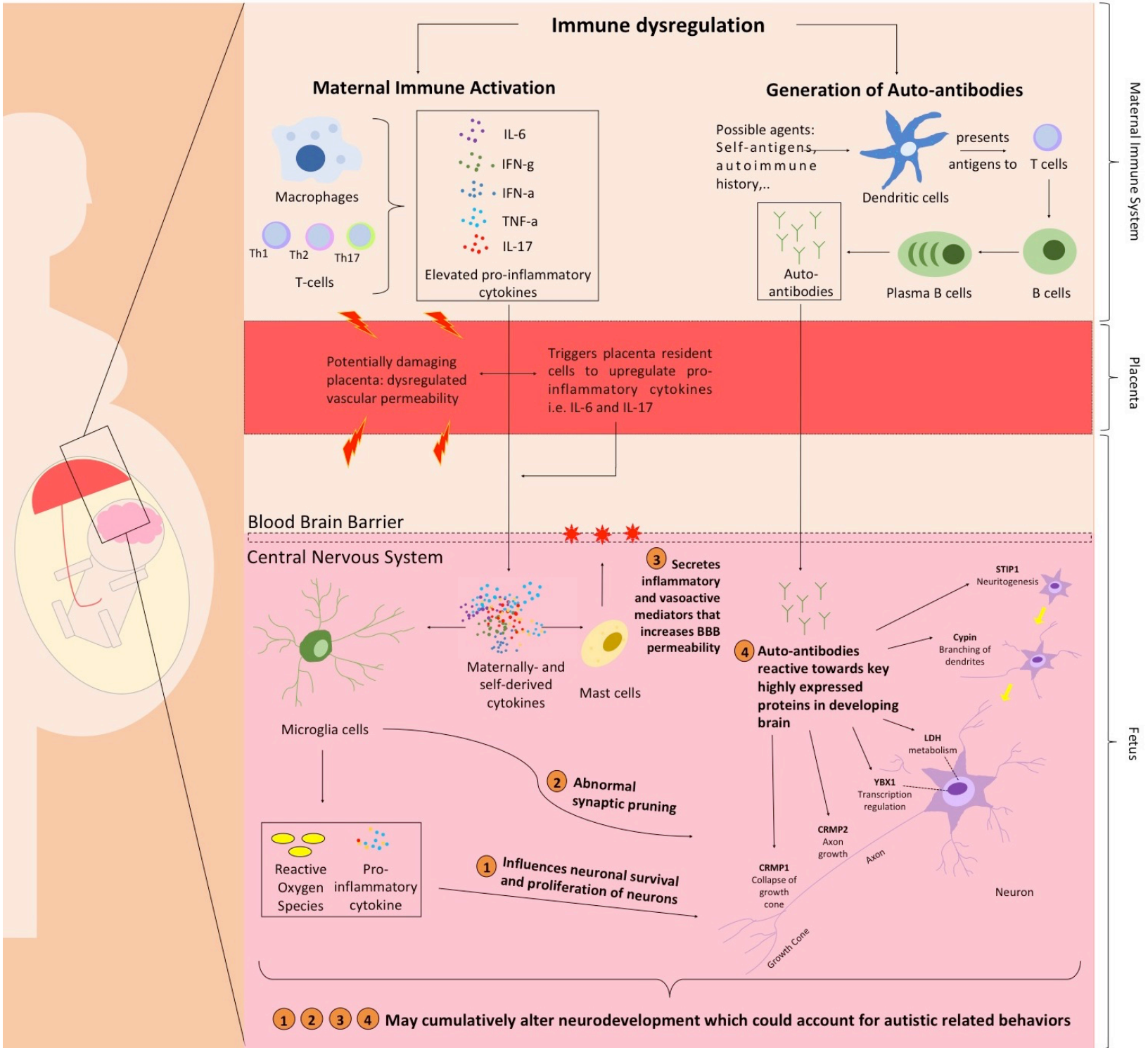
Figure 1. PRISMA Flow for Inclusion of Records in Systematic Review. Records were identified on *Pubmed* and *PsycInfo* databases from the following five searches: “Gut” AND “Brain” AND “Autism”; “Immune” AND “Brain” AND “Autism”; “Microbiome” AND “Autism”; “Gut” AND “Brain” AND “Immune” AND “Autism”; “Gut” AND “Brain” AND “Inflammation” AND “Autism”. Records written in English were screened for content relevant to connections across the gut-brain axis, immune system, inflammation processes and autism. A total of 358 records were included for qualitative analysis.

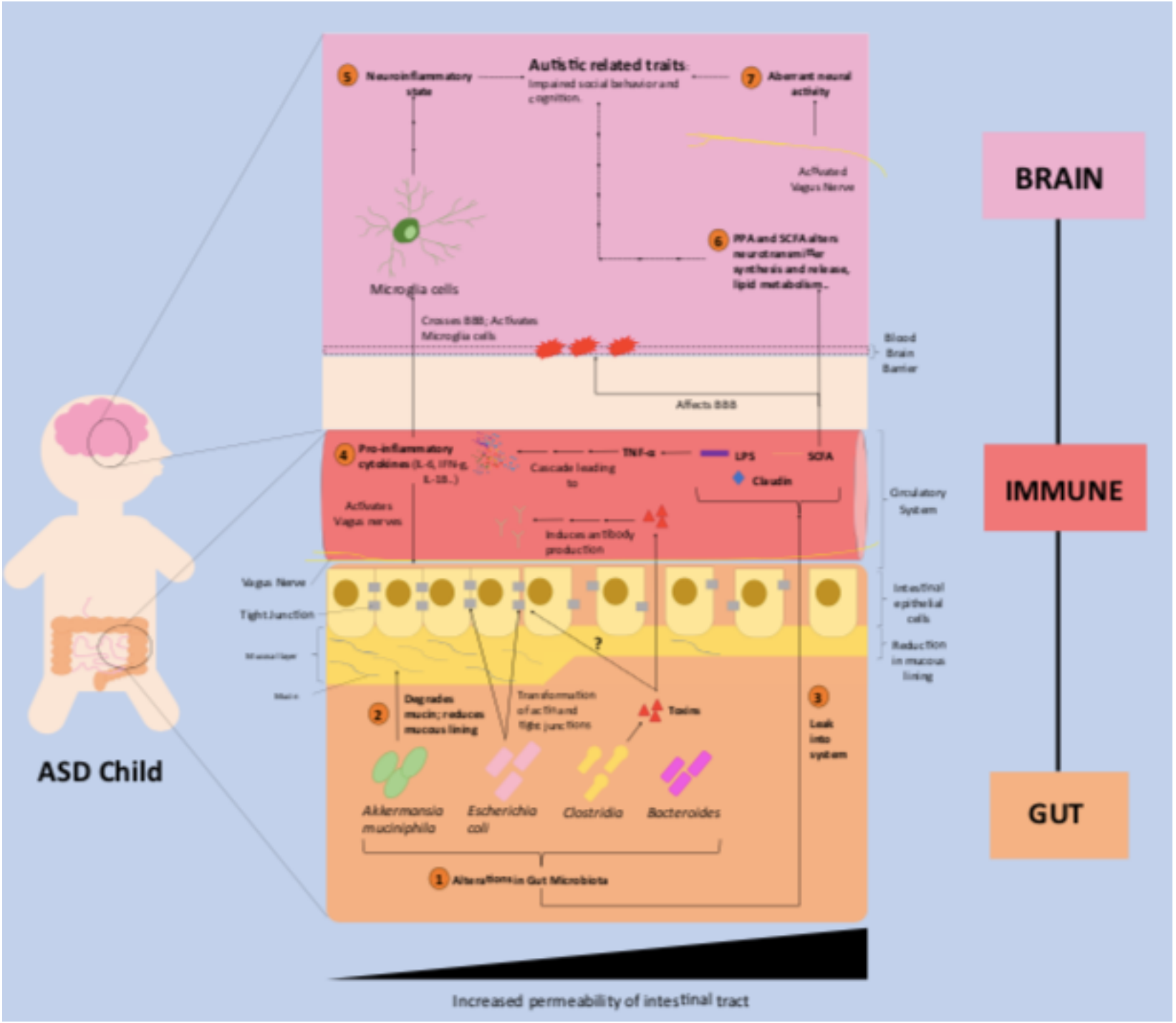
Figure 2. Schematic diagram consolidating the main mechanisms underlying Autism Spectrum Disorder (ASD) in the prenatal phase. (1) Maternal immune activation (MIA) generates maternally-derived cytokines that crosses the fetal blood brain barrier (BBB), leading to excessive production of reactive oxygen species (ROS) and pro-inflammatory cytokines from microglial cells, influencing neuronal survival and proliferation; (2) Dysfunction in microglial cells contribute to abnormal synaptic pruning; (3) Maternally-derived cytokines induces mast cells to secrete inflammatory and vasoactive substances, which further corrodes BBB integrity; (4)

Maternal autoantibodies crosses the BBB and targets highly expressed proteins in the fetal brain.

Figure 3. Schematic diagram consolidating Gut-Immune-Brain mechanisms underlying Autism Spectrum Disorder (ASD) in the postnatal phase. (1) Alteration in composition of gut microbiota; (2) Microbiota degrades mucin, further compromising the intestinal gut mucosa; (3) Compromised BBB allows bacterial metabolites (e.g. short chain fatty acids; SCFA), toxin, and bacterial components (e.g. lipopolysaccharides; LPS) to leak into the bloodstream; (4) Elevated levels of pro-inflammatory cytokines cross the BBB, which causes microglial dysregulation; (5) Dysfunction of microglial cells contribute to neuroinflammatory state in the child's brain; (6) Propionic acid (PPA) and SCFA compromises the BBB and influence neurotransmitter and metabolic functions; (7) Pro-inflammatory cytokines activate the vagus nerve, which leads to aberrant neural activity. (Dashed arrows indicate probable mechanisms that are involved which ought to be further investigated).


Identification
Screening
Included
Records identified through search on *Pubmed* database (*n* = 662)
Records identified through search on *PsycInfo* database (*n* = 269)
Full-text citations only, and duplicates removed (*n* = 380)
Records screened for title and abstract (*n* = 551)
Records removed after screening (*n* = 151)
Records included for qualitative analysis in this systematic review (*n* = 358)
Search Terms:
1. "Gut" AND "Brain" AND "Autism"
2. "Immune" AND "Brain" AND "Autism"
3. "Microbiome" AND "Autism"
4. "Gut" AND "Brain" AND "Immune" AND "Autism"
5. "Gut" AND "Brain" AND "Inflammation" AND "Autism"
Criteria for Screening of Records:
a) Records in English
b) Records that discuss the link between the gut-brain axis and immune system in ASD
c) Records that discuss the link between the immune system and inflammation processes in ASD


Immune dysregulation
Maternal Immune Activation
Generation of Auto-antibodies
Macrophages
Th1
Th2
Th17
T-cells
IL-6
IFN-g
IFN-a
TNF-a
IL-17
Elevated pro-inflammatory cytokines
Possible agents: Self-antigens, autoimmune history,..
Dendritic cells
presents antigens to
T cells
Auto-antibodies
Plasma B cells
B cells
Potentially damaging placenta: dysregulated vascular permeability
Triggers placenta resident cells to upregulate pro-inflammatory cytokines i.e. IL-6 and IL-17
Blood Brain Barrier
Central Nervous System
3 Secretes inflammatory and vasoactive mediators that increases BBB permeability
Maternally- and self-derived cytokines
Mast cells
Microglia cells
4 Auto-antibodies reactive towards key highly expressed proteins in developing brain
STIP1 Neuritogenesis
Cypin Branching of dendrites
LDH metabolism
YBX1 Transcription regulation
CRMP2 Axon growth
CRMP1 Collapse of growth cone
Axon
Growth Cone
Neuron
2 Abnormal synaptic pruning
Reactive Oxygen Species
Pro-inflammatory cytokine
1 Influences neuronal survival and proliferation of neurons
1 2 3 4 May cumulatively alter neurodevelopment which could account for autistic related behaviors
Maternal Immune System
Placenta
Fetus


Autistic related traits:
Impaired social behavior and cognition.
5 Neuroinflammatory state
7 Aberrant neural activity
Activated Vagus Nerve
6 PPA and SCFA alters neurotransmitter synthesis and release, lipid metabolism..
Microglia cells
Crosses BBB; Activates Microglia cells
Blood Brain Barrier
Affects BBB
4 Pro-inflammatory cytokines (IL-6, IFN-g, IL-1B..)
Cascade leading to
TNF-α
LPS
SCFA
Claudin
Activates Vagus nerves
Induces antibody production
Circulatory System
Vagus Nerve
Tight Junction
Mucosal layer
Mucin
Intestinal epithelial cells
Reduction in mucous lining
?
2 Degrades mucin; reduces mucous lining
Transformation of actin and tight junctions
Toxins
3 Leak into system
Akkermansia muciniphila
Escherichia coli
Clostridia
Bacteroides
1 Alterations in Gut Microbiota
ASD Child
BRAIN
IMMUNE
GUT
Increased permeability of intestinal tract