Immune Profile Associated with Newborns Later Diagnosed with a Neurodevelopmental Disorder

By

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Abstract

A combination of exposure to environmental and biological factors such as stress, age, genetics are well-known contributing elements of neurodevelopmental disorders (NDD) and autism spectrum disorder (ASD). It is only recently that immune molecules such as immune cells and cytokines/chemokines may take pivotal role in ASD progression. Increasing evidence suggest their critical function in mediating neurodevelopment and maintaining brain homeostasis. Aberrant levels of these immune molecules can alter the dynamics of neuro-immune interaction and their function, leading to impairments in neurodevelopment and progress to ASD diagnosis. The aim of this dissertation is to provide further evidence of immune dysregulation in children with ASD, particularly in peripheral cytokines/chemokines levels, and identify potential cytokine/chemokine profiles as predictors of neonatal ASD.

The first chapter of this thesis is an introduction and provides background information and the diagnostic methods for ASD. This chapter aims to identify immune molecules, particularly the cytokines/chemokines/growth factors, that are important factors to potentially separate children with ASD from those that are typically developing. The chapter further puts forward the concept that these factors have promise as a tool in augmenting ASD diagnostic methods.

The second chapter provides epidemiological evidence of abnormal neonatal cytokine/chemokine levels in children with ASD and developmental delay (DD) compared to those that are typically developing (TD), and further describes the relationship between aberrant cytokine/chemokine levels and their ASD associated behaviors. Some of the novel findings in this chapter includes the identification of two chemokines as early predictors of ASD and DD. These immune profiles in children with ASD and DD can help scrutinize the biological difference between ASD and DD development from TD controls.

The third chapter expands upon previously published study by Heuer et al. regarding whether there exists an interaction effect of sex and diagnosis (ASD and DD) in neonatal cytokine/chemokine levels. Unexpected novel findings suggest that 1) at birth, regardless of diagnosis, male and female children are born with different levels of peripheral cytokines/chemokines, and that 2) the inflammatory

chemokine, macrophage migration inhibitory factor (MIF), has an interaction effect in females and males depend on the diagnosis.

The fourth chapter describes how maternal SARS-CoV-2 (COVID-19) infection during pregnancy impacts the peripheral immune system and neurodevelopmental outcome in newborn offspring. Some of the novel findings include that 1) this is one of the first studies to determine the effect of COVID-19 infection during pregnancy in child immunity and neurodevelopmental outcomes, and that 2) newborn immune response to *in utero* exposure to COVID-19 is different by sex.

The final chapter focuses on a work-in-progress study of the impact of maternal immune and metabolic dysregulation in child neurodevelopmental outcome. Specifically, we are looking at maternal gestational inflammatory conditions and metabolic system during pregnancy adversely impacts child neurodevelopment. Using a longitudinal approach, we aim to characterize existing maternal immune/metabolic dysregulation throughout pregnancy and evaluate whether specific patterns associate with specific neurodevelopmental outcomes in the child.

Taken together, the studies included herein provide evidence of dysregulated peripheral immune system in early life of children later diagnosed with NDD and ASD, particularly in the immune signaling molecules, compared to those that are typically developing. These results hint at possible mechanisms regarding the differences in neuro-immune pathology of NDD or ASD compared to healthy neurodevelopment as well as mechanisms of severity for the spectrum of behaviors associated with NDD in the context of neuroimmune dysregulation. Future studies on functional mechanisms of select cytokine/chemokine in brain development will be important to develop new therapeutic interventions.

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Chapter 1:

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that causes developmental disabilities in communication, social interaction, and behaviors [1]. The incidence of ASD cases has tripled over the past two decades and currently, 1 in 36 children in the United States are diagnosed with ASD [2], adding to global estimate of 65/10,000 including both children and adults [3]. While some of the increase in the number of ASD cases can be attributed to increased public awareness and changes in diagnostic/screening criteria, considering this significant trend and a broad range of ASD symptoms/signs [4], it is of greater importance and urgency to find and improve precise screening/diagnostic tools for ASD. Currently, the traditional method to diagnose ASD depends only on behavior and developmental history of the child, and because of this, it is not until age of two or three years that a child can be reliably diagnosed with ASD. The current diagnostic tools naturally raise the concerns of subjectivity and sensitivity; thus, identifying additional methods that can accurately assess risk factors of ASD at earlier time points could advance the current behavioral methods and provide bases of preventive measures of developing ASD.

Multiple lines of research have sought to determine etiological and risk factors of ASD in order to discover alternative ASD diagnostic methods. Genetic factors are one of the predominant pathologies for ASD in which epidemiological studies indicate high heritability (60-90%) between siblings, twins, and even cousins [5-7]. Studies have shown the association between certain genes or mutations/variations related to functional and structural aspects in brain development in ASD [7-9]. Air pollutants, heavy metals, and other environmental toxicants are also thought to contribute to ASD [7]. Maternal and paternal factors such as their age, drug usage, health status including infection, diabetes, asthma, allergy, depression, anxiety and stress were all found to be associated with ASD [10]. While there is as yet no single unifying cause of ASD identified, combinations of numerous factors are thought to be associated with ASD. These advances will help the discovery of complementary methods for a behavior-based diagnosis [11, 12].

In the past few years, more research suggests that immune molecules have potential as strong biological predictors of ASD [13-15] and could someday serve as additional diagnostic markers. During the neurodevelopment, the processes of neurogenesis, neuronal migration, synaptogenesis, angiogenesis, and brain maturation heavily depend on molecules and receptors most commonly associated with the immune system [16]. For example, during early embryogenesis, major histocompatibility complex (MHC) class I molecules, toll-like receptors (TLRs), and complement proteins regulate and refine synaptic plasticity, neural growth and proliferation of neural progenitor cells (NPCs), promote neurogenesis, as well as inducing migration and survival of neurons, respectively [16-20]. Thus, maintaining homeostasis of neuro-immune crosstalk is necessary for healthy neurodevelopment and a significant disruption in this process would lead to impairments in neurodevelopment, including ASD [16, 21].

Cytokines and chemokines are also one of the essential immune molecules that are involved in cell survival, proliferation, differentiation, axonal growth and synaptogenesis in the developing brain [22] as a variety of cells within the central nervous system (CNS) produce these signaling molecules [23]. In fact, select cytokines/chemokines structurally and functionally resemble neurotrophins and other growth factors related to brain functions [24]. For example, interleukin (IL)-6, which can pass through the placenta [25], is a pro-inflammatory cytokine that functions as a neuropoietic factor in the brain in which it regulates self-renewing of neuronal precursors and cell survival [26]. IL-1β is another cytokine that crosses the blood-brain barrier (BBB) of the fetal brain and helps to promote proliferation of NPCs in certain regions of the brain [27, 28] as well as regulating the formation of excitatory synapses [29].

Due to the constant dynamics of CNS cytokines/chemokines levels in the absence of infection, a comparison between the level of neonatal cytokines/chemokines/growth factors in children with typically developing and those with neurodevelopmental disorders such as ASD can serve as an index of predicting abnormal brain development. Similarly, a comparison between the level of maternal cytokines/chemokines/growth factors in women whose children are typically developing and those with children diagnosed with ASD can provide information on what cytokines/chemokines/growth factors

required for healthy fetal brain development *in utero*. Indeed, increasing epidemiological evidence indicates altered levels of cytokines/chemokines in children with ASD compared to those without ASD, and predictably, similar patterns of results are seen when comparing mothers with children with ASD *versus* those with children that are typically developing. For example, elevated levels of peripheral IL-1β is associated with ASD, including newborns and children with age of 2 to 18 [30-32]. Maternal IL-1β, particularly during the first trimester of pregnancy, are associated with 2.3-fold increase in odds of ASD with intellectual disabilities [33]. Increased levels of IL-4 in children are associated with increased odds of ASD and ASD-related behaviors [30], and mothers of children with ASD have altered IL-4 levels in serum and amniotic fluid during mid-pregnancy compared to those with children that are typically developing [34-36]. Higher levels of peripheral cytokines IL-6 and IL-8 are also associated with an ASD diagnosis in children whether it is in newborns [37] or children at the age of 2-9 years compared to those that are typically developing [38-41]. Other chemokines such as macrophage chemoattractant protein 1 (MCP-1) in amniotic fluid [42], cerebellum, brain homogenates, cerebrospinal fluid [43], and neonatal bloodspots [44], macrophage inflammatory protein 1 (MIP-1) in plasma [39, 40, 45], and macrophage-derived chemokine (MDC) in sera [46] and anterior cingulate gyrus [43] are related to ASD where increased levels are observed in children with ASD compared to those that are typically developing.

These epidemiological research findings indicate possible pathological patterns of the type and level of cytokines/chemokines in children later diagnosed with ASD and their mothers compared to those that are neurotypical. Such patterns could potentially serve as an early predictor of ASD. Most importantly, in the context of development, the differences in the profiles of cytokines/chemokines in children with ASD could stem from prenatal and maternal risk factors and any confounding factors that attribute to risk of abnormal fetal development can cause these discrepancies in the immune profiles of children with neurodevelopmental disorders. Thus, it is essential to consider maternal health conditions particularly in identifying the strongest predictors of ASD, and the identified biological signatures could

potentially provide precise assessment for ASD risk at early time points, including severity, and could increase the efficacy of monitoring to therapeutic interventions.

The current dissertation aims to further provide epidemiological evidence in different study cohorts of altered immune profiles, particularly in the concentration and types of peripheral cytokines/chemokines in children with ASD or delayed development (DD) compared to those that are typically developing (TD). Further evidence of association between altered neonatal immune profiles and subsequent diagnosis of ASD, DD, or TD will be provided. The findings herein are focused to support and emphasize that the factors typically associated with the immune system indeed plays a critical role in fetal brain development, and that screening small immune molecules such as cytokines/chemokines/growth factors could help us better understand the neuroimmune-pathology of ASD development in early life.

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Chapter 2:

Neonatal Chemokine Markers Predict Subsequent Diagnosis of Autism Spectrum Disorder and Delayed Development

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Highlights

- Newborns later diagnosed with autism spectrum disorder (ASD) and delayed development (DD) have lower levels of cytokines and chemokines at birth compared to those with typical development (TD).
- Our exploratory analysis suggests that CTACK (CCL27) and MPIF-1 (CCL23) are the strongest predictors of ASD compared to TD and DD, respectively.
- Higher neonatal levels of CTACK were associated with a 60% decrease in the odds of ASD relative to TD while higher levels of MPIF-1 were associated with a 138% increase in the odds of ASD relative to DD.

Abstract

Immune dysregulation has been found to be related to a diagnosis of autism spectrum disorder (ASD). However, investigations in very early childhood examining immunological abnormalities such as altered neonatal cytokine/chemokine profiles in association with an aberrant developmental trajectory, are sparse. We assessed neonatal blood spots from 398 children, including 171 with ASD, which were subdivided according to severity (121 severe, 50 mild/moderate) and cognitive/adaptive levels (144 low-functioning, 27 typical to high-functioning). The remainder were 69 children with developmental delay (DD), and 158 with typical development (TD), who served as controls in the Childhood Autism Risks from Genetics and the Environment (CHARGE) study. Exploratory analysis suggested that, in comparisons with TD and DD, CTACK (CCL27) and MPIF-1 (CCL23), respectively, were independently associated with ASD. Higher neonatal levels of CTACK were associated with decreased odds of ASD compared to TD (odds ratio [OR]= 0.40, 95% confidence interval [Cl] 0.21, 0.77), whereas higher levels of MPIF-1 were associated with increased odds of ASD (OR= 2.38, 95% Cl 1.42, 3.98) compared to DD but not to TD. MPIF-1 was positively associated with better scores in several developmental domains. Dysregulation of chemokine levels in early life can impede normal immune and neurobehavioral development, which can lead to diagnosis of ASD or DD. This study collectively suggests that certain peripheral chemokines at

birth are associated with ASD progression during childhood and that children with ASD and DD have distinct neonatal chemokine profiles that can differentiate their diagnoses.

Keywords: Autism spectrum disorder, delayed development, neonatal blood spot, neonatal cytokines, neonatal chemokines, neurodevelopment

Introduction

Autism spectrum disorders (ASD) is composed of a group of complex neurodevelopmental disorders that are characterized clinically by deficits in communication, social interactions, and restricted or stereotyped behaviors [1]. The latest reported prevalence is as high as one out of every 54 children in the U.S. [2]. While ASD can sometimes be reliably diagnosed by the age of two, the ability to accurately diagnose ASD depends on behavioral assessments and developmental history [3, 4]. Biological signatures or biomarkers could provide a method for early ASD risk assessment, advancing the identification of at-risk children earlier than current behavioral methods [5]. Effective biomarkers could also facilitate classification of disease by severity and behavior, increasing the efficacy of monitoring response to therapeutic intervention. As reviewed by Hughes et al., numerous findings have supported links of the child's immune dysfunction with their ASD diagnosis, as well as with the immune profile of their mothers [6]. Cytokines and chemokines have the potential to serve as biomarker candidates, as alterations in their profile provide an overview of immune system status. Growing evidence demonstrates that unique cytokine/chemokine profiles in individuals with ASD can be associated with symptom severity, aberrant behaviors, and impaired cognitive/adaptive function [7-12]. However, the heterogeneity in individuals' demographics, cytokine/chemokine profiles, and prior study designs are hindering the determination of immune biomarkers as predictors of ASD. For example, some studies have demonstrated increased plasma levels of monocyte chemoattractant protein-1 (MCP-1) and RANTES in ASD patients [9, 12], whereas others observed decreased levels of RANTES in the newborn blood samples of children with ASD [11] or found comparable levels of MCP-1 in newborn bloodspots from control and ASD groups

[8]. In the neonatal blood spot study by Krakowiak et al., IL-1β was positively associated with mild to moderate ASD [7]; however, in the study by Masi et al., severe ASD was associated with decreased level of IL-1β in females only [10]. Possible explanations for these differences range from phenotypic heterogeneity, a polygenic etiology, a difference in time from birth to sample collection, and advances in technology. Although identifying a consistent postnatal biomarker for ASD remains challenging, it is promising that research on peripheral or neonatal cytokines and chemokines as predictors of ASD is growing.

Previous findings by our group demonstrated that neonatal IL-1β and IL-4 are independently associated with ASD, using newborn blood spots archived by Childhood Autism Risks from Genetics and the Environment (CHARGE) [7]. Children with severe ASD were more likely to have elevated IL-4 levels, whereas mild to moderate ASD symptoms were associated with increased levels of IL-1β. IL-4 was also a marker for the differentiation between children with severe ASD and those with mild to moderate ASD. Both cytokines were correlated with behavioral and developmental scores [7]. As an extension of this previous work, the current study utilized an expanded sample set of archived dried neonatal blood spot samples, as well as a broader array of cytokines and chemokines to investigate cytokine/chemokine levels from children later diagnosed with ASD or with developmental delay (DD) without autism, compared to children with typical development (TD). We further subdivided ASD individuals into groups based on symptom severity and developmental and adaptive functions to identify predictors of ASD.

Methods and materials

Participants

Archived neonatal blood spot samples corresponding to 398 children who enrolled in the Childhood Autism Risks from Genetics and the Environment (CHARGE) study [13] between April 2003 and May 2009 with confirmed diagnoses (171 with ASD, 69 with DD, and 158 with TD) were used for

cytokine/chemokine analysis. The CHARGE study is a population-based case-control study investigating risk factors for neurodevelopmental disorders, with participants from three groups: children with ASD, DD, and general population controls with TD. Eligible children were 2-5 years old, born in California, lived with a biological parent who spoke English or Spanish, and resided in selected regional center catchment areas at the time of recruitment. Consent was acquired from parents prior to participation. The CHARGE Study protocol was approved by the institutional review boards (IRB) at the University of California, Davis and Los Angeles, as well as the State of California Committee for the Protection of Human Subjects.

Diagnostic Confirmation

Diagnostic confirmation of the children (2-5 years of age) was performed at the University of California, Davis, MIND (Medical Investigation of Neurodevelopmental Disorders) Institute. Cognitive and adaptive functions were evaluated with Mullen Scales of Early Learning (MSEL) and Vineland Adaptive Behavior Scales (VABS), respectively. The Autism Diagnostic Interview-Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS) confirmed a diagnosis of ASD. The Social Communication Questionnaire was used to screen controls for ASD; children with scores ≥15 were evaluated with ADI-R and ADOS. Children with ASD (n=171) were subdivided according to 2-subgroups by severity and cognitive/adaptive functions. Using ADOS comparison scores and DSM-IV criteria, children showing a score of ≥7 were grouped into severe ASD symptoms (ASD_{sev} [n=121]) and children exhibiting a score of <7 were categorized as mild/moderate symptoms (ASD_{mild} [n=50]). Controls with TD had no prior diagnosis of ASD or DD, and their composite scores on MSEL and VABS were ≥70. Controls with DD had composite scores <70 on MSEL and/or VABS. Children with ASD who were within the typical cognitive and adaptive developmental range (both MSEL and VABS composite standard scores of ≥70) were grouped as typical to high-functioning ASD (ASD_{hi} [n=27]). Those with cognitive and/or adaptive delays were grouped as low-functioning ASD (ASD_{lo} [n=144]). Children who met the criteria for ASD were reclassified to the ASD group.

Behavioral and Developmental Assessments

Aberrant Behavior Checklist

Maladaptive behavior assessment was performed using the Aberrant Behavior Checklist (ABC) whose subscales included irritability (15 items), lethargy/social withdrawal (16 items), stereotypy (7 items), and hyperactivity (16 items). Each item was rated on a 4-point Likert scale ranging from 0 (not at all a problem) to 3 (problem severe in degree).

Mullen Scales of Early Learning

MSEL is a standardized assessment used to determine cognitive development in young children. The scales include visual reception (nonverbal cognitive ability), fine motor, receptive language (language comprehension), and expressive language (language production). For each scale and composite, developmental quotients were calculated (by age equivalent/chronological age x 100) to overcome the floor effect.

Vineland Adaptive Behavior Scales

VABS is a standardized assessment to determine the level of personal and social skills needed for everyday living. The domains are communication, daily living skills, socialization, and motor skills. Developmental quotients were calculated as described above.

Blood Spot Specimen collection

Capillary blood was collected within 48 h of birth by heel stick method and spotted onto standardized filter paper for testing of various disorders as part of the Genetic Disease Screening Program. The remaining blood spots were stored at -20^{o}C by the California Department of Public Health (CDPH).

Blood Spot Elution

Three 3 mm punches of dried blood spot specimen were put into a single well in a 96-well plate and stored at -80ºC until elution. In each well, 200 µl of elution buffer (phosphate-buffered saline, 0.5% bovine serum albumin, and protease inhibitors [Complete Protease Inhibitor Cocktail, Roche Diagnostics Corporation, Indianapolis, Indiana]) was added. Plates were placed on a plate shaker overnight at 4ºC and the eluates analyzed immediately following elution.

Total protein concentration

Following elution, a small 4µl aliquot was used for bicinchoninic acid assay (BCA) (Thermo Scientific, Rockford, IL) to determine total protein and to normalize cytokine/chemokine levels against blood sample quantity variation [8].

Cytokine and Chemokine Measurement

Blood spot cytokine and chemokine levels were measured using Bio-Plex Luminex (Bio-Rad, Hercules, CA) assays. Using a 40-plex chemokine panel, cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-16, TNF-α and chemokines IL-8, 6Ckine (CCL21), BCA-1 (CXCL13), CTACK (CCL27), ENA78 (CXCL5), eotaxin (CCL11), eotaxin-2 (CCL24), eotaxin-3 (CCL26), fractalkine (CX3CL2), GCP-2 (CXCL6), GM-CSF, Gro-α (CXCL1), Gro-β (CXCL2), I-309 (CCL1), IP-10 (CXCL10), I-TAC (CXCL11), MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), MDC (CCL22), MIF, MIG (CXCL9), MIP-1α (CCL3), MIP-1δ (CCL15), MIP-3α (CCL20), MIP-3β (CCL19), MPIF-1 (CCL23), SCYB16 (CXCL16), SDF-1α/β (CXCL12), TARC (CCL17), and TECK (CCL25) were measured according to the manufacturer's directions. IL-12p70 and IL-13 (Bio-Rad) were also included as individual analytes to expand the 40-plex panel. Briefly, the blood spot eluate (50 µl) was incubated with fluorescent-labeled capture antibody-coated beads in a 96-well plate (shaking) for 1 h at RT. The sample-bead mix was removed, washed, and biotinylated detection antibodies added for 30 min at RT with shaking. The reaction mixture was then incubated with streptavidin-phycoerythrin at RT for 10 min (shaking). After

washing, the beads were resuspended in sheath fluid on the plate shaker for 5 min. The plates were read on a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using Bio-Plex Manager software (Bio-Rad Laboratories). A five-parameter curve was used to calculate final concentrations (pg/ml). Reference samples were run on each plate for assay consistency. All samples were run blinded to child developmental outcome.

Statistical Analysis

Statistical analyses were performed on cytokine/chemokine levels in blood spot eluates from 398 children. Each immune marker was normalized for sampling variation in blood collection by dividing total protein content in the eluate (as determined by BCA assay). Cytokine/chemokine concentrations that fell below the minimum level of detection (MLD) were assigned a value of MLD/$\sqrt{2}$, and all cytokines/chemokines were natural log-transformed to normalize the distribution. GM-CSF, IL-12p70 and IL-13 levels were not detectable for >25% of the samples and were excluded from further analysis. All remaining cytokines/chemokines were above the MLD for ≥97% of the samples (Supplementary Table 1). Analyses were carried out using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina).

Primary analyses examined the associations between individual cytokine/chemokine levels and child diagnosis using multinomial logistic regression. Mild and severe ASD cases were also examined separately in relation to DD and TD groups. Covariates in initial model fitting included mother's education attainment, gestational age, child's sex, age (hours) at blood spot collection, and years between blood spot collection and elution. Akaike information criterion (AIC) was used to select best model fit [14].

The cytokines/chemokines was also modeled together as predictors and adjusted for covariates in a series of binary models (outcomes were ASD vs. TD, ASD vs. DD, DD vs. TD, ASD_{sev} vs. ASD_{mild} , ASD_{sev} vs. TD, ASD_{mild} vs. TD, ASD_{sev} vs. DD, ASD_{mild} vs. DD, ASD_{hi} vs. ASD_{lo}, ASD_{hi} vs. TD, ASD_{lo} vs. TD, ASD_{hi} vs. DD and ASD_{lo} vs. DD) using the Least Absolute Shrinkage and Selection Operator (LASSO) variable selection method [15] to identify a parsimonious subset of cytokines and chemokines

that was most strongly associated with the child's diagnosis. For example, if several cytokines were highly correlated with one another and associated with a particular diagnosis, LASSO selected one of those cytokines for inclusion in a model. Adjustments for multiple comparisons were not performed for these exploratory analyses and to preclude failing to detect immune markers that show promise in their ability to differentiate between diagnostic groups.

Secondary analyses were stratified by child's diagnosis and examined associations between the LASSO-selected cytokines/chemokines and developmental/behavioral domains measured by ABC, MSEL and VABS.

Results

Participant demographics

TD controls were frequency-matched to cases with ASD in 4:1 male-to-female ratio to ensure similar proportions of male/female participants in both groups (Table 1). This was not the case for the DD group where 70% were male, with 30% were female. In terms of birth season, fewer children with ASD and DD were born in the spring compared to children with TD (18% and 17% vs. 29%). Birth year was statistically different between populations, with ASD participants weighted toward the later years in the study (2003-2006), while DD and TD participants were distributed more toward the middle of the study, peaking in 2003. The average gestational age for all three groups was similar (TD 39.3 weeks, ASD 39.6 weeks, DD 39.1 weeks). However, newborn age at the time of blood spot collection was statistically different among the three groups. The maternal education status of those in the DD group demonstrated fewer mothers with a bachelor's degree compared to mothers of children with TD and those in the ASD group (35% vs. 56% and 50%). There were no differences in regional catchment areas among the three groups as the TD controls were frequency-matched to a projected distribution of ASD cases for the regional center catchment area. In addition, no differences were found between cases with ASD and controls with TD or DD in terms of race/ethnicity, years between blood spot collection and elution,

maternal age at delivery, or maternal allergies and asthma. The cases and controls were evenly distributed across the assay plates such that even representation of each study population was run on each plate.

Associations between neonatal cytokine and chemokine levels and child diagnosis

Comparison in total groups: ASD vs. TD, DD vs. TD and ASD vs. DD

We conducted multinomial logistic regression models adjusted for maternal education attainment, gestational age, child's age at blood spot collection, and years from blood spot collection to elution to determine the associations between individual cytokines/chemokines and ASD, regardless of symptom severity, compared to DD and TD. Overall, children with ASD and DD had significantly lower neonatal concentrations of nearly all cytokines and chemokines, and none were significantly higher, compared with concentrations in TD children (Fig. 1A, Table 2A). No sex differences were observed in cytokine/chemokine levels.

MDC (CCL22) and MPIF-1 (CCL23) emerged as the only chemokines whose concentrations at birth differed significantly between ASD and DD. Higher levels of MDC and MPIF-1 were each associated with a two-fold increased likelihood of having an ASD vs. DD diagnosis (MDC: OR=1.96, 95% Cl 1.09, 3.53; MPIF-1: OR=1.97, 95% Cl 1.26, 3.09) (Fig. 1A, Table 2A). MDC and MPIF-1 concentrations were also significantly higher in TD than DD but did not differentiate between ASD and TD.

Neonatal levels of Gro-β (CXCL2), IL-4 and MCP-4 (CCL13), were significantly decreased in ASD compared to TD but did not differ between DD and TD (Table 2A). Meanwhile, GCP-2 (CXCL6) was significantly decreased in DD compared to TD but not differ between ASD and TD (Table 2A). In addition, significant decreases in the levels of chemokines MIP-1δ (OR=0.54, 95% Cl 0.31, 0.96), MIP-3α (OR=0.39, 95% Cl 0.16, 0.94) and MIP-3β (OR=0.61, 95% Cl 0.41, 0.91) were noted only in DD compared to TD, while level of MIP-1α was significantly lower in both ASD vs TD (OR=0.36, 95% Cl 0.15, 0.88) and DD vs TD (OR=0.34, 95% Cl 0.12, 0.99) (Table 2A).

Comparison by symptom severity: ASD_{sev} vs. ASD_{mild}, ASD_{sev}/ASD_{mild} vs. TD and ASD_{sev}/ASD_{mild} vs. DD

We subdivided ASD cases into severe and mild/moderate groups based on symptom severity (ASD_{sev} and ASD_{mild}) to examine differences in ASD subgroups and to compare the subgroups with TD and DD groups in models adjusted for the same covariates as described earlier. None of the neonatal cytokines and chemokines differed significantly between ASD_{sev} and ASD_{mild} (Fig. 1B, Supplementary Table 2). Therefore, we observed similar trends in association between individual cytokine/chemokine levels and diagnosis for comparisons between subgroups ASD_{sev} and ASD_{mild} with TD and DD. Overall, regardless of the symptom severity, both ASD subgroups had lower level of cytokines and chemokines compared to TD (Table 2B-1). However, some cytokines/chemokines, namely, 6Ckine (CCL21), eotaxin-3 (CCL26), Gro-β (CXCL2), I-TAC (CXCL11), MCP-2 (CCL8; OR=0.47, 95% Cl 0.26), MCP-4 (CCL13), TARC (CCL17) and TNFα were significantly decreased in ASD_{sev} compared to TD but did not differ when comparing ASD_{mild} to TD (Table 2B-1). Meanwhile, IFN-γ, IL-6 and MIP-1α (CCL3) levels were significantly decreased in ASD_{mild} compared to TD but did not differ between ASD_{sev} and TD (Table 2B-1).

In contrast, the levels of most cytokines and chemokines did not differ significantly when we compared the two subgroups of ASD with DD except for MDC (CCL22) and MPIF-1 (CCL23) (Table 2B-2). Higher levels of MPIF-1 were associated with a 1.9-fold higher likelihood of ASD_{sev} (OR=1.87, 95% CI 1.16, 3.00) and a 2.3-fold higher likelihood of ASD_{mild} relative to DD (OR=2.26, 95% CI 1.23, 4.16) (Table 2B-2). MDC was the only chemokine that differentiated between ASD_{sev} and ASD_{mild} compared to DD; higher neonatal levels of MDC were associated with a 2.3-fold higher likelihood of ASD_{mild} relative to DD (OR=2.30, 95% CI 1.03, 5.16) but did not differ significantly between ASD_{sev} and DD (Table 2B-2).

Comparison by cognitive and adaptive development: ASD_{hi} vs. ASD_{lo}, ASD_{hi}/ASD_{lo} vs. TD and ASD_{hi}/ASD_{lo} vs. DD

Next, we subdivided the ASD group into typically-to-high- (ASD_{hi}) and low-functioning (ASD_{lo}) subgroups according to the children's cognitive and adaptive development level based on the MSEL and VABS scores to examine associations between cytokine/chemokine levels and cognitive/adaptive function in children with ASD. Most neonatal cytokines and chemokines did not differ significantly between ASD_{hi} and ASD_{lo} except for MCP-1 (CCL2) (Fig. 1C, Table 2C-1). Higher levels of MCP-1 were associated with a 3.2-fold higher likelihood of ASD_{hi} relative to ASD_{lo} (OR=3.18, 95% CI 1.23, 8.26) (Table 2C-1).

For comparisons of the ASD subgroups with TD, we observed significantly lower levels of cytokines/chemokines 6Ckine (CCL21), CTACK (CCL27), eotaxin (CCL11), I-309 (CCL1), MIF, SDF-1α/β (CXCL12), TECK (CCL25), TNFα, in both ASD_{hi} and ASD_{lo} relative to TD (Table 2C-1). Meanwhile, the levels of cytokines/chemokines BCA-1 (CXCL13), eotaxin-3 (CCL26), IL-2, IL-6, IP-10 (CXCL10), I-TAC (CXCL11), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), MIG (CXCL9), MIP-1α (CCL3), and TARC (CCL17) were significantly lower in ASD_{lo}, but not ASD_{hi}, relative to TD (Table 2C-1). The levels of two chemokines, GCP-2 (CXCL6) and Gro-β (CXCL2), were only significantly lower in ASD_{hi} relative to TD (Table 2C-1).

Comparisons of ASD_{hi} and ASD_{lo} to the DD group revealed significantly higher neonatal levels of MDC (CCL22) and MPIF-1 (CCL23) in one or both ASD subgroups relative to DD that were not observed relative to TD (Fig. 1C, Table 2C-2). Higher levels of MPIF-1 were associated with 2.6-fold higher odds of ASD_{hi} (OR= 2.63, 95% Cl 1.19, 5.79) and 1.9-fold higher odds of ASD_{lo} (OR= 1.88, 95% Cl 1.19, 2.97) relative to DD (Table 2C-2). Higher levels of MDC were associated with a 2.1-fold higher likelihood of ASD_{lo} (OR= 2.12, 95% Cl 1.14, 3.92), but MDC levels did not differ significantly between ASD_{hi} and DD (Table 2C-2). Higher concentrations of MCP-1 were associated with an a 3.4-fold higher likelihood of ASD_{hi} relative to DD (OR= 3.42, 95% Cl 1.25, 9.39), not observed between ASD_{lo} and DD (Table 2C-2). MCP-1 concentrations differed significantly between ASD_{hi} and ASD_{lo} as described earlier.

Interestingly, we did not observe significant differences in MCP-1 levels for either ASD subgroup relative to TD (Table 2C-1).

Identifying the strongest subset of predictors associated with ASD and DD: LASSO variable selection

After observing the significant differences in numerous cytokine/chemokine concentrations among children with ASD, DD, and TD, we conducted an exploratory analysis using LASSO to identify the cytokines and chemokines that were most strongly associated with child diagnosis, taking the entire cytokine/chemokine profile into account. Chemokines CTACK (CCL27), MIF and MPIF-1 (CCL23) emerged as the strongest predictors of child diagnosis after conducting a series of binary models with outcomes: ASD vs. TD, ASD vs. DD, and DD vs. TD. No predictive immune markers were identified when comparing models of the following binary outcomes: ASD_{sev} vs. ASD_{mild} and ASD_{hi} vs. ASD_{lo}. Models that compared these ASD subgroups with DD and TD produced the same marker selection results as the models that included ASD as a combined group. In addition, no sex differences were observed in peripheral CTACK, MIF and MPIF-1 levels.

We conducted multinomial logistic regression with chemokines CTACK (CCL27), MIF and MPIF-1 (CCL23) as predictors and adjusted for maternal education attainment, gestational age, child's age at blood spot collection, and years from blood spot collection to elution to determine associations with child diagnosis. Higher neonatal levels of CTACK were independently associated with a 60% decrease in the odds of ASD relative to TD (OR 0.40; 95% Cl 0.21, 0.77) (Table 3A), indicating that lower CTACK levels at birth were associated with ASD. CTACK levels did not differentiate between ASD and DD or between DD and TD. Decreased newborn levels of CTACK were particularly associated with ASD_{sev}, ASD_{hi} and ASD_{lo} compared with TD (Table 3B-C). In contrast, higher neonatal levels of MPIF-1 were associated with a 138% increase in the odds of ASD relative to DD (OR 2.38; 95% Cl 1.42, 3.98) and a 60% decrease in the odds of DD relative to TD (OR=0.40, 95% CI 0.24, 0.68) (Table 3A). However, MPIF-1 levels did not differ significantly between ASD and TD. We also observed similar

significant associations between MPIF-1 levels and all ASD subgroups (ASD_{sev}, ASD_{mild}, ASD_{hi} and ASD_{lo}) when compared to DD (Table 3B-C). MIF no longer differed significantly between ASD and TD or between DD and TD, after adjusting for CTACK and MPIF-1 levels. Therefore, from the panel of 39 cytokines/chemokines examined, CTACK emerged as the strongest predictor of ASD relative to TD as well as MPIF-1 for DD relative to TD. No new associations emerged when ASD was divided into subsets based on symptom severity or cognitive/adaptive development level.

Associations between CTACK and MPIF-1 levels and development among children with ASD

To assess whether neonatal CTACK and MPIF-1 concentrations were independently associated with behavioral or developmental patterns evaluated at age 2-5 years, the MSEL and VABS scores for each domain were individually modeled using linear regression adjusted for maternal education level. Increased MPIF-1 levels were associated with better scores on nearly all developmental domains examined (MSEL: fine motor, receptive language, expressive language, composite; VABS: communication, daily living skills, socialization, motor skills, and composite) among children with ASD, indicating less severe behaviors and impairments (Table 4). Meanwhile, CTACK was not significantly associated with any domains.

We also examined associations between neonatal CTACK and MPIF-1 levels and cognitive and adaptive development levels within the TD and DD groups; however, neither set of models revealed any significant associations between MPIF-1 or CTACK levels and the developmental domains (data not shown). ABC scales (irritability, lethargy, stereotypy, hyperactivity) were not associated with these chemokines within any diagnostic groups (data not shown).

Discussion

This study aimed to expand upon previously published work [7] using a larger sample size, expanding the study population to include children with developmental delay without ASD, and an increased repertoire of analytes (42 vs.17) to better assess neonatal blood spots for additional immune predictors of risk for

ASD and/or developmental delay. This study further examined a possible connection between potential early markers for cognitive and adaptive development levels of children diagnosed with ASD. Our findings suggest that children diagnosed with ASD or DD have lower overall neonatal cytokine/chemokines levels compared to those with TD, and the cytokine/chemokine profiles of children with ASD differ from those with DD. In addition, our exploratory analysis with immune markers identified by the LASSO variable selection method demonstrates that children with ASD were more likely to have decreased neonatal levels of CTACK relative to children with TD and higher levels of MPIF-1 relative to children with DD.

The previous study by Krakowiak et al. reported on IL-1β and IL-4 as early markers of ASD, where children with ASD had elevated levels of these cytokines depending upon their symptom intensity. Elevated IL-4 was associated with increased odds of severe ASD whereas IL-1β was associated with increased odds of mild/moderate ASD [7]. In the current study, although these two analytes were included in the study, neither IL-1β nor IL-4 were associated with an ASD diagnosis. Rather, lower levels of these cytokines were associated with children with DD compared to those with TD. Inconsistencies in the findings from this study and the previous one may be attributed to several notable methodological differences related to the measurement of immune markers, study population size and composition, and analytic approach. The two studies used cytokine/chemokine multiplex kits from different vendors, and the current panel had an expanded repertoire of 25 more analytes (compared to 17 analytes total in the earlier study) that included CTACK and MPIF-1, which were not previously measured. In addition, the immune markers in the current study had better detection rates compared to our prior study. For example, the current study had nearly all cytokines and chemokines detectable for ≥97% of the samples and only three analytes (GM-CSF, IL-12p70, IL-13) were below minimum detection levels, while previous study had one-third of the cytokines/chemokines (6 of 17) undetectable for >25% of the samples, with below-detection values imputed by multiple imputation methods. The current study was also larger with significantly more samples for the control groups, thus, providing more statistical power to detect differences among diagnostic groups and subgroups. Further, participant characteristics differed with

regard to the geographic distribution (regional center catchment areas), racial/ethnic composition and other sociodemographic characteristics within diagnostic groups between the two study cohorts' area was different. Additionally, the elapsed time before neonatal blood spot collection for the DD group was greater compared to that between the ASD and TD groups. Finally, the LASSO variable selection method was novel to the current study as we wished to expand our analytic approach. Thus, several notable differences in the quality and quantity of variables such as number of immune markers, study population size and demographic characteristics, and data analysis methods between the two related studies may have collectively contributed to contrasting results.

As previous studies have not examined CTACK levels in newborn blood samples [7, 8, 16, 17], the association between lower CTACK levels at birth and a higher likelihood of severe, low- and typical-to-high-functioning ASD relative to TD is a novel finding in this study. CTACK, an isoform of CCL27, is best known for its role in skin inflammation and lymphocyte trafficking, particularly the cutaneous lymphocyte-associated (CLA^+) memory T cells [18]. Numerous findings depict CTACK function in delayed-type hypersensitivity reactions and atopic dermatitis in both human and animal models [19, 20]. Recent studies have suggested that the function of CTACK is not restricted to the skin, but rather this chemokine may play a pivotal role in homeostasis and immune surveillance in the brain. Serum CCL27 levels have been shown to be increased in individuals diagnosed with multiple sclerosis (MS) [21], while a follow-up study by Blatt et al. suggested that infiltrating T cells into the central nervous system (CNS) in MS patients may be of cutaneous origin [22].

While these studies infer the possible role of T cells in the brain regarding CTACK production, secretion of CTACK may be independent of T cells but dependent on glial and non-neuronal cells that are present in the brain beginning in the early neurodevelopmental stages. For example, human astrocytes and neurons are capable of expressing CCL27 [23] and its receptor, CCR10 [24], suggesting both autocrine and paracrine effects of the chemokine within the CNS. In addition, expressions of CCL27 and CCR10 is abundant in the dentate gyrus (DG) of the hippocampus [25-27], cerebral cortex, and other limbic structures [25] in the adult brain, indicating a critical role of CTACK in brain function throughout the

lifespan. Immunologically, it is still possible that expression of CCL27 in the brain can trigger or modulate chemotaxis of memory T cells to these brain regions as the number of infiltrated T cells is at its peak during embryonic day 16 in the developing mouse brain [28]. The holistic view of T cell involvement in early neurodevelopment is still unclear; however, increasing evidence using rodent models depicts the importance of T cell infiltration in maintaining and/or modulating CNS development [29-33]. This emphasizes our observation that CTACK is indeed important in proper neurodevelopment, and deficient levels of CTACK could impede healthy neurodevelopment. Interestingly, neonatal levels of CTACK did not differ between children diagnosed with DD compared to those with TD, suggesting that the neurodevelopmental outcomes of DD and ASD are immunologically distinct. These findings provide support for the potential importance of sufficient levels CTACK in early life for healthy neurodevelopment, and thus the molecular basis of neuroimmune and neurobehavioral mechanisms involving cells that produce CTACK and express its cognate receptor in the CNS should be further examined.

The developmental characteristics of ASD and DD differ from each other in that each of the groups displays specific patterns of impairment in communication, cognition, and behaviors based on standard diagnostic tests. However, ASD and DD both lie under the spectrum of neurodevelopmental disorders, and a diagnosis of DD or ASD can change over time. As the behavioral intervention program is designed to address deficits specific to ASD, and because it is critical to identify these children as early as possible [34, 35], it is important to find biomarkers with the potential to differentiate between ASD and DD cases. Here, we found that neonatal cytokine and chemokine profiles of children with ASD differ from those with DD, particularly that chemokines MDC and MPIF-1 were significantly lower in children with DD than those with ASD and TD. We did not see these differences when comparting ASD to TD subjects. This finding suggests that a significant reduction in these two chemokines in the early neonatal period. Specifically, our exploratory analysis determined that MPIF-1 was the strongest candidate to differentiate between the ASD and DD diagnostic groups, including the subgroups of ASD. Higher neonatal concentrations of peripheral MPIF-1 were associated with more than a two-fold higher

likelihood of ASD compared to DD. This is of interest as a lower level of this chemokine at birth could indicate a potential role for MPIF-1 in development of executive and cognitive function, deficits in which are hallmark features of developmental delay. Of interest for future studies would be to design a study to compare neonatal samples from ASD with intellectual deficits to DD without ASD in the context of these differentiating chemokines.

With respect to function, immunologically, MPIF-1 (as indicated by its name myeloid progenitor inhibiting factor 1), inhibits colony formation of bone marrow myeloid immature progenitors and their activity. MPIF-1, also known as CCL23 and MIP-3, can modulate the immune response by promoting and directing the migration of mature immune cells such as activated T lymphocytes, macrophages, and granulocytes to local sites of injury [36-38], while simultaneously reducing the number of cells in the hematopoietic progenitor pool and inducing production of granulocytes and monocytes [39]. This may suggest a supportive role for MPIF-1 during development. In addition, the interaction of MPIF-1 with its chemokine receptor, CCR1, can stimulate pro-inflammatory cytokine production, including IL-1β, TNFα, and MIP-1α [38], although in the current study, the levels of these were not elevated in newborns later diagnosed with ASD or DD, but rather were lower or did not differ from children with TD. Interestingly, in the CNS, the CCL23-CCR1 interaction can induce angiogenesis by promoting the migration of endothelial cells through upregulation of matrix metalloproteinases in the endothelium [38, 40]. Blood vessel formation is critical in development and neuroplasticity, and either hypo- or hyper-angiogenesis can disrupt proper blood flow to the brain, which could ultimately affect neurodevelopmental outcome.

Recently, Azmitia et al. reported persistent angiogenesis in postmortem cortex, brainstem, and cerebellum of children and young adults with ASD and proposed that the heightened neuronal activity noted in some individuals with ASD was an outcome of sustained splitting angiogenesis [41]. Mulligan and Trauner found that more than half of the ASD patients in their study exhibited abnormal epileptiform electroencephalogram (EEG) activity [42], which has been considered as a method of early ASD diagnosis [43, 44]. Increased neuronal connectivity is closely related to pruning and myelination of axons, and heightened EEG activity could mean initial overgrowth and early maturation of brain white matter in

ASD, possibly resulting in altered behavior [45]. In fact, children with ASD exhibit overconnectivity rather than underconnectivity as is shown by elevation of fractional anisotropy at age of six months, followed by a reduction below that of age-matched controls at 24 months [46]. Our observation of positive associations between MPIF-1 and MSEL cognitive and VABS adaptive scores in children with ASD may potentially support the modulation in angiogenesis thereby affecting neuroplasticity and neurodevelopment. The contribution of MPIF-1 in angiogenesis and neuronal activity in the ASD participants in the current study is still unknown; however, the fact that all subgroups of ASD (ASD_{sev}, ASD_{mild}, ASD_{hi}, and ASD_{lo}) had significantly higher levels of MPIF-1 than DD demonstrates the importance of MPIF-1 homeostasis during brain development and the potential of MPIF-1 as a checkpoint for ASD versus DD. In addition, numerous ligands for CCR1 other than MPIF-1 (i.e., MIP-1α [CCL3], RANTES [CCL5], MCP-3 [CCL7], MCP-4 [CCL13], MIP-1δ [CCL15]) [47] and the global expression of CCR1 (immune/neuronal cells, tissues) [39, 48, 49] should be taken into account when trying to better understand the mechanistic and functional role of MPIF-1 in ASD.

Conclusions

Our data collectively suggest that chemokine levels measured in children shortly after birth can serve as early predictors of abnormal immune and neuroimmune development associated with ASD and DD. Lower peripheral levels of select cytokines and chemokines in both ASD and DD groups compared to TD suggest the importance of homeostatic cytokine/chemokine levels in normal neurodevelopment. The differences in neonatal chemokine and cytokine profiles provide support for addressing the mechanisms between the immune and neuronal systems during gestation. Questions regarding the function of CTACK and MPIF-1 in brain development and their potential role in neuroplasticity should be further investigated, perhaps using a rodent model to elaborate on the effect of these select chemokines on neurodevelopment. Furthermore, continued investigation of very early immune molecule predictors of ASD and DD risk as well as understanding their functional role in neurodevelopment will be necessary to elucidate mechanistic pathways of immune dysregulation in these neurodevelopmental disorders.

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Declaration of interests

Dr. Van de Water has a patent application involving the maternal autoantibody-related (MAR) ASD peptides described herein and has a UC Davis based startup company focusing on the development of the MAR-ASD autoantibody profile as a risk assessment for a child developing ASD. All other authors have no conflicts of interest to declare.

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Table 1. Participant demographic and clinical characteristics, N=398

	ASD (n=171)		***DD (n=69)***		***TD (n=158)***		
	n	***%***	***n***	***%***	***n***	***%***	***P-value[g]***
Sex[a]							0.03
Male	144	84	48	70	128	81	
Female	27	16	21	30	30	19	
Race/Ethnicity							0.22
White	90	53	29	42	81	51	
Hispanic	53	31	28	41	42	27	
Other[b]	28	16	12	17	35	22	
Season of birth[c]							0.33
Winter	45	26	19	28	37	23	
Spring	30	18	12	17	45	29	
Summer	47	27	18	26	35	22	
Fall	49	29	20	29	41	26	
Birth year							0.02
2000-2001	18	10	2	3	9	6	
2002	24	14	17	25	36	23	
2003	44	26	22	32	54	34	
2004	49	29	20	29	43	27	
2005-2006	36	21	8	11	16	10	
Maternal education							0.004
High school or less	25	14	22	32	22	14	
Some college/Vocational degree	61	36	23	33	48	30	
Bachelor's degree	85	50	24	35	88	56	
Maternal allergies or asthma[d]	103	61	39	57	92	58	0.82
Regional Center catchment area[a]							0.45
Alta, Far Northern, and Redwood Coast	71	42	31	45	70	44	
North Bay	21	12	6	9	19	12	
East Bay, San Andreas, and Golden Gate	44	26	13	19	44	28	
Valley Mountain, Central Valley, and selected Southern CA regions[f]	35	20	19	27	25	16	
	Mean	***SD***	***Mean***	***SD***	***Mean***	***SD***	***P-value[g]***
Maternal age at delivery	31.3	5.3	30.9	6.4	31.0	5.4	0.81
Gestational age (weeks)[e]	39.6	2.1	39.1	2.0	39.3	1.6	0.11
Age (hours) at blood spot collection	29.1	8.8	33.1	8.4	28.7	8.9	0.001
Age (months) at study enrollment for child[a]	44.0	9.5	46.5	8.7	42.8	10.0	0.03
Years between collection and elution	12.1	1.3	12.2	1.1	12.3	1.1	0.13

[a]TD controls were frequency-matched to a projected distribution of ASD cases on age, sex, and regional center catchment area

[b]Includes Black/African American, American Indian/Alaska Native, Asian, Pacific Islander/Native Hawaiian, Multi-racial

[c]Months grouped by season as follows: Winter = December to February, Spring = March to May, Summer = June to August, Fall = September to November

[d]1 participant (ASD) was missing maternal allergies/asthma; allergies include the following types: environmental (e.g., seasonal, pet, mold), food, skin, medication, or other

[e]2 participants were missing gestational age (1 ASD, 1 TD)

[f] Southern California regions include Los Angeles, Kern, Orange, San Diego, Tri-counties, and Inland

[g]*P*-values for categorical and continuous variables calculated with Chi-square test and one-way analysis of variance (ANOVA), respectively

Table 2A. Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD, DD, and TD[a]

	ASD vs. TD			*DD vs. TD*			*ASD vs. DD*		
Cytokine/ chemokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
6Ckine (CCL21)	0.51	0.33, 0.78	0.002	0.34	0.20, 0.59	0.0001	1.49	0.91, 2.44	0.12
BCA-1 (CXCL13)	0.54	0.31, 0.93	0.03	0.41	0.21, 0.82	0.01	1.30	0.68, 2.51	0.43
CTACK (CCL27)	0.36	0.21, 0.62	0.000 2	0.35	0.18, 0.67	0.002	1.05	0.60, 1.81	0.88
ENA78 (CXCL5)	0.98	0.79, 1.21	0.84	0.82	0.64, 1.05	0.12	1.19	0.93, 1.52	0.16
Eotaxin (CCL11)	0.26	0.11, 0.63	0.003	0.25	0.09, 0.70	0.01	1.02	0.49, 2.12	0.95
Eotaxin-2 (CCL24)	0.88	0.69, 1.11	0.28	0.78	0.59, 1.04	0.09	1.13	0.86, 1.48	0.38
Eotaxin-3 (CCL26)	0.56	0.37, 0.83	0.005	0.51	0.31, 0.85	0.01	1.09	0.68, 1.75	0.71
Fractalkine (CX3CL1)	0.56	0.32, 0.99	0.04	0.44	0.22, 0.87	0.02	1.28	0.69, 2.35	0.43
GCP-2 (CXCL6)	0.60	0.34, 1.06	0.08	0.46	0.23, 0.93	0.03	1.25	0.69, 2.28	0.46
Gro-α (CXCL1)	0.66	0.38, 1.14	0.14	0.68	0.34, 1.36	0.27	0.97	0.50, 1.89	0.93
Gro-β (CXCL2)	0.63	0.42, 0.95	0.03	0.60	0.36, 1.01	0.05	1.04	0.65, 1.68	0.86
I-309 (CCL1)	0.34	0.17, 0.67	0.002	0.31	0.13, 0.72	0.01	1.11	0.49, 2.53	0.80
IFN-γ	0.67	0.46, 0.96	0.03	0.59	0.36, 0.95	0.03	1.14	0.71, 1.82	0.59
IL-1β	0.69	0.39, 1.23	0.21	0.50	0.25, 1.00	0.05	1.37	0.71, 2.65	0.34
IL-2	0.51	0.28, 0.91	0.02	0.41	0.20, 0.84	0.01	1.25	0.64, 2.42	0.51
IL-4	0.51	0.26, 0.99	0.048	0.45	0.20, 1.01	0.05	1.15	0.54, 2.45	0.72
IL-6	0.56	0.34, 0.94	0.03	0.48	0.26, 0.90	0.02	1.17	0.67, 2.05	0.58
IL-8 (CXCL8)	0.91	0.59, 1.39	0.65	0.83	0.49, 1.40	0.48	1.10	0.64, 1.87	0.73
IL-10	0.59	0.35, 0.99	0.046	0.47	0.26, 0.86	0.01	1.25	0.75, 2.10	0.39
IL-16	0.74	0.40, 1.35	0.32	0.53	0.27, 1.01	0.07	1.38	0.77, 2.48	0.28
IP-10 (CXCL10)	0.34	0.18, 0.66	0.001	0.41	0.18, 0.92	0.03	0.84	0.39, 1.79	0.65

	ASD vs. TD			*DD vs. TD*			*ASD vs. DD*		
Cytokine/ chemokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
I-TAC (CXCL11)	0.62	0.41, 0.93	0.02	0.46	0.27, 0.79	0.01	1.33	0.80, 2.21	0.28
MCP-1 (CCL2)	0.88	0.58, 1.35	0.56	0.71	0.42, 1.21	0.20	1.22	0.73, 2.06	0.45
MCP-2 (CCL8)	0.49	0.28, 0.83	0.01	0.36	0.19, 0.71	0.003	1.33	0.75, 2.39	0.33
MCP-3 (CCL7)	0.47	0.27, 0.82	0.01	0.42	0.22, 0.80	0.01	1.13	0.66, 1.92	0.66
MCP-4 (CCL13)	0.57	0.36, 0.90	0.02	0.69	0.39, 1.23	0.21	0.82	0.48, 1.42	0.48
MDC (CCL22)	0.69	0.41, 1.14	0.15	0.35	0.19, 0.65	0.001	1.96	1.09, 3.53	0.03
MIF	0.35	0.16, 0.78	0.01	0.30	0.13, 0.69	0.005	1.19	0.79, 1.80	0.40
MIG (CXCL9)	0.55	0.35, 0.86	0.01	0.42	0.25, 0.73	0.002	1.30	0.80, 2.12	0.29
MIP-1α (CCL3)	0.36	0.15, 0.88	0.02	0.34	0.12, 0.99	0.047	1.04	0.38, 2.85	0.94
MIP-1δ (CCL15)	0.76	0.47, 1.23	0.27	0.54	0.31, 0.96	0.03	1.40	0.85, 2.30	0.19
MIP-3α (CCL20)	0.48	0.22, 1.09	0.08	0.39	0.16, 0.94	0.04	1.25	0.67, 2.37	0.48
MIP-3β (CCL19)	0.74	0.53, 1.03	0.07	0.61	0.41, 0.91	0.01	1.22	0.84, 1.75	0.29
MPIF-1 (CCL23)	0.72	0.49, 1.06	0.10	0.37	0.23, 0.59	<.0001	1.97	1.26, 3.09	0.003
SCYB16 (CXCL16)	0.68	0.44, 1.04	0.07	0.69	0.41, 1.17	0.17	0.99	0.61, 1.61	0.96
SDF-1α/β (CXCL12)	0.51	0.31, 0.85	0.01	0.39	0.21, 0.71	0.002	1.31	0.80, 2.14	0.29
TARC (CCL17)	0.65	0.45, 0.93	0.02	0.54	0.34, 0.84	0.0004	1.21	0.80, 1.84	0.38
TECK (CCL25)	0.34	0.17, 0.71	0.004	0.28	0.12, 0.65	0.003	1.21	0.64, 2.28	0.56
TNF-α	0.43	0.22, 0.83	0.01	0.38	0.19, 0.79	0.01	1.12	0.64, 1.71	0.61

[a] Multinomial logistic regression models were adjusted for maternal education attainment, gestational age, child's age at blood spot collection, and years from blood spot collection to elution; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); OR represents the fold change in the odds of having one diagnosis relative to another diagnosis or no

diagnosis for every 1-unit increase in the ln-transformed cytokine/chemokine (or for every *e*-fold increase in cytokine/chemokine levels); 398 participants comprised the following groups: 171 ASD, 69 DD and 158 TD; OR = adjusted odds ratio, CI = confidence interval, P = *P*-value

Table 2B-1. Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD (severe, mild to moderate symptoms) and TD[a]

	ASD_{sev} *vs. TD*			ASD_{mild} *vs. TD*		
Cytokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
6Ckine (CCL21)	0.49	0.30, 0.78	0.002	0.56	0.31, 1.02	0.06
BCA-1 (CXCL13)	0.56	0.30, 1.01	0.06	0.49	022, 1.09	0.08
CTACK (CCL27)	0.36	0.20, 0.63	0.0004	0.38	0.19, 0.79	0.01
ENA78 (CXCL5)	1.06	0.82, 1.36	0.67	0.86	0.65, 1.13	0.27
Eotaxin (CCL11)	0.26	0.10, 0.68	0.01	0.25	0.08, 0.74	0.01
Eotaxin-2 (CCL24)	0.88	0.68, 1.14	0.33	0.88	0.63, 1.22	0.44
Eotaxin-3 (CCL26)	0.55	0.35, 0.85	0.01	0.59	0.33, 1.04	0.07
Fractalkine (CX3CL1)	0.60	0.33, 1.11	0.10	0.49	0.23, 1.05	0.06
GCP-2 (CXCL6)	0.66	0.35, 1.23	0.19	0.51	0.24, 1.07	0.08
Gro-α (CXCL1)	0.68	0.37, 1.24	0.21	0.63	0.29, 1.33	0.22
Gro-β (CXCL2)	0.62	0.40, 0.97	0.04	0.66	0.37, 1.18	0.16
I-309 (CCL1)	0.36	0.17, 0.74	0.01	0.30	0.12, 0.80	0.02
IFN-γ	0.71	0.47, 1.05	0.09	0.58	0.34, 0.98	0.04
IL-1β	0.72	0.38, 1.36	0.31	0.63	0.28, 1.42	0.27
IL-2	0.54	0.29, 1.02	0.06	0.45	0.20, 1.01	0.05
IL-4	0.52	0.25, 1.07	0.08	0.49	0.19, 1.24	0.13
IL-6	0.60	0.34, 1.05	0.07	0.49	0.24, 0.98	0.04
IL-8 (CXCL8)	0.87	0.54, 1.39	0.55	1.00	0.53, 1.86	0.99
IL-10	0.59	0.34, 1.02	0.06	0.60	0.30, 1.21	0.16
IL-16	0.72	0.37, 1.37	0.31	0.79	0.34, 1.86	0.59
IP-10 (CXCL10)	0.36	0.18, 0.74	0.005	0.30	0.12, 0.74	0.01

	ASD_{sev} vs. TD			ASD_{mild} vs. TD		
Cytokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
I-TAC (CXCL11)	0.58	0.36, 0.91	0.02	0.73	0.40, 1.34	0.31
MCP-1 (CCL2)	0.77	0.49, 1.23	0.27	1.25	0.64, 2.45	0.52
MCP-2 (CCL8)	0.47	0.26, 0.83	0.01	0.54	0.26, 1.13	0.10
MCP-3 (CCL7)	0.47	0.26, 0.85	0.01	0.46	0.22, 0.97	0.04
MCP-4 (CCL13)	0.52	0.31, 0.85	0.01	0.72	0.37, 1.37	0.31
MDC (CCL22)	0.64	0.37, 1.12	0.12	0.81	0.38, 1.69	0.57
MIF	0.37	0.16, 0.86	0.02	0.32	0.13, 0.77	0.01
MIG (CXCL9)	0.57	0.35, 0.93	0.02	0.51	0.28, 0.94	0.03
MIP-1α (CCL3)	0.42	0.16, 1.07	0.07	0.24	0.07, 0.85	0.03
MIP-1δ (CCL15)	0.76	0.45, 1.30	0.32	0.76	0.39, 1.48	0.42
MIP-3α (CCL20)	0.51	0.21, 1.20	0.12	0.44	0.16, 1.21	0.11
MIP-3β (CCL19)	0.74	0.52, 1.05	0.09	0.76	0.48, 1.19	0.23
MPIF-1 (CCL23)	0.69	0.45, 1.04	0.07	0.83	0.47, 1.45	0.51
SCYB16 (CXCL16)	0.64	0.41, 1.02	0.06	0.77	0.42, 1.42	0.41
SDF-1α/β (CXCL12)	0.55	0.32, 0.96	0.03	0.43	0.22, 0.85	0.01
TARC (CCL17)	0.65	0.44, 0.97	0.03	0.65	0.39, 1.08	0.10
TECK (CCL25)	0.38	0.17, 0.83	0.02	0.28	0.11, 0.70	0.01
TNF-α	0.42	0.21, 0.83	0.01	0.46	0.20, 1.06	0.07

[a] Multinomial logistic regression models were adjusted for maternal education attainment, gestational age, child's age at blood spot collection, and years from blood spot collection to elution; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); OR represents the fold change in the odds of having one diagnosis relative to another diagnosis or no diagnosis for every 1-unit increase in the ln-transformed cytokine/chemokine (or for every *e*-fold

increase in cytokine/chemokine levels); 398 participants comprised the following groups: 121 ASD (severe), 50 ASD (mild), and 158 TD; ASD severity was defined using ADOS severity scores, where ≥7 indicated severe and <7 indicated mild to moderate symptoms; OR = adjusted odds ratio, CI = confidence interval, P = *P*-value

[b] ASD_{sev} vs ASD_{mild} results are in the Supplementary Table 2.

Table 2B-2. Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD (severe, mild to moderate symptoms) and DD[a]

	ASD_{sev} *vs. DD*			ASD_{mild} *vs. DD*		
Cytokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
6Ckine (CCL21)	1.43	0.84, 2.42	0.19	1.64	0.85, 3.14	0.14
BCA-1 (CXCL13)	1.35	0.67, 2.74	0.40	1.20	0.51, 2.83	0.68
CTACK (CCL27)	1.02	0.57, 1.83	0.94	1.10	0.54, 2.25	0.80
ENA78 (CXCL5)	1.29	0.98, 1.70	0.07	1.05	0.78, 1.40	0.77
Eotaxin (CCL11)	1.04	0.48, 2.26	0.92	0.97	0.37, 2.58	0.96
Eotaxin-2 (CCL24)	1.13	0.85, 1.51	0.41	1.13	0.79, 1.61	0.51
Eotaxin-3 (CCL26)	1.07	0.65, 1.76	0.78	1.15	0.62, 2.16	0.65
Fractalkine (CX3CL1)	1.37	0.70, 2.67	0.36	1.12	0.52, 2.42	0.78
GCP-2 (CXCL6)	1.39	0.70, 2.74	0.35	1.07	0.52, 2.23	0.85
Gro-α (CXCL1)	1.00	0.49, 2.05	0.995	0.92	0.40, 2.13	0.84
Gro-β (CXCL2)	1.02	0.61, 1.70	0.93	1.09	0.59, 2.03	0.78
I-309 (CCL1)	1.17	0.49, 2.80	0.73	1.00	0.34, 2.90	0.996
IFN-γ	1.21	0.74, 1.99	0.45	0.99	0.55, 1.81	0.98
IL-1β	1.44	0.70, 2.95	0.32	1.26	0.54, 2.95	0.59
IL-2	1.33	0.65, 2.70	0.44	1.09	0.46, 2.61	0.84
IL-4	1.17	0.52, 2.64	0.70	1.10	0.41, 2.99	0.85
IL-6	1.25	0.68, 2.31	0.47	1.02	0.50, 2.08	0.97
IL-8 (CXCL8)	1.05	0.59, 1.86	0.87	1.21	0.60, 2.42	0.60
IL-10	1.25	0.72, 2.17	0.43	1.27	0.63, 2.58	0.50
IL-16	1.34	0.71, 2.53	0.37	1.48	0.64, 3.45	0.36
IP-10 (CXCL10)	0.89	0.40, 1.99	0.77	0.73	0.28, 1.94	0.53

	ASD_{sev} vs. DD			ASD_{mild} vs. DD		
Cytokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
I-TAC (CXCL11)	1.24	0.72, 2.12	0.44	1.57	0.80, 3.10	0.19
MCP-1 (CCL2)	1.07	0.62, 1.86	0.80	1.74	0.83, 3.65	0.15
MCP-2 (CCL8)	1.28	0.69, 2.38	0.43	1.47	0.67, 3.22	0.33
MCP-3 (CCL7)	1.14	0.64, 2.01	0.66	1.11	0.54, 2.28	0.77
MCP-4 (CCL13)	0.75	0.42, 1.33	0.33	1.03	0.50, 2.13	0.93
MDC (CCL22)	1.84	0.98, 3.43	0.06	2.30	1.03, 5.16	0.04
MIF	1.29	0.74, 2.26	0.37	1.10	0.68, 1.77	0.71
MIG (CXCL9)	1.36	0.80, 2.31	0.26	1.21	0.64, 2.27	0.56
MIP-1α (CCL3)	1.27	0.41, 3.91	0.68	0.73	0.21, 2.60	0.63
MIP-1δ (CCL15)	1.40	0.81, 2.44	0.23	1.39	0.71, 2.72	0.33
MIP-3α (CCL20)	1.31	0.64, 2.68	0.46	1.15	0.49, 2.67	0.75
MIP-3β (CCL19)	1.21	0.82, 1.78	0.34	1.24	0.77, 2.00	0.37
MPIF-1 (CCL23)	1.87	1.16, 3.00	0.01	2.26	1.23, 4.16	0.01
SCYB16 (CXCL16)	0.93	0.56, 1.56	0.79	1.13	0.59, 2.16	0.72
SDF-1α/β (CXCL12)	1.42	0.82, 2.47	0.22	1.12	0.60, 2.10	0.72
TARC (CCL17)	1.21	0.77, 1.90	0.40	1.21	0.70, 2.09	0.50
TECK (CCL25)	1.37	0.65, 2.86	0.41	1.01	0.47, 2.19	0.97
TNF-α	1.09	0.69, 1.71	0.71	1.21	0.62, 2.37	0.57

[a] Multinomial logistic regression models were adjusted for maternal education attainment, gestational age, child's age at blood spot collection, and years from blood spot collection to elution; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); OR represents the fold change in the odds of having one diagnosis relative to another diagnosis or no diagnosis for every 1-unit increase in the ln-transformed cytokine/chemokine (or for every *e*-fold

increase in cytokine/chemokine levels); 398 participants comprised the following groups: 121 ASD (severe), 50 ASD (mild), and 69 DD; ASD severity was defined using ADOS severity scores, where ≥7 indicated severe and <7 indicated mild to moderate symptoms; OR = adjusted odds ratio, CI = confidence interval, P = *P*-value

Table 2C-1. Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD (typical to high-functioning, low-functioning) and TD[a]

	ASD_{hi} *vs.* ASD_{lo}			ASD_{hi} *vs. TD*			ASD_{lo} *vs. TD*		
Cytokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
6Ckine (CCL21)	0.86	0.42, 1.80	0.70	0.45	0.21, 0.95	0.04	0.52	0.34, 0.81	0.004
BCA-1 (CXCL13)	1.00	0.37, 2.72	0.99	0.54	0.20, 1.46	0.22	0.54	0.30, 0.95	0.03
CTACK (CCL27)	0.90	0.43, 1.92	0.79	0.34	0.15, 0.76	0.01	0.37	0.21, 0.64	0.0004
ENA78 (CXCL5)	0.85	0.60, 1.21	0.38	0.86	0.61, 1.22	0.39	1.01	0.80, 1.27	0.94
Eotaxin (CCL11)	0.78	0.27, 2.27	0.65	0.21	0.06, 0.75	0.02	0.27	0.11, 0.67	0.01
Eotaxin-2 (CCL24)	0.88	0.59, 1.34	0.56	0.79	0.52, 1.20	0.27	0.89	0.70, 1.15	0.38
Eotaxin-3 (CCL26)	0.93	0.46, 1.87	0.83	0.52	0.26, 1.07	0.08	0.57	0.37, 0.86	0.01
Fractalkine (CX3CL1)	0.74	0.31, 1.76	0.50	0.44	0.18, 1.08	0.07	0.60	0.33, 1.07	0.08
GCP-2 (CXCL6)	0.57	0.25, 1.32	0.19	0.39	0.16, 0.92	0.03	0.68	0.37, 1.25	0.21
Gro-α (CXCL1)	0.89	0.35, 2.26	0.80	0.60	0.23, 1.54	0.29	0.68	0.38, 1.20	0.18
Gro-β (CXCL2)	0.72	0.37, 1.40	0.33	0.48	0.24, 0.95	0.04	0.67	0.44, 1.03	0.07
I-309 (CCL1)	0.81	0.24, 2.74	0.74	0.28	0.08, 0.97	0.04	0.35	0.17, 0.71	0.004
IFN-γ	0.74	0.38, 1.45	0.38	0.52	0.26, 1.01	0.05	0.70	0.48, 1.02	0.07
IL-1β	1.19	0.40, 3.52	0.76	0.80	0.27, 2.38	0.69	0.68	0.37, 1.23	0.20
IL-2	0.88	0.31, 2.49	0.81	0.46	0.16, 1.31	0.15	0.52	0.28, 0.95	0.03
IL-4	0.75	0.24, 2.34	0.62	0.40	0.13, 1.28	0.12	0.54	0.27, 1.07	0.08
IL-6	0.85	0.36, 2.02	0.71	0.49	0.20, 1.19	0.12	0.58	0.34, 0.98	0.04
IL-8 (CXCL8)	0.89	0.41, 1.93	0.77	0.82	0.38, 1.76	0.62	0.93	0.59, 1.46	0.74
IL-10	0.95	0.42, 2.12	0.89	0.57	0.24, 1.32	0.19	0.60	0.35, 1.02	0.06
IL-16	1.05	0.38, 2.90	0.93	0.77	0.27, 2.18	0.62	0.73	0.39, 1.37	0.33
IP-10 (CXCL10)	1.36	0.41, 4.50	0.61	0.45	0.13, 1.49	0.19	0.33	0.17, 0.65	0.001

	ASD_{hi} vs. ASD_{lo}			ASD_{hi} vs. TD			ASD_{lo} vs. TD		
Cytokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
I-TAC (CXCL11)	1.37	0.62, 3.00	0.44	0.80	0.37, 1.75	0.58	0.59	0.38, 0.91	0.02
MCP-1 (CCL2)	3.18	1.23, 8.26	0.02	2.43	0.94, 6.25	0.07	0.76	0.49, 1.19	0.23
MCP-2 (CCL8)	0.96	0.42, 2.19	0.91	0.47	0.20, 1.12	0.09	0.49	0.28, 0.86	0.01
MCP-3 (CCL7)	0.92	0.40, 2.14	0.84	0.44	0.18, 1.07	0.07	0.48	0.27, 0.84	0.01
MCP-4 (CCL13)	1.33	0.59, 3.03	0.49	0.73	0.32, 1.67	0.45	0.55	0.34, 0.87	0.01
MDC (CCL22)	0.69	0.30, 1.61	0.39	0.51	0.22, 1.18	0.12	0.73	0.43, 1.25	0.25
MIF	0.95	0.47, 1.92	0.89	0.34	0.13, 0.90	0.03	0.36	0.16, 0.80	0.01
MIG (CXCL9)	0.85	0.41, 1.77	0.66	0.48	0.23, 1.03	0.06	0.57	0.36, 0.90	0.02
MIP-1α (CCL3)	0.59	0.14, 2.43	0.46	0.23	0.05, 1.01	0.05	0.39	0.16, 0.98	0.045
MIP-1δ (CCL15)	1.17	0.47, 2.91	0.73	0.87	0.35, 2.17	0.76	0.74	0.45, 1.23	0.25
MIP-3α (CCL20)	0.84	0.30, 2.37	0.74	0.42	0.13, 1.35	0.15	0.50	0.22, 1.16	0.11
MIP-3β (CCL19)	0.97	0.58, 1.62	0.90	0.72	0.42, 1.23	0.23	0.75	0.53, 1.05	0.09
MPIF-1 (CCL23)	1.40	0.67, 2.93	0.38	0.96	0.46, 2.02	0.91	0.69	0.46, 1.03	0.07
SCYB16 (CXCL16)	1.12	0.55, 2.29	0.76	0.75	0.36, 1.56	0.44	0.67	0.43, 1.04	0.07
SDF-1α/β (CXCL12)	0.76	0.36, 1.62	0.48	0.41	0.18, 0.91	0.03	0.54	0.32, 0.91	0.02
TARC (CCL17)	0.92	0.49, 1.70	0.78	0.60	0.32, 1.14	0.12	0.66	0.45, 0.96	0.03
TECK (CCL25)	0.85	0.33, 2.19	0.73	0.30	0.10, 0.87	0.03	0.35	0.17, 0.75	0.01
TNF-α	0.94	0.46, 1.89	0.85	0.41	0.17, 0.98	0.046	0.43	0.22, 0.85	0.02

[a] Multinomial logistic regression models were adjusted for maternal education attainment, gestational age, child's age at blood spot collection, and years from blood spot collection to elution; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); OR represents the fold change in the odds of having one diagnosis relative to another diagnosis or no diagnosis for every 1-unit increase in the ln-transformed cytokine/chemokine (or for every *e*-fold increase in cytokine/chemokine levels); 398 participants comprised the following groups: 27 ASD

(high), 144 ASD (low) and 158 TD; Mullen Scales of Early Learning (MSEL) and Vineland Adaptive Behavior Scales (VABS) composite standard scores were used to define high/low cognitive and adaptive development levels, where both MSEL and VABS scores of ≥70 indicated typical to high-function and a score of <70 on either MSEL or VABS indicated low-function; OR = adjusted odds ratio, CI = confidence interval, P = *P*-value

Table 2C-2. Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD (typical to high-functioning, low-functioning) and DD[a]

	ASD_{hi} vs. DD			ASD_{lo} vs. DD		
Cytokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
6Ckine (CCL21)	1.32	0.61, 2.86	0.48	1.53	0.92, 2.56	0.10
BCA-1 (CXCL13)	1.31	0.45, 3.79	0.62	1.30	0.66, 2.56	0.44
CTACK (CCL27)	0.97	0.43, 2.18	0.93	1.07	0.60, 1.89	0.82
ENA78 (CXCL5)	1.05	0.73, 1.51	0.81	1.23	0.95, 1.59	0.12
Eotaxin (CCL11)	0.83	0.27, 2.58	0.75	1.06	0.50, 2.26	0.87
Eotaxin-2 (CCL24)	1.02	0.66, 1.57	0.94	1.15	0.87, 1.52	0.33
Eotaxin-3 (CCL26)	1.03	0.48, 2.19	0.94	1.11	0.68, 1.80	0.68
Fractalkine (CX3CL1)	1.01	0.41, 2.50	0.98	1.36	0.72, 2.58	0.35
GCP-2 (CXCL6)	0.84	0.38, 1.88	0.67	1.47	0.75, 2.88	0.27
Gro-α (CXCL1)	0.88	0.32, 2.43	0.81	1.00	0.50, 1.98	0.99
Gro-β (CXCL2)	0.80	0.39, 1.63	0.53	1.11	0.67, 1.83	0.68
I-309 (CCL1)	0.93	0.25, 3.47	0.92	1.15	0.49, 2.67	0.75
IFN-γ	0.88	0.42, 1.84	0.74	1.19	0.74, 1.93	0.47
IL-1β	1.59	0.51, 4.98	0.42	1.34	0.68, 2.63	0.39
IL-2	1.12	0.37, 3.38	0.85	1.27	0.64, 2.51	0.49
IL-4	0.90	0.27, 3.04	0.86	1.21	0.55, 2.63	0.64
IL-6	1.02	0.41, 2.54	0.96	1.20	0.68, 2.14	0.53
IL-8 (CXCL8)	1.00	0.44, 2.27	0.996	1.12	0.65, 1.95	0.68
IL-10	1.20	0.52, 2.80	0.67	1.27	0.74, 2.17	0.38
IL-16	1.44	0.52, 4.01	0.48	1.37	0.74, 2.55	0.31
IP-10 (CXCL10)	1.10	0.30, 3.95	0.89	0.81	0.37, 1.75	0.58

	ASD_{hi} vs. DD			ASD_{lo} vs. DD		
Cytokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
I-TAC (CXCL11)	1.74	0.74, 4.05	0.20	1.27	0.75, 2.14	0.37
MCP-1 (CCL2)	3.42	1.25, 9.39	0.02	1.08	0.63, 1.83	0.79
MCP-2 (CCL8)	1.29	0.53, 3.15	0.57	1.35	0.74, 2.48	0.33
MCP-3 (CCL7)	1.05	0.43, 2.55	0.91	1.14	0.66, 1.99	0.63
MCP-4 (CCL13)	1.05	0.43, 2.57	0.91	0.79	0.45, 1.38	0.41
MDC (CCL22)	1.46	0.62, 3.41	0.38	2.12	1.14, 3.92	0.02
MIF	1.15	0.60, 2.21	0.68	1.21	0.77, 1.91	0.41
MIG (CXCL9)	1.14	0.53, 2.45	0.74	1.34	0.81, 2.23	0.26
MIP-1α (CCL3)	0.68	0.15, 3.03	0.62	1.17	0.40, 3.41	0.78
MIP-1δ (CCL15)	1.60	0.63, 4.08	0.32	1.37	0.82, 2.29	0.23
MIP-3α (CCL20)	1.09	0.39, 3.07	0.87	1.30	0.66, 2.55	0.45
MIP-3β (CCL19)	1.19	0.68, 2.06	0.54	1.23	0.84, 1.79	0.29
MPIF-1 (CCL23)	2.63	1.19, 5.79	0.02	1.88	1.19, 2.97	0.01
SCYB16 (CXCL16)	1.09	0.50, 2.36	0.83	0.97	0.59, 1.60	0.91
SDF-1α/β (CXCL12)	1.05	0.49, 2.25	0.90	1.38	0.82, 2.32	0.23
TARC (CCL17)	1.12	0.58, 2.17	0.73	1.23	0.80, 1.90	0.35
TECK (CCL25)	1.06	0.41, 2.78	0.90	1.26	0.64, 2.47	0.51
TNF-α	1.06	0.52, 2.15	0.87	1.14	0.73, 1.78	0.58

[a] Multinomial logistic regression models were adjusted for maternal education attainment, gestational age, child's age at blood spot collection, and years from blood spot collection to elution; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); OR represents the fold change in the odds of having one diagnosis relative to another diagnosis or no diagnosis for every 1-unit increase in the ln-transformed cytokine/chemokine (or for every *e*-fold

increase in cytokine/chemokine levels); 398 participants comprised the following groups: 27 ASD (high), 144 ASD (low), and 69 DD; Mullen Scales of Early Learning (MSEL) and Vineland Adaptive Behavior Scales (VABS) composite standard scores were used to define high/low cognitive and adaptive development levels, where both MSEL and VABS scores of ≥70 indicated typical to high-function and a score of <70 on either MSEL or VABS indicated low-function; OR = adjusted odds ratio, CI = confidence interval, P = *P*-value

Table 3A. Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD, DD, and TD in one model, N=398[a]

Cytokine or	***ASD vs. TD***			***ASD vs. DD***			***DD vs. TD***		
Chemokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
CTACK	**0.40**	**(0.21, 0.77)**	**0.01**	0.49	(0.21, 1.14)	0.10	0.82	(0.35, 1.94)	0.65
MPIF-1	0.95	(0.62, 1.45)	0.81	**2.38**	**(1.42, 3.98)**	**0.001**	**0.40**	**(0.24, 0.68)**	**0.001**
MIF	0.59	(0.24, 1.48)	0.26	1.21	(0.65, 2.25)	0.56	0.49	(0.18, 1.34)	0.17

[a] Multinomial logistic regression model was adjusted for maternal education, gestational age, child's age at blood spot collection, and years from blood spot collection to elution; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); OR represents the fold change in the odds of having one diagnosis relative to another diagnosis or no diagnosis for every 1-unit increase in the ln-transformed cytokine/chemokine (or for every *e*-fold increase in cytokine/chemokine levels); 398 participants comprised the following groups: 171 ASD, 69 DD and 158 TD; OR = adjusted odds ratio, CI = confidence interval

Table 3B. Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD (severe, mild to moderate symptoms), DD, and TD in one model, N=398[a]

Cytokine or	***ASD_{sev} vs. ASD_{mild}***			***ASD_{sev} vs. TD***			***ASD_{mild} vs. TD***		
Chemokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
CTACK	0.83	0.32, 2.12	0.70	**0.38**	**0.49, 0.77**	**0.01**	0.46	0.18, 1.15	0.10
MPIF-1	0.79	0.42, 1.49	0.46	0.89	0.56, 1.41	0.61	1.13	0.60, 2.10	0.71
MIF	1.53	0.64, 3.66	0.34	0.69	0.26, 1.80	0.44	0.45	0.15, 1.30	0.14
Cytokine or	***ASD_{sev} vs. DD***			***ASD_{mild} vs. DD***			***DD vs. TD***		
Chemokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
CTACK	0.46	0.19, 1.11	0.08	0.55	0.19, 1.60	0.27	0.82	(0.35, 1.94)	0.65
MPIF-1	**2.23**	**1.29, 3.84**	**0.004**	**2.83**	**1.42, 5.64**	**0.003**	**0.40**	**(0.24, 0.68)**	**0.001**
MIF	1.44	0.65, 3.21	0.37	0.94	0.45, 1.98	0.88	0.49	(0.18, 1.34)	0.17

[a] Multinomial logistic regression model was adjusted for maternal education, gestational age, child's age at blood spot collection, and years from blood spot collection to elution; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); OR represents the fold change in the odds of having one diagnosis relative to another diagnosis or no diagnosis for every 1-unit increase in the ln-transformed cytokine/chemokine (or for every *e*-fold increase in cytokine/chemokine levels); 398 participants comprised the following groups: 121 ASD-severe, 50 ASD-mild, 69 DD and 158 TD; OR = adjusted odds ratio, CI = confidence interval

Table 3C. Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD (high, low cognitive and adaptive functioning), DD, and TD in one model, N=398[a]

Cytokine or	***ASD_{hi} vs. ASD_{lo}***			***ASD_{hi} vs. TD***			***ASD_{lo} vs. TD***		
Chemokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
CTACK	0.67	0.21, 2.09	0.49	**0.29**	**0.09, 0.91**	**0.03**	**0.43**	**0.22, 0.84**	**0.01**
MPIF-1	1.64	0.72, 3.72	0.24	1.45	0.63, 3.32	0.38	0.89	0.57, 1.37	0.59
MIF	0.93	0.33, 2.60	0.89	0.56	0.16, 1.94	0.36	0.60	0.24, 1.52	0.28
Cytokine or	***ASD_{hi} vs. DD***			***ASD_{lo} vs. DD***			***DD vs. TD***		
Chemokine	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
CTACK	0.35	0.10, 1.26	0.11	0.52	0.22, 1.23	0.14	0.82	(0.35, 1.94)	0.65
MPIF-1	**3.64**	**1.50, 8.84**	**0.004**	**2.22**	**1.31, 3.76**	**0.003**	**0.40**	**(0.24, 0.68)**	**0.001**
MIF	1.14	0.41, 3.16	0.80	1.22	0.62, 2.37	0.56	0.49	(0.18, 1.34)	0.17

[a] Multinomial logistic regression model was adjusted for maternal education, gestational age, child's age at blood spot collection, and years from blood spot collection to elution; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); OR represents the fold change in the odds of having one diagnosis relative to another diagnosis or no diagnosis for every 1-unit increase in the ln-transformed cytokine/chemokine (or for every *e*-fold increase in cytokine/chemokine levels); 398 participants comprised the following groups: 27 ASD-high, 144 ASD-low, 69 DD and 158 TD; OR = adjusted odds ratio, CI = confidence interval

Table 4. Developmental characteristics of 2-5-year-old children with ASD in relation to their neonatal CTACK and MPIF-1 concentrations, N=171[a]

	CTACK			***MPIF-1***		
	β	***95% CI***	***P-value***	***β***	***95% CI***	***P-value***
Mullen Scales of Early Learning						
Visual Reception	-4.16	-12.94, 4.61	0.35	6.03	-1.16, 13.21	0.10
Fine Motor	-3.96	-10.91, 2.98	0.26	**7.64**	**1.95, 13.33**	**0.01**
Receptive Language	-7.09	-16.76, 2.57	0.15	**11.48**	**3.56, 19.40**	**0.005**
Expressive Language	-4.60	-13.19, 3.99	0.29	**8.85**	**1.82, 15.89**	**0.01**
Composite	-4.95	-12.65, 2.74	0.21	**8.50**	**2.20, 14.80**	**0.01**
Vineland Adaptive Behavior Scales						
Communication	-5.70	-12.87, 1.47	0.12	**7.26**	**1.39, 13.12**	**0.02**
Daily Living Skills	-2.97	-7.68, 1.74	0.21	**4.83**	**0.97, 8.68**	**0.01**
Socialization	-2.63	-9.21, 3.95	0.43	**6.74**	**1.36, 12.12**	**0.01**
Motor Skills	-5.66	-12.91, 1.59	0.13	**7.04**	**1.11, 12.96**	**0.02**
Composite	-4.34	-9.64, 0.96	0.11	**6.46**	**2.12, 10.80**	**0.004**

[a]Linear regression models were adjusted for maternal education (≤High school, Some college vs. ≥Bachelor degree) and CTACK or MPIF-1 (both were included in one model); β-coefficient (estimate) represents the change in developmental quotient (DQ) for a 1-unit increase in ln-transformed chemokine (pg/mg total protein), with a higher DQ indicating a better developmental outcome; DQ is defined as the developmental age divided by chronological age and multiplied by 100, with Mean = 100 and Standard Deviation = 15; CI = confidence interval

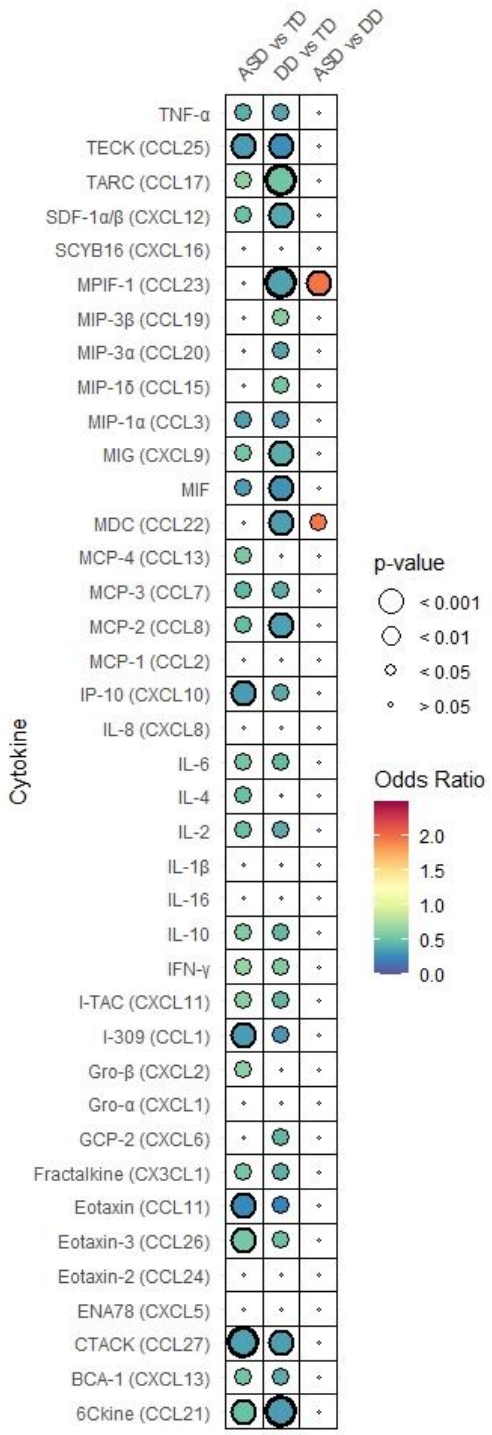

Figure 1A. Adjusted odds ratio plot comparing neonatal cytokine and chemokine concentrations in ASD, DD, and TD. Odds ratio is depicted by the heat map with highest ORs in red to lowest in blue. Each figure has its own heat map. Relative P-value is depicted by circle size. P-values that are below 0.001 are bolded.

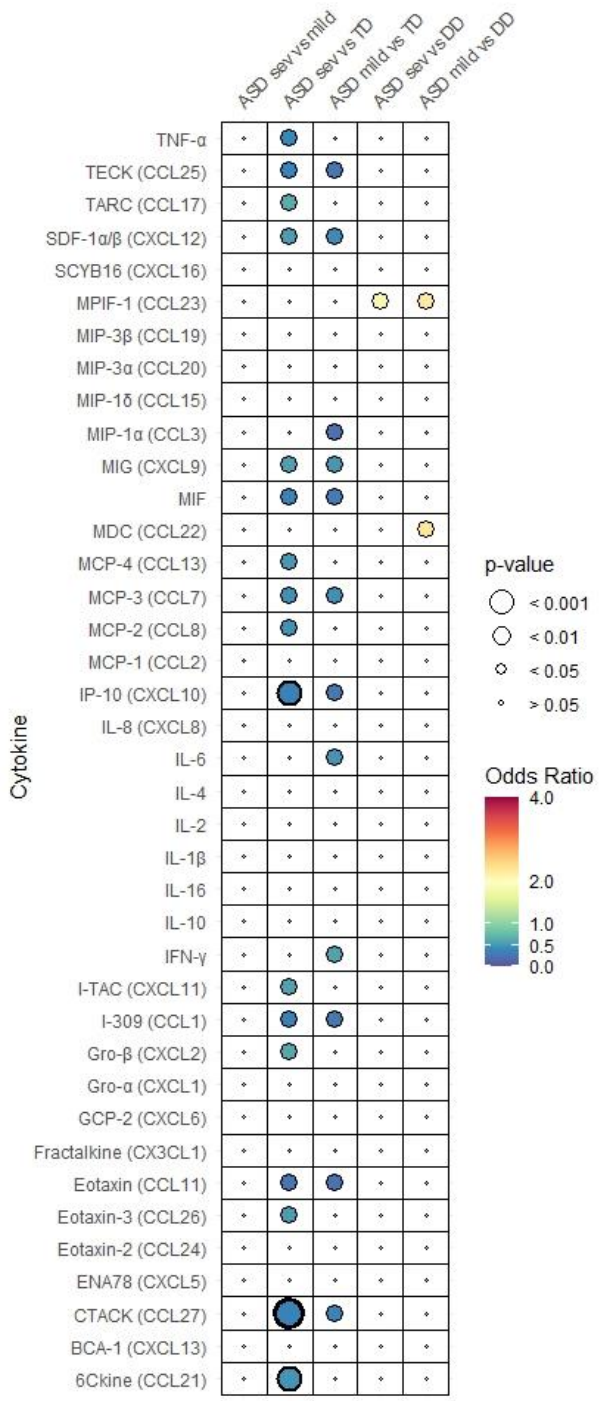

Figure 1B. Adjusted odds ratio plot comparing neonatal cytokine and chemokine concentrations in subgroups of ASD (ASD_{sev}, ASD_{mild}), DD, and TD. Odds ratio is depicted by the heat map with highest ORs in red to lowest in blue. Each figure has its own heat map. Relative P-value is depicted by circle size. P-values that are below 0.001 are bolded.

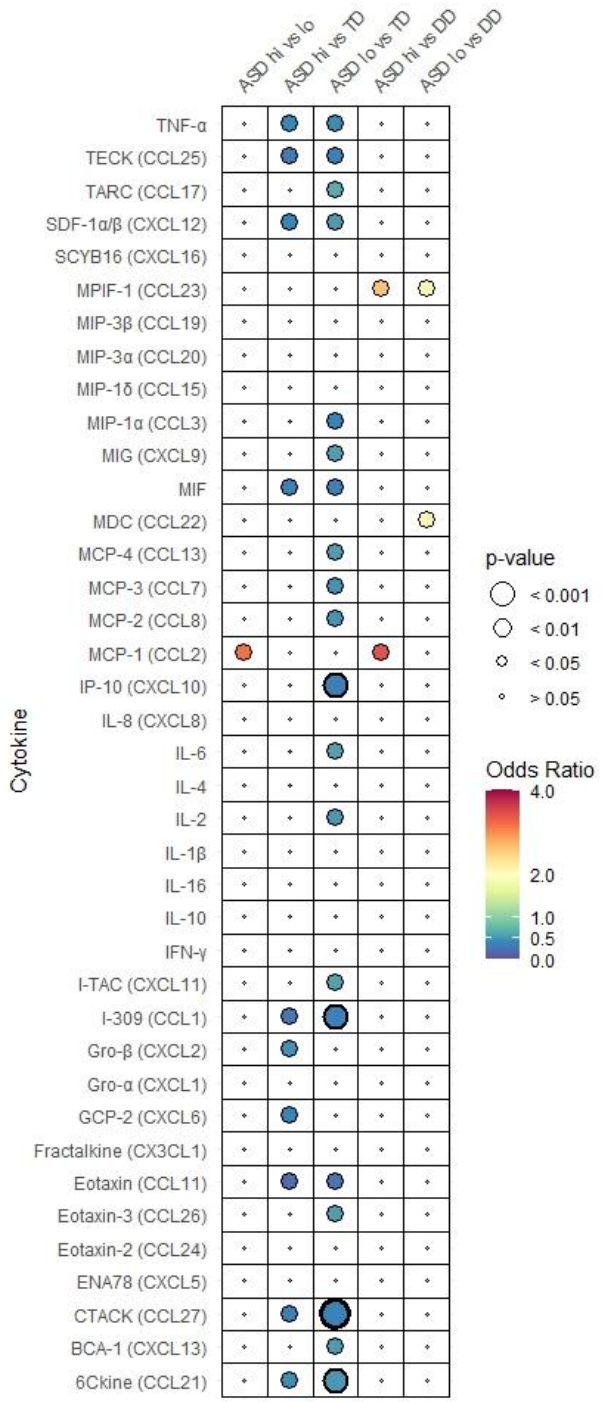

Figure 1C. Adjusted odds ratio plot comparing neonatal cytokine and chemokine concentrations in subgroups of ASD (ASD_{hi}, ASD_{lo}) DD, and TD. Odds ratio is depicted by the heat map with highest ORs in red to lowest in blue. Each figure has its own heat map. Relative P-value is depicted by circle size. P-values that are below 0.001 are bolded.

Supplementary Table 1. Median concentrations and ranges of cytokines and chemokines in eluted blood spots, N=398

	ASD (n=171)			
Cytokine[a]	***Med***	***Min***	***Max***	***<MDL[b]***
6Ckine (CCL21)	48.44	6.47	216.69	0.0
BCA-1 (CXCL13)	1.05	0.10	11.87	0.0
CTACK (CCL27)	17.26	0.91	53.86	0.0
ENA78 (CXCL5)	68.00	0.33	230.03	1.8
Eotaxin (CCL11)	6.59	0.07	12.94	0.6
Eotaxin-2 (CCL24)	2.43	0.03	10.99	2.9
Eotaxin-3 (CCL26)	2.55	0.04	10.83	0.6
Fractalkine (CX3CL1)	16.67	0.73	40.30	0.0
GCP-2 (CXCL6)	10.50	1.48	29.87	0.0
GM-CSF	0.75	0.01	3.69	23.4
Gro-α (CXCL1)	38.85	3.88	100.59	0.0
Gro-β (CXCL2)	52.63	4.47	145.49	0.0
I-309 (CCL1)	6.77	0.90	15.51	0.0
IFN-γ	0.62	0.09	1.89	0.0
IL-1β	1.98	0.16	5.52	0.0
IL-2	0.79	0.04	2.15	0.6
IL-4	0.98	0.05	2.34	0.6
IL-6	1.96	0.03	11.81	0.6
IL-8 (CXCL8)	7.13	1.65	46.62	0.0
IL-10	5.45	0.06	13.88	0.6
IL-16	367.64	30.02	787.48	0.0
IP-10 (CXCL10)	1.39	0.07	6.68	0.6
I-TAC (CXCL11)	21.18	1.07	121.37	0.0
MCP-1 (CCL2)	8.33	0.56	33.32	0.0
MCP-2 (CCL8)	0.92	0.07	5.03	0.0
MCP-3 (CCL7)	7.72	0.09	20.28	0.6
MCP-4 (CCL13)	1.51	0.06	4.11	0.0

	ASD (n=171)			
Cytokine[a]	***Med***	***Min***	***Max***	***<MDL[b]***
MDC (CCL22)	16.87	2.81	48.03	0.0
MIF	46485.24	681.55	93637.94	0.0
MIG (CXCL9)	17.10	1.10	50.20	0.0
MIP-1α (CCL3)	0.63	0.09	1.26	0.0
MIP-1δ (CCL15)	181.03	11.99	921.51	0.0
MIP-3α (CCL20)	1.24	0.01	3.95	0.6
MIP-3β (CCL19)	30.58	1.25	153.55	0.0
MPIF-1 (CCL23)	10.39	0.71	38.23	0.0
SCYB16 (CXCL16)	9.91	0.79	29.93	0.0
SDF-1α/β (CXCL12)	46.73	0.38	111.89	0.6
TARC (CCL17)	26.43	1.97	106.34	0.0
TECK (CCL25)	175.90	10.89	322.81	0.0
TNF-α	3.57	0.01	8.06	0.0
IL-12p70	0.03	0.00	0.22	36.3
IL-13	0.00	0.00	0.22	50.3

[a]Cytokine and chemokine concentrations are presented in pg/mg total protein; values below minimum detection limit (MDL) were replaced by MDL/√2 and then adjusted for total protein; none of the cytokines or chemokines were above the maximum detection limit.

[b]MDL = minimum detection limit; presented at percent below MDL

Supplementary Table 1. Median concentrations and ranges of cytokines and chemokines in eluted blood spots, continued, N=398

	DD (n=69)			
Cytokine[a]	***Med***	***Min***	***Max***	***<MDL***[b]
6Ckine (CCL21)	46.58	0.93	154.00	0.0
BCA-1 (CXCL13)	1.02	0.10	3.35	1.4
CTACK (CCL27)	17.04	0.23	55.65	0.0
ENA78 (CXCL5)	59.67	0.37	209.31	5.8
Eotaxin (CCL11)	6.17	1.80	13.84	0.0
Eotaxin-2 (CCL24)	2.29	0.04	19.61	2.9
Eotaxin-3 (CCL26)	2.41	0.13	13.24	1.4
Fractalkine (CX3CL1)	15.39	0.57	49.91	1.4
GCP-2 (CXCL6)	9.83	0.12	31.74	1.4
GM-CSF	0.58	0.01	3.87	36.2
Gro-α (CXCL1)	37.82	1.08	131.50	0.0
Gro-β (CXCL2)	46.55	0.66	196.12	1.4
I-309 (CCL1)	6.24	1.95	17.59	0.0
IFN-γ	0.58	0.08	2.19	0.0
IL-1β	1.88	0.06	4.62	1.4
IL-2	0.75	0.12	2.00	1.4
IL-4	0.93	0.17	3.62	1.4
IL-6	1.96	0.10	5.19	1.4
IL-8 (CXCL8)	8.23	0.07	29.09	1.4
IL-10	4.99	0.19	16.36	1.4
IL-16	357.13	1.43	811.87	0.0
IP-10 (CXCL10)	1.48	0.62	4.36	0.0
I-TAC (CXCL11)	20.64	0.78	102.83	0.0
MCP-1 (CCL2)	7.88	0.11	20.83	0.0
MCP-2 (CCL8)	0.95	0.04	2.14	1.4
MCP-3 (CCL7)	7.14	0.27	25.18	1.4
MCP-4 (CCL13)	1.66	0.03	6.38	1.4

	DD (n=69)			
Cytokine[a]	***Med***	***Min***	***Max***	***<MDL[b]***
MDC (CCL22)	15.33	0.13	52.91	1.4
MIF	43932.59	8.17	84176.80	0.0
MIG (CXCL9)	15.17	0.26	56.02	1.4
MIP-1α (CCL3)	0.59	0.06	10.00	1.4
MIP-1δ (CCL15)	179.38	0.24	506.78	1.4
MIP-3α (CCL20)	1.13	0.04	2.42	1.4
MIP-3β (CCL19)	27.61	0.29	196.94	1.4
MPIF-1 (CCL23)	8.54	0.14	32.71	1.4
SCYB16 (CXCL16)	10.05	0.07	35.36	1.4
SDF-1α/β (CXCL12)	44.41	1.19	121.92	1.4
TARC (CCL17)	22.10	0.24	208.43	1.4
TECK (CCL25)	166.68	2.96	363.81	1.4
TNF-α	3.37	0.01	12.09	1.4
IL-12p70	0.01	0.00	0.16	39.1
IL-13	0.00	0.00	0.18	66.7

[a]Cytokine and chemokine concentrations are presented in pg/mg total protein; values below minimum detection limit (MDL) were replaced by MDL/√2 and then adjusted for total protein; none of the cytokines or chemokines were above the maximum detection limit.

[b]MDL = minimum detection limit; presented at percent below MDL

Supplementary Table 1. Median concentrations and ranges of cytokines and chemokines in eluted blood spots, continued, N=398

	TD (n=158)			
Cytokine[a]	***Med***	***Min***	***Max***	***<MDL***[b]
6Ckine (CCL21)	57.55	17.65	177.28	0.0
BCA-1 (CXCL13)	1.12	0.42	3.32	0.0
CTACK (CCL27)	19.80	6.80	53.21	0.0
ENA78 (CXCL5)	73.14	0.31	211.64	3.2
Eotaxin (CCL11)	7.10	3.71	13.04	0.0
Eotaxin-2 (CCL24)	2.59	0.04	11.14	1.9
Eotaxin-3 (CCL26)	3.07	0.66	11.50	0.0
Fractalkine (CX3CL1)	16.86	2.99	44.58	0.0
GCP-2 (CXCL6)	10.33	4.51	29.77	0.0
GM-CSF	0.74	0.01	3.13	28.5
Gro-α (CXCL1)	39.31	16.31	90.17	0.0
Gro-β (CXCL2)	56.79	16.59	185.63	0.0
I-309 (CCL1)	7.48	3.62	16.14	0.0
IFN-γ	0.81	0.21	1.99	0.0
IL-1β	1.90	0.79	15.00	0.0
IL-2	0.84	0.36	2.01	0.0
IL-4	1.00	0.41	2.28	0.0
IL-6	2.14	0.58	10.92	0.0
IL-8 (CXCL8)	7.77	2.72	45.75	0.0
IL-10	5.96	0.40	14.90	0.0
IL-16	372.77	123.34	743.10	0.0
IP-10 (CXCL10)	1.55	0.87	3.73	0.0
I-TAC (CXCL11)	23.19	7.68	165.58	0.0
MCP-1 (CCL2)	7.97	1.26	32.54	0.0
MCP-2 (CCL8)	1.07	0.06	2.56	0.0
MCP-3 (CCL7)	8.50	3.04	20.34	0.0
MCP-4 (CCL13)	1.60	0.38	4.67	0.0

	TD (n=158)			
Cytokine[a]	***Med***	***Min***	***Max***	***<MDL[b]***
MDC (CCL22)	17.27	3.50	54.64	0.0
MIF	49414.64	30834.17	98462.56	0.0
MIG (CXCL9)	18.64	3.92	51.41	0.0
MIP-1α (CCL3)	0.65	0.42	17.81	0.0
MIP-1δ (CCL15)	195.55	74.09	567.08	0.0
MIP-3α (CCL20)	1.27	0.62	2.30	0.0
MIP-3β (CCL19)	34.88	0.15	160.24	0.0
MPIF-1 (CCL23)	11.51	1.89	34.12	0.0
SCYB16 (CXCL16)	11.01	2.80	31.74	0.0
SDF-1α/β (CXCL12)	53.31	15.21	116.19	0.0
TARC (CCL17)	29.34	4.46	124.04	0.0
TECK (CCL25)	197.29	91.04	350.86	0.0
TNF-α	3.72	1.42	10.93	0.0
IL-12p70	0.05	0.00	0.15	28.5
IL-13	0.00	0.00	0.17	55.1

[a]Cytokine and chemokine concentrations are presented in pg/mg total protein; values below minimum detection limit (MDL) were replaced by MDL/√2 and then adjusted for total protein; none of the cytokines or chemokines were above the maximum detection limit.

[b]MDL = minimum detection limit; presented at percent below MDL

Supplementary Table 2. Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD severe and ASD mild to moderate symptoms

	ASD_{sev} *vs.* ASD_{mild}		
Cytokine	***OR***	***95% CI***	***P***
6Ckine (CCL21)	0.87	0.47, 1.60	0.66
BCA-1 (CXCL13)	1.13	0.50, 2.53	0.77
CTACK (CCL27)	0.93	0.48, 1.80	0.83
ENA78 (CXCL5)	1.23	0.91, 1.67	0.18
Eotaxin (CCL11)	1.07	0.36, 2.69	0.88
Eotaxin-2 (CCL24)	1.00	0.72, 1.41	0.99
Eotaxin-3 (CCL26)	0.93	0.52, 1.65	0.80
Fractalkine (CX3CL1)	1.22	0.58, 2.59	0.60
GCP-2 (CXCL6)	1.30	0.61, 2.74	0.50
Gro-α (CXCL1)	1.09	0.50, 2.37	0.83
Gro-β (CXCL2)	0.94	0.53, 1.66	0.82
I-309 (CCL1)	1.17	0.44, 3.12	0.75
IFN-γ	1.22	0.71, 2.10	0.47
IL-1β	1.14	0.50, 2.63	0.75
IL-2	1.21	0.53, 2.76	0.65
IL-4	1.06	0.42, 2.72	0.90
IL-6	1.24	0.62, 2.46	0.55
IL-8 (CXCL8)	0.87	0.45, 1.69	0.68
IL-10	0.98	0.50, 1.93	0.96
IL-16	0.91	0.39, 2.12	0.82
IP-10 (CXCL10)	1.21	0.50, 2.94	0.67
I-TAC (CXCL11)	0.79	0.42, 1.48	0.46
MCP-1 (CCL2)	0.62	0.31, 1.24	0.18
MCP-2 (CCL8)	0.87	0.42, 1.80	0.70
MCP-3 (CCL7)	1.02	0.51, 2.04	0.95
MCP-4 (CCL13)	0.73	0.38, 1.40	0.34
MDC (CCL22)	0.80	0.37, 1.70	0.56
MIF	1.18	0.63, 2.22	0.61
MIG (CXCL9)	1.12	0.61, 2.07	0.71

	ASD_{sev} vs. ASD_{mild}		
Cytokine	***OR***	***95% CI***	***P***
MIP-1α (CCL3)	1.73	0.50, 5.99	0.39
MIP-1δ (CCL15)	1.01	0.51, 2.00	0.98
MIP-3α (CCL20)	1.15	0.47, 2.77	0.76
MIP-3β (CCL19)	0.97	0.62, 1.52	0.90
MPIF-1 (CCL23)	0.83	0.47, 1.46	0.51
SCYB16 (CXCL16)	0.83	0.46, 1.51	0.54
SDF-1α/β (CXCL12)	1.27	0.67, 2.40	0.47
TARC (CCL17)	1.01	0.60, 1.68	0.98
TECK (CCL25)	1.35	0.60, 3.04	0.47
TNF-α	0.90	0.46, 1.74	0.75

Multinomial logistic regression models were adjusted for maternal education attainment, gestational age, child's age at blood spot collection, and years from blood spot collection to elution; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); OR represents the fold change in the odds of having one diagnosis relative to another diagnosis or no diagnosis for every 1-unit increase in the ln-transformed cytokine/chemokine (or for every *e*-fold increase in cytokine/chemokine levels); 121 ASD (severe), 50 ASD (mild); ASD severity was defined using ADOS severity scores, where ≥7 indicated severe and <7 indicated mild to moderate symptoms; OR = adjusted odds ratio, CI = confidence interval, P = *P*-value

Chapter 3:

Neonatal Immune Signatures Differ by Sex Regardless of Neurodevelopmental Disorder Status: Macrophage Migration Inhibitory Factor (MIF) Alone Reveals a Sex by Diagnosis Interaction Effect

(Published in Brain, Behavior, and Immunity—Short Communications, 2023)

Highlights

- At birth, female and male newborns are born with different immune profiles regardless of neurodevelopmental outcome.
- Differences in neonatal concentration of macrophage migration inhibitory factor in females and males depend on diagnosis.
- Sex should be considered as a key factor in understanding the mechanisms to developing immune and neuroimmune systems.

Abstract

Immune dysregulation, including aberrant peripheral cytokine/chemokine levels, is implicated in neurodevelopmental disorders (NDD) such as autism spectrum disorder (ASD). While the diagnosis of ASD is more common in males compared to females, sex effects in immune dysregulation related to neurodevelopment remain understudied. The aim of this exploratory study was to determine whether there are sex-specific effects in neonatal immune dysregulation with respect to an ASD or delayed development (DD) diagnosis. We utilized the data from the Early Markers for Autism study, a population based case-control study of prenatal and neonatal biomarkers of ASD. The immune profile of newborns later diagnosed with ASD (n= 482, 91 females), DD (n= 140, 61 females) and sex-matched general population controls (GP; n= 378, 67 females) were analyzed using neonatal bloodspots (NBS) via 42-plex multiplex assay. Multiple linear regression analysis was performed to identify whether sex was associated with differences in cytokine/chemokine levels of children with ASD, DD, and GP. A sex by diagnosis interaction effect was observed only for the chemokine macrophage migration inhibitory factor (MIF), with males displaying higher levels of NBS MIF than females in the GP control group ($p= 0.02$), but not in ASD ($p= 0.52$) or DD ($p= 0.29$) groups. We found that regardless of child diagnosis, newborn blood spot eluates from females had a significantly higher concentration than males with the same diagnosis of the chemokines granulocyte chemotactic protein 2 (GCP-2; $p< 0.0001$), macrophage inflammatory

protein 2-alpha (GROβ; p= 0.002), interferon-inducible t-cell alpha chemoattractant (I-TAC; p< 0.0001), stromal cell-derived factor 1 alpha and beta (SDF-1α-β; p= 0.03), innate inflammatory chemokines interferon-gamma induced protein 10 (IP-10; p= 0.02), macrophage inflammatory protein 1-alpha (MIP-1α; p= 0.02), and the Th1-related pro-inflammatory cytokine interleukin-12 active heterodimer (IL-12p70; p= 0.002). In contrast, males had a higher concentration than females of secondary lymphoid-tissue chemokine (6CKINE; p= 0.02), monocyte chemotactic protein 1 (MCP-1; p= 0.005) and myeloid progenitor inhibitory factor 1 (MPIF-1; p= 0.03). Results were similar when analyses were restricted to NBS from DD and ASD further classified as ASD with intellectual disability (ID), ASD without ID, and DD (GCP-2, p= 0.007; I-TAC, p= 0.001; IP-10, p= 0.005; IL-12p70, p= 0.03 higher in females; MPIF-1, p= 0.03 higher in male). This study is the first to examine sex differences in neonatal cytokine/chemokine concentrations, and whether these differences are associated with neurodevelopmental outcomes. Results highlight the importance of considering sex as a critical factor in understanding the immune system as it relates to child development.

1. Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder with a 4:1 male-to-female ratio [1, 2]. Studies have repeatedly noted sexual dimorphism in both the neurophysiology and behavioral profiles of ASD. For example, global brain connectivity (e.g., cerebellar interactions with other regions of the brain including the cortex) in resting state [3], brain volume [4], brain cytokine/chemokine expression along with glial immunoreactivity activation [5], and the expression and total number of astrocyte- and microglia-related genes in post-mortem autistic brain [6] are different by sex. ASD-related externalizing behaviors are also more common in males compared with females (see review [2]). Despite these apparent anatomical and functional differences in the brain by sex, many studies still focus exclusively on males with ASD given the increased rate of an ASD diagnosis [2]. Consequently, remarkably little research has focused on the reasons for these sex differences in ASD [2]. This information deficit may not

only decrease the development and efficacy of therapeutic interventions, but also has the potential to reduce the precision of biomarker discovery. More studies are warranted that address the sex differences in ASD with respect to both behavior and biology.

Thus far, numerous studies have corroborated peripheral immune dysregulation in the child as well as maternal immune activation in the mother as key features in ASD with potential for predicting an ASD diagnosis [7-11]. As with the sex differences in both behavioral and neuroanatomical aspects of ASD, sex differences in the immune response may also play a role in ASD. For example, females have higher responses in both innate (e.g., toll-like receptor pathways, efficiency of antigen-presenting cells, activation of macrophages and neutrophils, number of natural killer cells) and adaptive (e.g., size of thymus, $CD4^+$ and $CD8^+$ cell counts, cytotoxic activity, B cell counts, antibody production) immunity than males [12]. However, whether this clear biological difference in immunity relates to the sexual dimorphism in ASD still remains understudied.

A better understanding of potential sex differences in immune markers could illuminate the sex-specific behaviors and molecular pathways involved in sex-skewed neurodevelopmental disorders (NDD) such as ASD [3, 13]. Therefore, the primary aim of the current study was to investigate whether child sex is an important factor in predicting the neonatal immune profile of children with ASD and DD compared to general population (GP) controls, and children with ASD with intellectual disability (ASD w/ ID) and ASD without intellectual disability (ASD w/out ID) compared to those with developmental delay (DD).

2. Materials and methods

2.1. Study population

The study population has been previously described in detail [7, 14]. Briefly, the Early Markers for Autism (EMA) study is a large population-based, case-control study, designed to investigate biological markers that contribute to autism risk using archived maternal and neonatal samples. The study sample included three groups of mother-child pairs: ASD, DD, and GP. All study participants were born between March 2000 and July 2003 in Southern California to mothers who participated in California's prenatal

screening program. Case status was determined via record linkage to the California Department of Developmental Services (DDS), which provides services for people with ASD and other developmental disabilities through Regional Centers (RC). GP controls were frequency- and case-matched with ASD in approximately 4:1 male-to-female ratio, whereas, delayed development (DD) was not case matched, having a nearly 1:1 male-to-female ratio. The GP control group was randomly selected from birth certificate files, and frequency matched to ASD cases by sex, birth month, and birth year. All study procedures were approved by the institutional review boards of the California Health and Human Services Agency and Kaiser Permanente of Northern California [7].

2.2. Diagnostic confirmation

Participant diagnostic validation was previously described in detail [7, 14]. Briefly, confirmation of the RC records for children who had received services for ASD or intellectual disability (ID) were reviewed by a developmental pediatrician. Final diagnosis of ASD was determined using the DMS-IV-TR criteria, and final classification of DD was determined using composite scores of standardized cognitive and adaptive test scores less than 70, found in RC records. Children with ASD were further categorized into two groups based on cognitive scores. Those with developmental/cognitive scores and adaptive composite scores below 70 were defined as having ASD w/ ID, and those with both developmental/cognitive scores and adaptive composite scores of 70 and above as ASD w/out ID. The final study population consisted of 482 children with ASD (91 females, 391 males), including 163 ASD w/ ID (30 females, 133 males), 292 ASD w/out ID (59 females, 233 males), and 27 ASD with ID information not available; 140 children with DD only (61 females, 79 males); and 378 GP children (67 females, 311 males).

2.3. Newborn bloodspot collection

Capillary blood samples collected within 72 hours of birth by the heel prick method were spotted onto a standardized filter paper for a newborn screening panel for endocrine, metabolic, and genetic disorders. Bloodspot specimens were then transported without temperature control by courier to a regional screening

laboratory for analysis. Any remaining specimens were catalogued and stored at -20ºC by the California Department of Public Health.

2.4. Newborn bloodspot elution

Dried bloodspot (DBS) specimens were received as three 3 mm punches per subject in a single well of 96 well plates. DBSs were stored at -80ºC until elution. Each sample received 200 μL of elution buffer containing 0.5% bovine serum albumin and 1 tablet of Roche Complete Protease Inhibitor Cocktail (Roche Diagnostics Operations, Indianapolis, IN) in 50 mL phosphate-buffered saline. The samples with elution buffer were eluted overnight at 4ºC in a shaker. 4 μL of eluates per each sample were used for bicinchoninic acid (BCA) assay to determine total protein concentration for normalization. About 40 μL were used for 42-plex Luminex and the remainder of eluates were stored in -80ºC.

2.5. Multiplex assay

Following overnight elution, the DBSs were assessed for 42 peripheral blood immune markers using Luminex Multiplex magnetic bead assays (Bio-Rad Laboratories, Hercules, CA). Two individual single-plex beads (IL-12p70 and IL-13) were mixed with 40-plex beads of Bio-Plex Pro Human Chemokine kit (Bio-Rad Laboratories), and the assay was run according to the manufacturer's directions. Details of the assay methods have been described previously [7]. The complete raw cytokine/chemokine data are available upon request from the corresponding author.

2.6. Statistical analysis

Descriptive statistics, including frequencies, medians, and standard deviations (SD) were used to summarize the socio-demographic and clinical variables and cytokine/chemokine concentrations. All cytokine/chemokine analytes were normalized to the eluate total protein levels based on BCA assay. Cytokines/chemokines that fell below minimum level of detection (MLD) were assigned with MLD/2 and

data were natural log transformed prior to analysis. Multiple linear regression models were fitted to examine potential differences in cytokine/chemokine concentrations by sex and whether diagnosis impacted these differences. Separate models were fit for each cytokine and chemokine, with the cytokine/chemokine as the outcome variable. In these analyses, we started with a model (Model 1) that included main effects for child sex (female/male), diagnosis (ASD, DD, or GP), and their interaction. If the interaction was significant, we estimated sex differences for each diagnosis group (ASD, DD, or GP) from the interaction model. If the sex-diagnosis interaction was not significant, we removed it from the model and refit the model with only main effects for child sex and diagnosis. All models accounted for covariates listed in the previous study that were pertinent to cytokine/chemokine levels, including child's gestational age (days) at birth, birth weight, birth season, birth year, age (in hours) at bloodspot collection, and ethnicity (defined as Hispanic if at least one parent was Hispanic), delivery method, maternal weight, age, education level (less than highschool graduate, highschool graduate, college, post-graduate), birthplace (US, Mexico, other) and race, and Bio-Plex Luminex plate number.

As we previously reported that children with ASD have distinct immune profiles when compared to children with DD (no ASD) [11], in secondary analyses (Model 2) restricted to the ASD and DD children, we subdivided ASD cases into ASD with intellectual disability (ASD w/ ID) and without intellectual disability (ASD w/out ID). We then followed a similar analytic strategy as above in Model 1, first evaluating sex by diagnosis (ASD w/ ID, ASD w/out ID, or DD) interactions in models adjusted for covariates and refitting main effect only models (child sex and diagnosis) if the interaction was not significant.

For each cytokine and chemokine we report the estimate (i.e., beta coefficient) for sex from the multiple linear regression model, representing the adjusted difference in concentration levels between females and males. To facilitate comparisons of the magnitude of the sex effects across the cytokines and chemokines, for all models we calculated standardized effect sizes (Cohen's *d*) that account for imbalanced groups and take covariates into account [15]. We used the following formula:

$$d = \frac{t(n_F + n_M)}{\sqrt{n_F n_M}\sqrt{df}}$$

where *t* is the *t* value obtained for sex (evaluating the female vs. male difference) from the multiple linear regression model, *df* is the degrees of freedom used for the *t* value, and n_F and n_M are the sample sizes for females and males in the respective model.

Models were validated using analytic and graphical techniques to ensure that assumptions (e.g., linearity, homoscedasticity, normality, independence) were adequately met. Tests were two-sided, with $\alpha = 0.05$. All analyses were conducted in SAS OnDemand version 9.4. (SAS Institute Inc., Cary, NC).

3. Results

Descriptive statistics for the participant demographic characteristics and for the raw cytokine/chemokine concentrations and their common nomenclature stratified by diagnosis and sex are presented in Supplementary Tables 1 and 2, respectively. Briefly, there were no differences in delivery method, child birth year and birth season, gestational age, maternal race, maternal weight, or age at bloodspot collection when comparing the ASD, DD, and GP study groups. Maternal education status, child ethnicity, maternal age, and child birth weight were significantly different across the ASD, DD, and GP study groups.

3.1. Sex by diagnosis interaction effect of macrophage migration inhibitory factor (MIF)

Our adjusted multiple regression analysis showed that a sex by diagnosis interaction effect was only present for the chemokine MIF. Sex differences evaluated in each diagnosis group revealed that females exhibited decreased levels of neonatal MIF compared to males only in the GP control group (female vs. male estimate [est.]= -0.09, SE= 0.04, p= 0.01). No differences in the level of MIF by sex were seen in the ASD (est= 0.02, SE= 0.03, p= 0.52) and DD groups (est= 0.05, SE= 0.05, p= 0.29; Table 1 and Figure 1A). Thus, sex differences in neonatal concentration of MIF depend on the child NDD status. Interestingly, the concentration of MIF across groups was more than two orders of magnitude higher than other immune markers measured herein (Supplementary Table 2).

3.2. Sex-specific effect on neonatal cytokine/chemokine levels among the total sample

After removing the non-significant sex by diagnosis terms from the models, 11 chemokines showed a main effect of sex across the diagnosis groups. Female children had higher levels of granulocyte chemotactic protein 2 (GCP-2; est.= 0.08, SE= 0.02, p< 0.0001), macrophage inflammatory protein 2-alpha (GROβ; est.= 0.10, SE= 0.03, p= 0.002,), interferon-inducible t-cell alpha chemoattractant (I-TAC; est.= 0.12, SE= 0.03, p< 0.0001), stromal cell-derived factor 1 alpha and beta (SDF-1α-β; est.= 0.03, SE= 0.02, p= 0.03), interferon-gamma induced protein 10 (IP-10; est.= 0.06, SE= 0.02, p= 0.02), macrophage inflammatory protein 1-alpha (MIP-1α; est.= 0.05, SE= 0.02, p= 0.02), MIP-3α (est= 0.04, SE= 0.02, p= 0.047), and interleukin-12 active heterodimer (IL-12p70; est.= 0.08, SE= 0.02, p= 0.002) than same-diagnosis male children across all groups of ASD, DD, and GP (Table 1). Effect sizes were relatively higher (d > 0.2) for chemokines GCP-2, GROβ, I-TAC, and IL-12p70 than for chemokines SDF-1α-β, IP-10, MIP-1α, and MIP-3α (d < 0.2; Fig. 1A). In the case of secondary lymphoid-tissue chemokine (6CKINE; est.= -0.06, SE= 0.03, p= 0.02), monocyte chemotactic protein 1 (MCP-1; est.= -0.10, SE= 0.03, p= 0.005) and myeloid progenitor inhibitory factor 1 (MPIF-1; est.= -0.10, SE= 0.05, p= 0.03), female newborns had lower concentrations than males with the same diagnosis (Table 1). Chemokine MCP-1 had relatively higher effect size (d > 0.2) than chemokines 6CKINE and MPIF-1 (d < 0.2; Fig. 1A). Thus, female children are born with different immune profiles than male children regardless of later diagnosis of NDD.

3.3. Sex-specific effects in neonatal cytokine/chemokine levels among children with ASD or DD

With previous findings of a distinct neonatal immune profile between DD and ASD [11], we examined ASD w/ID, ASD w/out ID, and DD in analyses adjusted for the same covariates as described above (section 2.6). We first evaluated sex by diagnosis (ASD w/ID, ASD w/out ID, and DD) interaction effects. Since none of the interactions were significant, we removed the sex by diagnosis terms from the

models and examined adjusted models with main effects for sex and diagnosis. Similar results were seen when comparing ASD, DD, GP, where GCP-2 (est.= 0.07, SE= 0.02, p= 0.007), I-TAC (est.= 0.13, SE= 0.04, p= 0.001), IP-10 (est.= 0.08, SE= 0.03, p= 0.005), and IL-12p70 (est.= 0.06, SE= 0.03, p= 0.03) concentrations were higher in females compared to males within the same diagnostic group of ASD w/ ID, ASD w/out ID, and DD (Table 1). The opposite trend was observed in MPIF-1, where females had lower levels of MPIF-1 (est.= -0.13, SE= 0.06, p= 0.03) than males in the same diagnosis group (Table 1). The effect sizes for the statistically significant chemokines ranged from small to medium (0.2-0.5, Fig. 1B). Thus, restricting the analysis to intellectual disability groups did not significantly change the results of sex-specific effects in neonatal immune profiles, corroborating our aforementioned findings.

4. Discussion

In the current exploratory study, we investigated the child sex and neurodevelopmental outcome as factors in predicting neonatal immune profile. Herein, we observed that female and male children, regardless of diagnosis, have distinct neonatal immune profiles. Further, the neonatal levels of the chemokine MIF differ by sex by diagnosis, suggesting its vital role in normal brain development.

Overall, our exploratory analyses demonstrated that female newborns display higher concentrations of neonatal chemokines that are known to be involved in neutrophil, monocyte, and naïve T cell recruitment compared to newborn male children, suggesting immunity differences by sex at birth. Biological sex is a critical factor that modulates the immune response both organically and temporally, as certain immune responses are predominantly displayed during particular developmental time points under the influence of endocrine, genetic, and environmental factors ([2, 12, 16], for review see [17]). Further, it is important to note that fundamental sex differences in the neonatal immune profile set the stage for potentially life long differences in immune function, potentially leading to distinct immune responses to foreign and self-antigens based on biological sex of the individual [12]. For example, activation of genes on the X chromosome that code for innate immune receptors, cytokine receptors, and transcription factors are all differentially expressed by sex [12]. Most innate and adaptive immune cells exert different effects

depending on the type of sex-related hormone exposure, through activation of the hormonal response element-promoter [18]. These immunologic differences could contribute to differences in immune resistance to and clearance of both bacterial and viral infections (see review [18]) as well as differences in susceptibility to autoimmune/inflammatory disorders ([12, 18]). Indeed, our findings corroborate that females may be born with stronger and more active innate immunity than males.

Our findings that female newborns have a higher concentration of several neonatal chemokines than male newborns including GCP-2, GROβ, and SDF-1 may support previous studies on the biological roles of these chemokines. Female fetuses display higher levels of estrogen during the early second trimester which induces a regulatory immune environment by altering endothelial GCP-2 expression and activating *CXCL1* or GROα in the placenta [19, 20]. In addition, estrogen can contribute to immune homeostasis through control of chemokine secretion such as SDF-1 in thymic epithelial cells [21], the expression of which is also detected in the developing brain, particularly in the meninges where SDF-1 guides neural stem cells to migrate, proliferate, and differentiate in different parts of the brain that play a role in the development of corpus callosum, cerebral cortex, and hippocampus [22]. In contrast, male fetuses induce more of an inflammatory placental environment [23], and our results also show that male newborns have higher levels of inflammatory 6CKINE, MCP-1, and MPIF-1 than female newborns. These discrepancies in neonatal chemokines may thus stem from hormonal differences. However, it is important to note that the select chemokines that displayed sex effects at birth (e.g., 6CKINE, GCP-2, GROβ, I-TAC, SDF-1, IP-10, MCP-1, MIP-1α, MPIF-1, IL-12p70) may not behave in their traditional immune roles as viral/bacterial inflammation is typically low in the newborn period. This suggests that these neonatal chemokines may be more related to developmental processes of recruiting cells for cell growth, cell survival, cell adhesion, angiogenesis and apoptosis [24].

To our knowledge, this is the first study to report MIF as a sole indicator of a sex-diagnosis interaction. We observed that male children have higher levels of neonatal MIF than female children only in the GP controls; that is, no sex differences in neonatal MIF levels were observed within the ASD and

DD groups. This suggests that MIF may be a necessary signaling molecule during neurodevelopment both in male and female children, and lower levels of MIF in female children may increase the risk of developing a neurodevelopmental disorder. A clear role for MIF in the developing CNS is largely unknown. However, one *in vitro* study of mouse neural stem/progenitor cells (NSPCs) from the ganglionic eminence and spinal cord-derived neurospheres at embryonic day 14.5 revealed autocrine activity of MIF in NSPCs suggesting that MIF can serve as a proliferation/survival factor of NSPCs without changing cell fate [25]. Abnormal neonatal levels of MIF have repeatedly been associated with an ASD diagnosis, although few studies describe a sex effect or a sex-diagnosis interaction for the chemokine MIF. For example, in a population-based case-control Childhood Autism Risks from Genetics and Environment (CHARGE) study, a study of 398 children, lower neonatal MIF levels were associated with ASD compared to typically developing children, while higher neonatal MIF levels were associated with higher odds of ASD than DD [11]. In addition, higher circulating MIF levels are associated with symptom severity in ASD children [26, 27], and a functional polymorphism in the promotor region of the gene coding for MIF is associated with ASD-related behaviors [27]. These studies collectively suggest a critical role for MIF in healthy neurodevelopment, and our study further suggests that child biologic sex is an important factor in the context of neuroimmune development. Of note, the traditional immune aspects of MIF function seems to be as important as the neuroimmune aspects, particularly in the newborns. It has been demonstrated clinically that at birth, neonates produce and require a higher concentration of neonatal MIF (10-fold higher than adults) to exert a powerful proinflammatory response to stave off neonatal infection and sustain overactivation of immune cells and overproduction of cytokines [28]. In the current study, the concentration of MIF was comparably higher than any other cytokine/chemokine across the three diagnostic groups with a concentration relevant to other newborn clinical studies [28-30]. This signifies a vital role of MIF in the developing immune and neuroimmune systems and further suggests that abnormal levels of neonatal MIF may impede normal development.

We did not anticipate the outcome of this study as it was not initially designed to examine the effects of sex in ASD as the controls were matched to the cases for age and biological sex (resulting in the

same 4:1 male to female ratio) and thus had a male-biased study design. However, even with this restriction, our exploratory findings emphasize the importance of considering the effect of biological sex in neonatal immune status in ASD. To our knowledge, this study is the first to highlight sex-specific immune differences at birth in the context of NDD. Nevertheless, future study designs with the intent of enrolling equal numbers of female participants are warranted to validate our preliminary findings. Moreover, a multidisciplinary investigation that includes genetic, endocrine, immune, and neuroimmune systems as well as longitudinal investigation of sex effects and the sex by diagnosis interaction for early immune differences should be performed to better understand the stability of these factors in NDD.

5. Conclusions

It is important to understand sex differences in immune signatures early in the newborn period have substantial potential to impact various systems throughout the body, including the developing brain. This exploratory study offers preliminary evidence that immune development may differ by sex via differentially expressed neonatal chemokines, and that the chemokine MIF has an interaction effect that may contribute to an ensuing diagnosis of an NDD, including ASD and DD. Our results underscore the importance of considering very early immune differences in NDD by biologic sex and provides the basis for future studies towards a better understanding of the sexual dimorphism in some NDDs.

Declaration of interest

The authors have no conflicts of interest to declare.

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Table 1. Adjusted sex differences (female vs. male) for neonatal cytokine and chemokine concentrations

	Model 1[a]			**Model 2**[b]		
	Estimate	*SE*	*p*-value	Estimate	*SE*	*p*-value
Chemokine						
6CKINE	-0.06	0.03	**0.02**	-0.06	0.04	0.11
BCA-1	-0.02	0.02	0.27	-0.02	0.03	0.50
CTACK	-0.03	0.03	0.22	-0.03	0.03	0.34
Fracktalkine	0.003	0.02	0.88	0.009	0.03	0.73
GCP-2	0.08	0.02	**<.0001**	0.07	0.02	**0.007**
GROa	0.02	0.03	0.43	-0.009	0.03	0.78
GROb	0.10	0.03	**0.002**	0.07	0.04	0.10
I-TAC	0.12	0.03	**<.0001**	0.13	0.04	**0.001**
SCYB16	0.03	0.03	0.42	0.05	0.04	0.22
SDF-1a/1b	0.03	0.02	**0.03**	0.02	0.02	0.28
TARC	-0.03	0.05	0.44	-0.004	0.06	0.94
TECK	0.004	0.01	0.76	0.006	0.02	0.67
Innate inflammatory chemokine						
IL-8	-0.04	0.04	0.31	-0.05	0.05	0.25
IP-10	0.06	0.02	**0.02**	0.08	0.03	**0.005**
MCP-1	-0.10	0.03	**0.005**	-0.07	0.04	0.11
MCP-2	0.02	0.02	0.24	0.04	0.02	0.11
MCP-3	-0.01	0.02	0.70	0.005	0.03	0.86
MDC	0.01	0.02	0.65	0.02	0.03	0.62
MIF (ASD)[c]	0.02	0.03	0.52	0.02	0.02	0.39
MIF (DD)[c]	0.05	0.05	0.29	-	-	-
MIF (GP)[c]	-0.09	0.04	**0.01**	-	-	-
MIG	-0.02	0.03	0.43	-0.003	0.04	0.93
MIP-1a	0.05	0.02	**0.02**	0.02	0.03	0.39
MIP-1d	-0.04	0.04	0.30	-0.01	0.05	0.79
MIP-3a	0.04	0.02	**0.047**	0.02	0.02	0.33
MIP-3b	-0.04	0.04	0.36	-0.009	0.05	0.86
MPIF-1	-0.10	0.05	**0.03**	-0.13	0.06	**0.03**
Innate inflammatory cytokine						
Eotaxin	-0.02	0.02	0.44	-0.01	0.03	0.78
I-309	0.0006	0.02	0.97	-0.008	0.02	0.72
IL-1b	-0.01	0.03	0.74	-0.007	0.03	0.84
IL-6	-0.06	0.03	0.08	-0.04	0.04	0.34
IL-16	0.02	0.03	0.40	-0.007	0.03	0.84
Regulatory						
IL-10	0.01	0.02	0.61	0.01	0.03	0.57
Th1-related						
IL-2	-0.01	0.03	0.70	0.004	0.03	0.91

IL-12p70	0.08	0.02	**0.002**	0.06	0.03	**0.03**

Abbreviations: SE= standard error, ASD= autism spectrum disorder, DD= delayed development, GP= general population. Full names of cytokines/chemokines in Supplementary Table 2.

[a]Model 1 was a multiple linear regression model fitted to a sample of 482 ASD (91 females, 391 males), 140 DD (61 females, 79 males) and 378 GP (67 females, 311 males) children using log transformed neonatal cytokine/chemokine concentrations as dependent variables, included terms for sex and diagnosis and was adjusted for child's gestational age at birth, birth weight, birth season, birth year, age at bloodspot collection and ethnicity, delivery method, maternal weight, age, education level (less than highschool graduate, highschool graduate, college, post-graduate), birthplace (US, Mexico, other) and race, and Bio-Plex Luminex plate number. Estimates represent the adjusted difference in log-transformed concentration levels between the females and males.

[b]Model 2 was a multiple linear regression model fitted to a subsample of children with neurodevelopment disorders (163 ASD w/ ID (30 females, 133 males), 292 ASD w/out ID (59 females, 233 males), and 140 DD (61 females, 79 males), using log transformed neonatal cytokine/chemokine concentrations as dependent variables, included terms for sex and diagnosis and was adjusted for the same variables as used in Model 1. Estimates represent the adjusted difference in log-transformed concentration levels between the females and males.

[c]For the chemokine macrophage migration inhibitory factor (MIF), Model 1 also included the significant interaction between sex and diagnosis (ASD, DD, GP); thus sex differences were estimated for each diagnosis. In Model 2, no significant interaction between sex and diagnosis was observed; the estimate represents the adjusted difference between the females and males in the same diagnosis group.

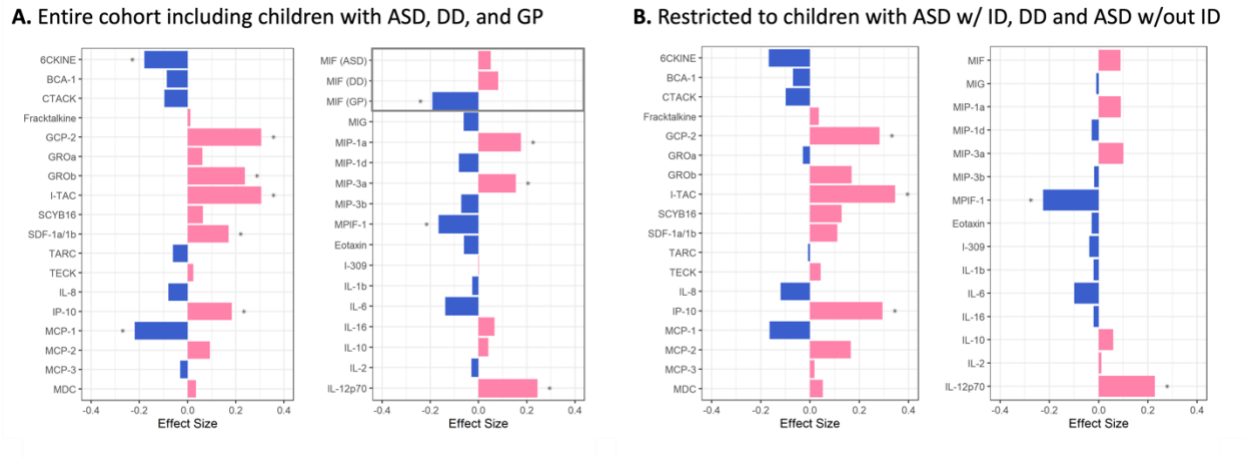

Figure 1. Sex differences in cytokine/chemokine levels at birth. Estimated effect sizes for female vs. male differences in chemokine concentration using the multiple regression models in the **(A)** entire EMA cohort (children with ASD, DD, and GP) and **(B)** restricted to children with intellectual disabilities (DD, ASD w/ ID) and ASD w/out ID. Bars represent the standardized effect sizes (Cohen's *d*) for the female vs. male difference in concentration calculated after accounting for imbalance in the groups and covariates. Cytokine/chemokines with higher concentrations in females than in males are shown in pink (to the right of center); those with higher concentrations in males than in females in blue (to the left). MIF was the only chemokine that demonstrated a sex by diagnosis interaction effect; separate bars are used to represent sex differences in each group (ASD, DD, and GP). Only in the GP control group male children have a higher concentration of this chemokine than female children. Cytokines/chemokines with significant sex difference ($p < 0.05$) are marked with asterisks (*).

Supplementary Table 1. Participant demographics stratified by diagnosis and sex, EMA Study

	ASD (n= 482)		DD (n= 140)		GP[1] (n= 378)	
	Males	Females	Males	Females	Males	Females
	n= 391 (81)	n= 91 (19)	n= 79 (56)	n= 61 (44)	n= 311 (82)	n= 67 (18)
Delivery method						
C-section	109 (28)	28 (31)	24 (30)	16 (26)	93 (30)	11 (16)
Vaginal	282 (72)	63 (69)	55 (70)	45 (74)	218 (70)	56 (84)
Birth year						
2000	74 (19)	13 (14)	20 (25)	13 (21)	64 (21)	9 (13)
2001	102 (26)	23 (25)	22 (28)	15 (25)	78 (25)	20 (30)
2002	156 (40)	43 (47)	31 (39)	22 (36)	124 (40)	30 (45)
2003	59 (15)	12 (13)	6 (8)	11 (18)	45 (14)	8 (12)
Birth season[2]						
Spring	112 (29)	36 (40)	23 (29)	20 (33)	97 (31)	22 (33)
Summer	107 (23)	24 (26)	19 (24)	13 (21)	87 (28)	18 (27)
Fall	91 (23)	14 (15)	15 (19)	14 (23)	71 (23)	13 (19)
Winter	81 (21)	17 (19)	22 (28)	14 (23)	56 (18)	14 (21)
Maternal education						
Less HS graduate	73 (19)	12 (13)	31 (39)	26 (43)	74 (24)	19 (28)
HS graduate	84 (21)	22 (24)	17 (22)	17 (28)	78 (25)	24 (36)
College	163 (42)	42 (46)	24 (30)	16 (26)	111 (36)	17 (25)
Post-graduate	71 (18)	15 (16)	7 (9)	2 (3)	48 (15)	7 (10)
Maternal race						
White	302 (77)	64 (70)	69 (87)	51 (84)	239 (77)	59 (88)
Asian	59 (15)	14 (15)	3 (4)	5 (8)	41 (13)	4 (6)
Other	30 (8)	13 (14)	7 (9)	5 (8)	31 (10)	4 (6)
Child ethnicity						
Hispanic	189 (84)	37 (79)	61 (59)	18 (49)	141 (77)	170 (88)
Non-Hispanic	202 (16)	54 (21)	42 (41)	19 (51)	43 (23)	24 (12)
Maternal age (yrs)	30 ± 6	30 ± 5	26 ± 6	28 ± 6	29 ± 5	28 ± 6
Maternal weight (Lbs)	151.9 ± 35.4	151.5 ± 38.2	152.4 ± 33.3	162.9 ± 40.8	148.3 ± 33.9	157.2 ± 32.7
Gestational age (days)	275 ± 13	275 ± 14	275 ± 16	277 ± 14	278 ± 13	276 ± 14

Age at bloodspot collection (hrs)	31.3 ± 11.9	32.4 ± 11.0	31.8 ± 13.0	34.4 ± 15.2	32.3 ± 11.7	30.3 ± 11.9
Birth weight (g)	3495.6 ± 516.3	3281.3 ± 476.1	3339.3 ± 537.1	3210.5 ± 548.3	3466.3 ± 474.3	3404.7 ± 508.6

Values are n (%) or mean ± SD. ASD, autism spectrum disorder; DD, developmental delay; GP, general population; HS, high school.

[1]GP controls were frequency-matched with ASD cases on age and sex

[2]Winter, December to February; Spring, March to May; Summer, June to August; Fall, September to November

[3]Child was considered Hispanic if at least one of the parents was Hispanic

Supplementary Table 2. Neonatal cytokine/chemokine level ranges by child diagnosis, EMA Study

		ASD					
		Males			Females		
		n= 391			n= 91		
Cytokine/ chemokine	**Alternative names**	Med	Min	Max	Med	Min	Max
C6KINE	Secondary Lymphoid-Tissue Chemokine (CCL21)	68.6	18.7	200.5	62.2	27.5	155.7
BCA-1	B Lymphocyte Chemoattractant (CXCL13)	1.2	0.6	3.7	1.2	0.6	2.1
CTACK	Cutaneous T Cell Attracting Chemokine (CCL27)	22.1	7.8	61.4	23.0	8.5	52.5
EOTAXIN-3	Macrophage Inflammatory Protein 4-alpha (CCL26)	5.2	1.5	12.6	5.2	2.3	13.3
FRACTALKINE	Fractalkine (CX3CL1)	13.2	5.9	28.6	13.8	6.5	27.9
GCP-2	Granulocyte Chemotactic Protein 2 (CXCL6)	8.3	2.8	31.4	8.5	4.6	20.2
GROa	GRO1 Oncogene (CXCL1)	43.5	12.9	156.6	41.7	20.6	118.4
GROb	Macrophage Inflammatory Protein 2-alpha (CXCL2)	52.6	12.2	189.4	52.2	17.0	156.0
I-309	Small Inducible Cytokine A1 (CCL1)	5.6	12.2	9.2	5.6	1.0	9.0
IL-1b	Interleukin-1 beta	0.4	0.03	1.0	0.4	0.1	0.8
IL-2	Interleukin-2	1.3	0.2	2.9	1.3	0.4	3.1
IL-6	Interleukin-6	1.3	0.05	7.6	1.2	0.1	3.6
IL-8	Interleukin-8 (CXCL8)	5.4	1.4	79.5	5.1	2.0	33.2
IL-10	Interleukin-10	2.9	1.0	7.1	3.0	1.4	5.5
IL-16	Interleukin 16	494.2	58.5	1351.5	448.8	112.1	1025.4
IP-10	Interferon-gamma Induced Protein 10 (CXCL10)	4.3	1.2	12.4	4.5	2.3	12.5

I-TAC	Interferon Inducible T-Cell alpha Chemoattractant (CXCL11)	3.4	0.9	13.4	4.2	1.3	14.5
MCP-1	Monocyte Chemotactic Protein 1 (CCL2)	7.3	2.2	63.9	6.7	1.8	25.2
MCP-2	Monocyte Chemotactic Protein 2 (CCL8)	0.9	0.2	2.4	0.9	0.4	2.1
MCP-3	Monocyte Chemotactic Protein 3 (CCL7)	8.2	2.0	17.1	8.4	3.1	19.8
MDC	Macrophage Derived Chemokine (CCL22)	21.5	7.5	47.6	21.9	6.3	45.1
MIF	Macrophage Migration Inhibitory Factor	79980.6	30901.8	381601.1	79831.3	38575.6	281259.0
MIG	Monokine Induced by Gamma Interferon (CXCL9)	14.1	4.3	39.6	14.3	5.6	33.1
MIP-1a	Macrophage Inflammatory Protein 1-alpha (CCL3)	0.7	0.3	7.9	0.7	0.3	1.2
MIP-1d	Macrophage Inflammatory Protein 5 (CCL15)	160.2	25.5	1714.4	167.6	64.9	620.7
MIP-3a	Macrophage Inflammatory Protein 3-alpha (CCL20)	1.0	0.3	3.0	1.1	0.4	1.8
MIP-3b	Macrophage Inflammatory Protein 3-beta (CCL19)	40.0	10.1	155.1	39.0	11.2	148.5
MPIF-1	Myeloid Progenitor Inhibitory Factor 1 (CCL23)	9.2	1.3	49.8	8.3	1.1	31.0
SCYB16	Small-Inducible Cytokine B16 (CXCL16)	9.0	2.6	36.6	9.5	2.6	24.1
SDF-1a/1b	Stromal Cell Derived Factor 1	35.2	15.4	56.9	35.4	16.7	50.5

	alpha and beta (CXCL12)						
TARC	Thymus and Activation Regulated Chemokine (CCL17)	29.7	6.9	138.8	30.8	7.1	123.5
TECK	Thymus Expressed Chemokine (CCL25)	144.1	84.6	267.5	145.5	95.1	255.7
IL-12p70	Interleukin-12 Active Heterodimer	0.3	0.1	0.6	0.3	0.1	0.6

All neonatal cytokine/chemokine levels are in pg/ml

Supplementary Table 2. Neonatal cytokine/chemokine level ranges by child diagnosis, EMA Study, continued

		DD					
		Males			Females		
		n= 79			n= 61		
Cytokine/ chemokine	**Alternative names**	Med	Min	Max	Med	Min	Max
C6KINE	Secondary Lymphoid-Tissue Chemokine (CCL21)	60.4	30.5	183.6	58.0	24.6	171.4
BCA-1	B Lymphocyte Chemoattractant (CXCL13)	1.2	0.5	2.2	1.1	0.4	1.9
CTACK	Cutaneous T Cell Attracting Chemokine (CCL27)	23.4	9.2	53.8	20.7	7.7	63.1
EOTAXIN-3	Macrophage Inflammatory Protein 4-alpha (CCL26)	5.4	2.0	8.8	4.7	1.9	9.6
FRACTALKINE	Fracktalkine (CX3CL1)	13.5	6.1	21.9	13.4	5.9	24.7
GCP-2	Granulocyte Chemotactic Protein 2 (CXCL6)	8.3	3.3	22.8	8.5	4.6	18.3
GROa	GRO1 Oncogene (CXCL1)	44.1	16.3	88.1	39.6	16.8	117.9
GROb	Macrophage Inflammatory Protein 2-alpha (CXCL2)	53.3	11.9	166.5	48.6	10.7	174.7
I-309	Small Inducible Cytokine A1 (CCL1)	5.5	3.6	9.1	5.5	3.2	8.7
IL-1b	Interleukin-1 beta	0.4	0.2	0.8	0.4	0.03	0.8
IL-2	Interleukin-2	1.3	0.4	2.6	1.3	0.3	2.4
IL-6	Interleukin-6	1.3	0.5	8.3	1.2	0.4	2.2
IL-8	Interleukin-8 (CXCL8)	5.7	1.4	19.3	5.0	1.8	21.8
IL-10	Interleukin-10	2.8	1.4	5.3	2.8	1.4	5.8
IL-16	Interleukin 16	451.0	99.5	971.4	408.5	76.4	1099.1

IP-10	Interferon-gamma Induced Protein 10 (CXCL10)	4.5	2.3	14.9	4.6	2.2	14.3
I-TAC	Interferon Inducible T-Cell alpha Chemoattractant (CXCL11)	3.6	1.1	11.1	4.2	1.1	9.9
MCP-1	Monocyte Chemotactic Protein 1 (CCL2)	7.9	1.9	32.6	6.8	1.8	20.5
MCP-2	Monocyte Chemotactic Protein 2 (CCL8)	0.9	0.5	2.0	0.9	0.3	1.7
MCP-3	Monocyte Chemotactic Protein 3 (CCL7)	8.2	3.8	15.1	7.6	3.5	16.0
MDC	Macrophage Derived Chemokine (CCL22)	19.6	7.7	44.5	19.6	8.3	53.7
MIF	Macrophage Migration Inhibitory Factor	72865.3	30273.0	344937.2	71959.2	43250.1	262618.2
MIG	Monokine Induced by Gamma Interferon (CXCL9)	14.4	4.8	29.5	13.3	4.7	30.9
MIP-1a	Macrophage Inflammatory Protein 1-alpha (CCL3)	0.7	0.4	10.0	0.7	0.3	9.6
MIP-1d	Macrophage Inflammatory Protein 5 (CCL15)	197.8	38.8	704.9	156.8	20.9	694.4
MIP-3a	Macrophage Inflammatory Protein 3-alpha (CCL20)	1.0	0.4	1.8	1.0	0.5	3.2
MIP-3b	Macrophage Inflammatory Protein 3-beta (CCL19)	41.5	9.7	102.7	36.1	10.4	108.2
MPIF-1	Myeloid Progenitor Inhibitory Factor 1 (CCL23)	7.7	1.9	29.2	7.1	0.7	19.3
SCYB16	Small-Inducible Cytokine B16 (CXCL16)	9.1	3.2	27.2	9.6	2.9	24.7

SDF-1a/1b	Stromal Cell Derived Factor 1 alpha and beta (CXCL12)	34.1	20.8	49.5	35.5	21.5	53.3
TARC	Thymus and Activation Regulated Chemokine (CCL17)	31.1	5.3	103.1	26.6	7.0	73.7
TECK	Thymus Expressed Chemokine (CCL25)	143.3	105.5	226.5	144.6	94.8	232.1
IL-12p70	Interleukin-12 Active Heterodimer	0.2	0.1	0.4	0.3	0.1	0.4

All neonatal cytokine/chemokine levels are in pg/ml

Supplementary Table 2. Neonatal cytokine/chemokine level ranges by child diagnosis, EMA Study, continued

		GP					
		Males			Females		
		n= 311			n= 67		
Cytokine/ chemokine	**Alternative names**	Med	Min	Max	Med	Min	Max
C6KINE	Secondary Lymphoid-Tissue Chemokine (CCL21)	65.0	23.2	214.5	61.5	23.0	135.6
BCA-1	B Lymphocyte Chemoattractant (CXCL13)	1.1	0.5	2.4	1.1	0.1	1.9
CTACK	Cutaneous T Cell Attracting Chemokine (CCL27)	22.3	7.1	67.1	21.3	6.1	56.3
EOTAXIN-3	Macrophage Inflammatory Protein 4-alpha (CCL26)	5.2	0.6	12.0	4.9	0.2	12.2
FRACTALKIN E	Fracktalkine (CX3CL1)	13.0	4.9	27.9	13.5	0.5	28.9
GCP-2	Granulocyte Chemotactic Protein 2 (CXCL6)	8.2	3.0	22.2	8.9	1.8	23.7
GROa	GRO1 Oncogene (CXCL1)	41.2	17.9	171.1	43.9	19.8	169.7
GROb	Macrophage Inflammatory Protein 2-alpha (CXCL2)	48.6	12.0	174.4	59.8	19.3	163.9
I-309	Small Inducible Cytokine A1 (CCL1)	5.5	1.0	9.4	5.6	1.2	8.0
IL-1b	Interleukin-1 beta	0.4	0.02	1.0	0.4	0.03	0.8
IL-2	Interleukin-2	1.3	0.3	3.3	1.2	0.1	2.1
IL-6	Interleukin-6	1.3	0.5	27.4	1.2	0.02	3.0
IL-8	Interleukin-8 (CXCL8)	5.0	1.4	158.8	5.3	2.2	20.9
IL-10	Interleukin-10	2.8	1.1	7.2	2.7	0.3	6.7
IL-16	Interleukin 16	476.4	60.3	1116.0	510.0	42.5	986.7
IP-10	Interferon-gamma Induced Protein 10 (CXCL10)	4.3	1.8	21.8	4.4	0.6	13.6

I-TAC	Interferon Inducible T-Cell alpha Chemoattractant (CXCL11)	3.6	0.8	13.4	4.1	0.4	11.5
MCP-1	Monocyte Chemotactic Protein 1 (CCL2)	7.3	1.5	28.8	6.8	0.8	18.1
MCP-2	Monocyte Chemotactic Protein 2 (CCL8)	0.9	0.3	4.1	0.9	0.1	1.6
MCP-3	Monocyte Chemotactic Protein 3 (CCL7)	8.1	2.4	18.8	7.7	0.7	13.5
MDC	Macrophage Derived Chemokine (CCL22)	20.5	7.3	77.5	19.6	4.5	47.0
MIF	Macrophage Migration Inhibitory Factor	77478.4	30615.9	2010501.6	66566.6	826.1	235863.5
MIG	Monokine Induced by Gamma Interferon (CXCL9)	13.4	5.2	45.1	12.4	2.7	30.7
MIP-1a	Macrophage Inflammatory Protein 1-alpha (CCL3)	0.7	0.3	6.6	0.8	0.1	6.9
MIP-1d	Macrophage Inflammatory Protein 5 (CCL15)	164.2	65.1	671.2	142.4	40.2	969.7
MIP-3a	Macrophage Inflammatory Protein 3-alpha (CCL20)	1.0	0.4	3.2	1.1	0.1	2.0
MIP-3b	Macrophage Inflammatory Protein 3-beta (CCL19)	37.5	9.7	174.2	33.8	4.2	105.6
MPIF-1	Myeloid Progenitor Inhibitory Factor 1 (CCL23)	8.8	1.2	49.5	9.8	0.3	24.1
SCYB16	Small-Inducible Cytokine B16 (CXCL16)	8.7	2.6	44.2	8.7	2.0	31.9
SDF-1a/1b	Stromal Cell Derived Factor 1	34.5	16.6	56.5	35.8	5.3	54.4

	alpha and beta (CXCL12)						
TARC	Thymus and Activation Regulated Chemokine (CCL17)	28.1	7.7	187.2	24.7	6.1	101.9
TECK	Thymus Expressed Chemokine (CCL25)	145.3	93.2	253.1	151.3	17.2	233.5
IL-12p70	Interleukin-12 Active Heterodimer	0.3	0.003	0.5	0.3	0.01	0.6

All neonatal cytokine/chemokine levels are in pg/ml

Chapter 4:

Neonatal cytokine/chemokine/growth factor response post *in-utero* exposure to COVID-19 and its association to later diagnosis of neurodevelopmental disorder

(*In preparation for submission*)

Abstract

Importance

Despite the prevalence and major concern of COVID-19 in maternal and offspring health, little is known about the impact of COVID-19 during pregnancy on newborn immunity and neurodevelopment.

Objective

To investigate the effects of maternal COVID-19 infection during pregnancy in the developing offspring's immune system and whether altered neonatal immunity due to *in-utero* exposure to COVID-19 leads to a higher risk of subsequent diagnosis of neurodevelopmental disorders (ND) in offspring.

Design

Newborn dried bloodspots from 545 children (277 female, 268 male) born at Kaiser Permanente Northern California (KPNC) from January 2020 to September 2021 were utilized to profile newborn immunity. Data on maternal COVID-19 infection, maternal health status, race, and socioeconomic status during pregnancy to delivery date, and newborn birth date, and birth year were obtained from KPNC EHR databases. COVID-19 infection was defined by COVID-19 laboratory test results (PCR and antibody).

Participants

Initial study started with 550 children with or without *in-utero* exposure to COVID-19. Five children were eliminated from analyses due to their exceeding range of age at bloodspot collection (> 72 h).

Exposures

Maternal COVID-19 infection during pregnancy.

Main outcomes and measures

Positive results for COVID-19 PCR and antibody tests defined maternal COVID-19 infection during pregnancy. The newborn immune profile was analyzed using a 42-plex Multiplex assay on extracted dried bloodspots sampled within 72 hours at birth. ICD-10 identified clinical diagnoses of ND at 18- and 21 months.

Results

Adjusted multiple linear regression models showed that newborns with *in-utero* exposure to COVID-19 (COVID+, N= 460) had significantly higher levels of GM-CSF (p= 0.004) and IL-22 (p= 0.04) than those that did not experience *in-utero* exposure to COVID-19 (COVID-, N= 85) newborns. Sex-stratified analysis showed sex-dependent reactivity to *in-utero* exposure to COVID-19. Within children who had *in-utero* exposure to COVID-19, those later diagnosed with ND had significantly lower levels of IP-10 (p= 0.03), FLT-3L (p=0.04), VEGF (p= 0.02), and IL-22 (p= 0.02) than children with no-ND diagnosis. Sex-stratified analysis within these children who had *in-utero* exposure to COVID-19 and later diagnosed with ND showed a difference in immune profile by sex.

Conclusions and relevance

Our novel results suggest that *in-utero* exposure to COVID-19 leads to altered immunity in newborns in a sex-dependent way, and that significantly decreased levels of select cytokines/chemokines/growth factors originating from maternal COVID-19 infection may impact healthy brain development with a potential subsequent diagnosis of ND in newborns.

Introduction

Numerous clinical and preclinical studies have implied that there are adverse effects associated with maternal immune activation during pregnancy. It is thought that maternal immune dysregulation, such as disruption in homeostatic cytokine/chemokine levels, can directly/indirectly impact the developing fetus, potentially leading to abnormal newborn immune profiles and neurodevelopmental disorders (ND) [1-4].

With the number of pregnant women positive for COVID-19 in the United States still increasing from approximately 226,000 from January 2020 to July 2022 [5], the various consequences of maternal COVID-19 infection during pregnancy in newborns remain largely unknown, particularly relating to the child's developing peripheral immune system and neuro-immune axis. However, to this date, the most recent studies have shown hints or evidence of association between prenatal COVID-19 exposure and neurodevelopmental outcome. For example, a retrospective Massachusetts hospitals study and the COGESTCOV-19 (Cohort of COVID-19 pregnant women and newborns: study of biological and psychological aspects related to neurodevelopment) study showed that children born to COVID-19-positive (COVID+) mothers had an approximately 2-fold increased risk of a diagnosis of altered neurodevelopment[6], and poorer motor development and interactive behaviors [7], respectively. An additional cohort study from Kuwait showed that infants were more likely to develop developmental delays when their mothers were COVID+ during the first and second trimesters than those whose mothers had COVID-19 infection during the third trimester [8]. Despite these epidemiological and clinical studies that support an association between maternal COVID-19 infection during pregnancy and offspring ND, it remains unclear what factors might cause progression to ND due to *in-utero* COVID-19 exposure in the offspring. Remarkably, little is known about the immune aspects of maternal COVID-19 infection during pregnancy and child ND in which transmission of maternal antibodies or maternal cytokines/chemokines are potent effectors that can impact fetal development.

One of the detrimental clinical outcomes of COVID-19 infection is the excessive and uncontrolled levels of circulating pro-inflammatory cytokines, known as a 'cytokine storm', along with immune cell hyperactivation, which can have a negative impact on the life of both pregnant women and the developing fetus [9]. As the vertical transmission of COVID-19 from mom to the fetus is rare [10-12], but specific maternal cytokines/chemokines can pass through the placenta and affect the fetus [13] or the placental environment itself [14], it is of interest whether the maternal immune response originating from COVID-19 infection during pregnancy impacts fetal immune development, including cytokine/chemokine profiles as well as concurrent neurodevelopment.

Using neonatal bloodspot samples of newborns with or without *in-utero* exposure to COVID-19, the current study aimed to investigate whether gestational exposure to COVID-19 impacts cytokine/chemokine/growth factor levels in children at birth, and whether these peripheral levels of neonatal cytokines/chemokines/growth factors are associated with later diagnosis of ND. This is the first study to examine the immunological impact of *in-utero* COVID-19 exposure on newborns and its relation to child ND diagnosis. Further, to our knowledge, this study utilizes the most up-to-date newborn participants born between the first and second wave of the COVID-19 pandemic.

Materials and methods

Participants

Participants had to meet several criteria to be enrolled in this current study: 1) women who were members of the Kaiser Permanente Northern California (KPNC), 2) pregnant in 2020, 3) delivered a liveborn baby, and 4) completed a COVID-19 pregnancy survey. From this group (N~40,000), we identified all women who had a positive COVID-19 PCR test anytime during pregnancy (positive controls) and those that did not have a diagnosis of COVID-19 in their medical record nor report that they had COVID-19 infection on the pregnancy survey (negative controls). Among this group, newborn bloodspots for subsets of COVID-19-positive women (n=465) and COVID-19-negative women (n=85) were obtained from the California Newborn Screening Program specimen archive maintained by the Genetics Diseases Branch (GDB), CDPH.

COVID-19 exposure definition

Maternal COVID-19 infection during pregnancy was ascertained from the COVID-19 laboratory test results (PCR and antibody) recorded in the maternal KPNC EHR during pregnancy.

Child neurodevelopmental outcome definition

All children had at least 21 months of follow-up to screen for a ND. A diagnosis of ND was defined by ICD-10 codes and their corresponding diagnostic categories: autism spectrum disorder (ASD; F84.x), developmental disorders (F80, F81, F82, F88, F89) including motor delay (F82), speech delay (F80, H93.25), and learning disorder (F81, R48), other behavioral disorders (F91, F93, F94, F98), cognitive disorders (F70-F73, F78-F79), and cerebral palsy (G80-G83). The KPNC's universal child developmental screening program screened children at the 18- and 24-month well-child visits using the Developmental Milestones Questionnaire from the validated Survey of Well-Being of Young Children (SWYC) and The Parent's Observation of Social Interactions (POSI) form of the SWYC. Those children who screened positive for developmental or ASD concerns underwent a secondary screening using the Ages of Stages Questionnaire (ASQ) and the Modified Checklist for Autism in Toddlers—Revised with Follow-up (MCHAT-R/F).

Dried neonatal bloodspot collection and elution

Dried newborn bloodspot specimens were obtained by the heel-stick method and spotted onto a standardized filter paper within 48-72 hours of birth. The dried bloodspot specimen was then sampled with 3mm punches, and three 3mm punches per sample were put into single wells in a 96-well plate and stored at -80^{o}C until elution. Upon elution, 200 μl of elution buffer, consisting of phosphate-buffered saline (PBS), 0.5% bovine serum albumin, and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics Corporation, Indianapolis, Indiana), was added to each well. Plates were placed on a plate shaker overnight at 4^{o}C. Eluates were analyzed via Luminex immediately following elution and a short incubation with protease inhibitors, dipeptidyl peptidase IV (DPPIV), and perfubloc in PBS.

Total protein concentration in dried neonatal bloodspot

4 μl aliquot per eluted sample was used for bicinchoninic acid assay (BCA, Thermo Scientific, Rockford, IL) for total protein concentration analysis. Cytokine/chemokine levels per each sample were normalized against each total protein concentration.

Cytokine/chemokine measurement

Cytokine/chemokine levels of bloodspots were measured using a 42-plex cytokine/chemokine/growth factor panel in Bio-Plex Luminex assay (Bio-Rad, Hercules, CA). Cytokines/chemokines/growth factors sCD40L, EGF, eotaxin, FGF-2, FLT-3L, G-CSF, GM-CSF, IFNα2, IFNγ, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-12p40, IL-12p70, IL-13, IL-15, IL-17E/IL-25, IL-18, IL-22, IL-27, IP-10, MCP-1, MCP-3, M-CSF, MDC, MIG, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, TGFα, TNFα, and VEGF, were measured following manufacturer's directions. Refer to Luminex methods in Kim et al. for detailed assay instructions [16]. The complete raw cytokine/chemokine/growth factor database is available upon request from the corresponding author. Before statistical analyses, each analyte concentration was normalized to total protein concentration based on BCA assay to standardize sampling variation in blood collection. Cytokine/chemokine/growth factor concentrations that fell below the lowest detection (LD) were assigned LD/2, and data were natural log-transformed before analyses. Five samples that were missing data or were above 72 h at the age of bloodspot collection were excluded from the analyses.

Statistical analysis

Statistical analyses were conducted using multiple linear regression models [15]. This framework allowed us to examine all the hypotheses of interest while accounting for the effects of other variables.

We first sought to determine potential differences in cytokine/chemokine/growth factor concentrations between neonates born to COVID+ mothers and those born to COVID- mothers. Thus, for each of the log-transformed cytokine/chemokine/growth factor variables, we fitted a model with a term

for maternal COVID-19 infection status (COVID+ or COVID-) and adjusted for child sex, birth season and year (a combination of birth year and months; Winter, January to March; Spring, April to June; Summer, July to September; Fall, October to December), age at bloodspot collection, maternal race, prepregnancy obesity status, length of child insurance, and Luminex plate number (Model 1). The maternal COVID-19 infection status term in this model estimates the adjusted mean concentration difference between children born to COVID+ mothers and those born to COVID- mothers.

Next, we examined whether the effects of *in-utero* exposure to COVID-19 exacerbate with pre-existing maternal health conditions such as gestational diabetes, asthma, infection defined as not COVID-19, and autoimmunity. Model 2 was fitted by further adjusting Model 1 for the aforementioned maternal health conditions.

To examine modification of the association between infection status and cytokine by child sex, we ran Model 1 (adjusted for age at bloodspot collection, birth season and year, maternal race, prepregnancy obesity status, length of child insurance, and Luminex plate number) separately for each sex (Model 3).

To identify whether potential differences in immune markers due to COVID-19 infection during pregnancy were associated with child's ND diagnosis in offspring, Model 4 was fit to data from only the children born to COVID+ mothers and included terms for child ND status, child sex, birth season and year (a combination of birth year and months; Winter, January to March; Spring, April to June; Summer, July to September; Fall, October to December), age at bloodspot collection, length of child insurance, length of follow-up (birth to end of June 2023 for all children), maternal gestational age, maternal infection defined as not COVID-19, and Luminex plate number. To further assess whether the association between altered neonatal immunity and ND diagnosis was sex-dependent, Model 5 was stratified by child sex and adjusted the same base covariates as Model 4.

Results

Participant demographics stratified by maternal COVID-19 infection status during pregnancy

There were no statistical differences in child diagnosis, child sex, birth weight, age at bloodspot collection, child insurance type, maternal race, maternal age, gestational age, maternal prepregnancy asthma, gestational diabetes, allergy, autoimmunity, and infection defined as not COVID-19 when comparing children with COVID+ mothers vs. those with COVID- mothers (Table 1). COVID+ mothers were more likely to be obese than COVID- mothers (p= 0.02), and a significant portion of the COVID+ newborns were born during 2021, whereas COVID- newborns were primarily born during 2020 (p< 0.0001). COVID- mothers were more likely to have their children enrolled in child insurance longer than COVID+ mothers (p <0.0001, Table 1).

The effects of *in-utero* exposure to COVID-19 on newborn immunity

We first wanted to see how maternal COVID-19 infection during pregnancy affects the immune system of the matching offspring. Our adjusted model (Model 1) showed that children born to COVID+ mothers were associated with having increased levels (~31%) of neonatal GM-CSF (estimated COVID+ vs. COVID- adjusted difference [est]= 0.27, standard error [SE]= 0.09, p=0.004) and (~17%) IL-22 (est= 0.16, SE= 0.07, p= 0.04), than those born to COVID- mothers (Table 3). Although statistically insignificant, a similar trend in results were seen for the T cell cytokines IL-2 (est= 0.47, SE= 0.25, p= 0.06) and IL-4 (est= 0.09, SE= 0.05, p= 0.06) (Table 2).

The effects of pre-existing maternal health conditions with COVID-19 infection during pregnancy on newborn immunity

When considering maternal health conditions that have the potential to exacerbate COVID-19 infection during pregnancy, such as gestational diabetes, asthma, infection defined as not COVID-19, and autoimmunity (Model 2), newborns born to COVID+ moms were associated with increased levels of GM-CSF (est= 0.26, SE= 0.09, p= 0.007) and increased levels of IL-22 (est= 0.16, SE= 0.07, p= 0.04) than those born to COVID- moms (Table 2).

Sex-dependent effects of *in-utero* COVID-19 exposure on newborn immunity

Next, we asked whether female and male newborns react differently to COVID-19 exposure *in-utero* (Model 3). Both male and female newborns born to COVID+ moms were associated with having higher neonatal levels of GM-CSF (male: est= 0.29, SE= 0.14, p= 0.04; female: e= 0.31, SE= 0.13, p= 0.01) than same-sex newborns born to COVID- moms. While there were no other immune markers than GM-CSF that had statistically noticeable differences in between male newborns born to COVID+ moms compared to those born to COVID- moms, female newborns exposed to COVID-19 *in-utero* were associated with higher neonatal levels of the T and B cell growth factor IL-7 (est= 0.16, SE= 0.08, p= 0.04) and the cytokine, IL-12p40 (est= 0.71, SE= 0.23, p= 0.006) than those who did not have COVID-19 exposure (Table 3).

Participant demographics of COVID-19-positive newborns and their mothers, stratified by diagnosis

Stratifying by child diagnosis, total length of follow-up was longer in newborns later diagnosed with ND compared to those with no-ND (p= 0.0004), length of follow-up until the first diagnosis or end of the study was different between ND vs. no-ND (p <.0001, Table 4). Child sex (p= 0.004), length of child insurance (p <.0001), and maternal gestational age (p= 0.02) were statistically different between children with ND vs. children with no-ND (Table 4). The rest of the demographic and clinical characteristics were statistically insignificant between children with ND vs. those with no-ND (Table 4).

Comparison of neonatal immune profiles of children with or without ND diagnosis among COVID-19-positive mothers

Next, we investigated whether altered immune profiles in newborns born to COVID+ mothers were associated with subsequent diagnosis of ND in newborns. We restricted the analysis to newborns born to COVID+ mothers. Our adjusted model (Model 4) showed that among children born to COVID+ mothers, children later diagnosed with ND were associated with having lower neonatal levels of the inflammatory

cytokines and chemokines IP-10 (est= -0.13, SE= 0.06, p= 0.03), FLT-3L (est= -0.12, SE= 0.06, p= 0.04), VEGF (est= -0.16, SE= 0.07, p= 0.02), and IL-22 (est= -0.19, SE= 0.08, p= 0.02) than no-ND children (Table 5).

Sex-dependent effects of *in-utero* COVID-19 exposure in children diagnosed with ND

Further, we assessed whether the associated immune profiles with a diagnosis of ND were sex-dependent (Model 5). Among the COVID+ population, male children with ND were associated with having lower neonatal levels of IL-1β (est= -0.61, SE= 0.29, p= 0.04) and IL-22 (est= -0.20, SE= 0.10, p= 0.049) whereas, female children with ND were associated with having lower neonatal levels of the growth factor VEGF (est= -0.31, SE= 0.12, p= 0.009) than same-sex no-ND children (Table 6).

Discussion

We investigated the cytokine/chemokine/growth factor-related neonatal response to *in-utero* exposure to COVID-19 and its relation to a later diagnosis of ND. Our findings show that newborns exhibit higher levels of various functions of cytokines/growth factors following exposure to COVID-19 *in-utero* that are sex-dependent, and that newborns with *in-utero* exposure to COVID-19 and ND diagnosis show lower levels of cytokines/chemokines/growth factors overall compared to those with exposure and no-ND diagnosis.

Our findings imply that maternal COVID-19 infection during pregnancy induce an increase in the growth factor/immunomodulatory GM-CSF and in the Th17 cytokine IL-22 in newborns at birth. The cytokines IL-2 and IL-4 that stimulate growth/development of effector T cells and regulate antibody production showed similar trends but did not meet statistical significance. This was an unexpected combination of cytokines as one might hypothesize that *in-utero* exposure to COVID-19 would primarily elicit induction of a more pro-inflammatory response. However, several studies indicate the association between these select cytokines/chemokines/growth factors with COVID-19 infection. For example, GM-CSF is one of the important and frequently addressed immunomodulatory cytokine/growth factor in lung

inflammation during COVID-19 [16, 17], and increased levels of circulating GM-CSF have been reported in patients with COVID-19 compared with healthy controls [18]. IL-22 is essential for host defense immunity in viral infections, but only at critical levels [19], and COVID-19 patients aged between 2 months and 16 years have shown increased serum IL-22 [20]. In a meta-analysis, IL-2 and IL-4 were also seen increased in COVID-19 patients .[21]. While it is unclear which of these altered neonatal immune profiles found in the current study are explicitly from the newborns and which are maternally derived, considering the observation of mostly positive estimate values throughout the cytokines/chemokines/growth factors panel in newborns, we can infer that maternal COVID-19 infection during pregnancy alters the newborn immune profile and induces heightened levels of cytokines/chemokines/growth factors shown at birth.

Previously, our group reported on sex as a critical factor in developing a neuroimmune system in which female newborns are born with a distinct immune system compared to male newborns regardless of ND outcome [22]. This current study also reveals a sex-dependent immunity in which sex-stratified analyses showed that female and male newborns react differently to *in-utero* exposure to COVID-19, where the direction and magnitude of estimate values differ by each neonatal cytokine/chemokine/growth factor. Particularly for this current study, the female newborns were driving the overall immunity difference between children with *in-utero* exposure to COVID-19 *versus* those without *in-utero* exposure to COVID-19. Epidemiological evidence points to a consistent male-bias vulnerability and severity in COVID-19 infection across ages [23-27] and general differences in innate and adaptive immune responses upon viral infection by sex [28-30]. Although not in the context of cytokines, Bordt et al. also reported sex-specific SARS-CoV-2-specific immunoglobulin G (IgG) titers and SARS-CoV-2-specific placental antibody transfer during maternal COVID-19 infection in which male fetus showed reductions in both of these criteria that was not seen in female fetus [31]. Our findings align with these results and corroborate sexually dimorphic immunity, particularly in peripheral cytokine/chemokine/growth factor molecules, upon COVID-19 exposure during pregnancy.

Recently, several studies reported on the risk of maternal COVID-19 infection in offspring neurodevelopment [14, 32-34]. However, these studies primarily focus on the odds of neurodevelopmental outcomes associated with maternal COVID-19 infection, and little is known about the association between immune profiles at birth and a later diagnosis of ND. To our knowledge, this is the first study to report a relationship between newborn cytokines/chemokines/growth factors immunity post *in-utero* exposure to COVID-19 and subsequent diagnosis of ND. We noted that a child born to a COVID+ mother and was later diagnosed with an ND were associated with reduced levels of several cytokines/chemokines/growth factors than those without ND. Furthermore, the associated patterns of neonatal cytokines/chemokines/growth factors to ND diagnosis were different by sex. Our results suggest a relationship between a deficit in important immune molecules crucial for orchestrating healthy neuroimmune development and neurodevelopmental outcome. Interestingly, some studies have reported similar results to our current study of children with ND having a strong association with lower levels of chemokines and cytokines than typically developing children. For example, a large population-based case-control study showed that newborns later diagnosed with ASD and delayed development had lower levels of IFN-γ, IP-10 and MIG than those with typically developing [35], and in an Italian study, lower levels of VEGF were detected in autistic patients [36]. Together with these findings, maintaining critical levels of cytokines/chemokines/growth factors during development may be necessary for healthy neurodevelopment.

While our study is the first to determine the association between in-utero exposure to COVID-19 and newborn immunity and its association with neurodevelopmental outcomes, several limitations should be noted. This study included many more cases of newborns born to COVID+ moms than those born to COVID- moms. Future studies with a case-matched design is necessary to validate our current findings and to conduct a robust analysis to better understand the relationship with corona virus exposure during pregnancy and child outcome. In addition, while we controlled for follow-up time points for all children, there is a possibility that ND diagnosis in newborns may change over time; therefore, having at least a two-year follow-up for every participant would likely increase the statistical robustness of the study.

Further, while we adjusted for major confounding factors related to maternal and newborn immunity, there may be other important factors that may have accounted for our results such as severity of maternal COVID-19 infection, infection length, infection frequency, the variant of SARS-Co-V-2 infection, and level of antibody against COVID-19 that can concurrently interact with cytokines/chemokines/growth factors to impact neurodevelopment. Lastly, future maternal-newborn dyad study with samples from the mothers, placenta, and newborns may help to better understand the source of cytokines/chemokines/growth factors post COVID-19 infection during pregnancy. Despite these limitations, we were able to determine a significant difference in newborn immunity in general between newborns born to COVID+ moms *versus* those born to COVID- moms, a sex-dependent immunity against *in-utero* exposure to COVID-19, and the difference and association between newborn immunity and a later diagnosis of ND.

Conclusions

Cytokines/chemokines/growth factors are key factors that fundamentally govern the early dynamics of both the innate and adaptive immune response to infection and shape the immune environment. Thus, by comparing both types and levels of select peripheral cytokines/chemokines/growth factors between newborns exposed to *in-utero* COVID-19 and those who were not, this study offers a better understanding of the impact of maternal immune response to infection during gestation and newborn immunity as well as the potential relationship between newborn immune status following *in-utero* exposure to COVID-19 and a subsequent diagnosis of ND.

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Table 1. Demographic and clinical characteristics of newborns and their mothers in the IMPaCT COVID-19 study, stratified by maternal COVID-19 status during pregnancy (N= 545)

	Maternal COVID-19 infection status during pregnancy		**P-value[a]**
	Yes (N=460)	No (N=85)	
Child sex, n (%)			0.45
Female	237 (52)	40 (47)	
Male	223 (48)	45 (53)	
Birth season and year[b], n (%)			**<.0001**
Winter 2020	33 (7)	8 (9)	
Spring 2020	4 (1)	19 (22)	
Summer 2020	30 (7)	17 (20)	
Fall 2020	54 (12)	15 (18)	
Winter 2021	83 (18)	4 (5)	
Spring 2021	119 (26)	6 (7)	
Summer 2021	112 (24)	11 (13)	
Fall 2021	25 (5)	5 (6)	
Birth weight (g), mean (SD)	3388.2 (502.1)	3351.9 (467.6)	0.40
Age (hrs) at bloodspot collection, mean (SD)	23 (7.8)	24.8 (7.6)	0.32
Child insurance type, n (%)			0.22
Commercial	399 (87)	73 (86)	
Government	56 (12)	9 (11)	
Unknown	5 (1)	3 (4)	
Child insurance length (mos), mean (SD)	17.6 (6.8)	19.7 (6.5)	**<.0001**
Maternal race, n (%)			0.40
White	150 (33)	31 (37)	
Hispanic	204 (44)	40 (47)	
Other	106 (23)	14 (16)	
Maternal age (yrs), mean (SD)	34.4 (6)	32.2 (5.6)	0.23
Gestational age (wks), mean (SD)	38.9 (1.4)	38.6 (2.2)	0.44
Maternal prepregnancy asthma, n (%)	72 (16)	12 (14)	0.72

Maternal gestational diabetes, n (%)	48 (10)	8 (9)	0.78
Maternal prepregnancy obesity, n (%)	161 (35)	19 (22)	**0.02**
Maternal prepregnancy allergy, n (%)	85 (18)	17 (20)	0.74
Maternal autoimmunity, n (%)	41 (9)	3 (4)	0.09
Maternal infection defined as not COVID-19 during pregnancy, n (%)	301 (65)	47 (55)	0.07

[a] Group differences were assessed using Chi-square tests for categorical variables and Wilcoxon's rank-sum tests for continuous variables.

[b] Months were grouped by season: Winter = December to February, Spring = March to May, Summer = June to August, Fall = September to November.

Table 2. Adjusted associations between neonatal cytokine/chemokine concentrations and maternal COVID infection status (N= 545)

		Model 1[a]			Model 2[b]		
Function	**Cytokine/ Chemokine/ growth factor**	**Estimate**	**SE**	**P**	**Estimate**	**SE**	**P**
Pro-inflammatory/ pro-inflammatory mediator	sCD40L	-0.04	0.10	0.70	-0.05	0.10	0.59
	Eotaxin	0.04	0.04	0.36	0.03	0.04	0.44
	GROα	0.06	0.06	0.31	0.05	0.06	0.39
	IFNγ	0.13	0.10	0.22	0.13	0.11	0.21
	IL-1α	0.01	0.14	0.92	-0.03	0.14	0.83
	IL-1β	-0.02	0.19	0.90	-0.02	0.19	0.93
	IL-2	0.47	0.25	0.06	0.47	0.25	0.07
	IL-3	0.12	0.09	0.20	0.10	0.09	0.29
	IL-5	-0.16	0.21	0.43	-0.22	0.21	0.30
	IL-6	0.09	0.18	0.61	0.07	0.18	0.71
	IL-7	0.06	0.06	0.26	0.05	0.06	0.38
	IL-8	0.08	0.08	0.33	0.07	0.08	0.40
	IL-12p70	-0.02	0.10	0.85	-0.03	0.10	0.73
	IL-15	0.01	0.06	0.88	0.00	0.06	0.99
	IL-17E/25	0.05	0.07	0.46	0.05	0.07	0.53
	IL-18	0.04	0.07	0.56	0.04	0.07	0.58
	IP-10	0.03	0.06	0.60	0.02	0.06	0.67
	MIP-1b	0.01	0.06	0.80	0.01	0.06	0.80
	MCP-1	0.04	0.08	0.62	0.02	0.08	0.79
	MCP-3	0.04	0.04	0.40	0.03	0.04	0.51
	MDC	-0.02	0.06	0.77	-0.03	0.06	0.66
	MIG	0.06	0.06	0.33	0.05	0.06	0.42
	RANTES	0.04	0.07	0.50	0.03	0.07	0.62
	TNFα	0.00	0.06	0.96	-0.01	0.06	0.85
Growth/proliferation factor	EGF	-0.03	0.20	0.87	-0.03	0.20	0.86
	FGF-2	-0.09	0.10	0.38	-0.10	0.10	0.35
	FLT-3L	0.05	0.05	0.39	0.04	0.06	0.45
	G-CSF	-0.01	0.22	0.95	-0.03	0.22	0.88
	GM-CSF	0.27	0.09	**0.004**	0.26	0.09	**0.007**
	M-CSF	0.06	0.07	0.43	0.05	0.08	0.51
	PDGF AB/BB	0.01	0.06	0.82	0.01	0.06	0.92

	TGFα	0.06	0.11	0.55	0.06	0.11	0.56
	VEGF	0.09	0.06	0.14	0.10	0.06	0.12
Anti-inflammatory	IL-1RA	0.03	0.08	0.70	0.03	0.08	0.70
Repair	IL-22	0.16	0.07	**0.04**	0.16	0.07	**0.04**
	PDGF AA	0.03	0.06	0.60	0.03	0.06	0.65
Pro- and anti-inflammatory	IL-9	0.08	0.05	0.14	0.07	0.05	0.21
	IL-27	0.24	0.34	0.48	0.19	0.34	0.57
	IFNa2	-0.01	0.06	0.91	-0.01	0.06	0.85
Regulatory	IL-4	0.09	0.05	0.06	0.09	0.05	0.06
	IL-12p40	0.28	0.17	0.10	0.24	0.17	0.15
	IL-13	0.07	0.16	0.67	0.02	0.17	0.88

[a] Estimates represent adjusted differences between newborns born to COVID+ mothers (n=460) and those born to COVID- mothers (n=85) from multiple linear regression models fitted to natural log-transformed neonatal cytokine/chemokine/growth factor concentration. The model included a term for maternal COVID-19 infection status during pregnancy and was adjusted for child sex, birth season and year, age at bloodspot collection, maternal race, prepregnancy obesity status, length of child insurance, and Luminex plate number.

[b] Estimates represent adjusted differences between newborns born to COVID+ mothers (n=460) and those born to COVID- mothers (n=85) from multiple linear regression models fitted to natural log-transformed neonatal cytokine/chemokine/growth factor concentration. The model included a term for maternal COVID-19 infection status during pregnancy, all covariates used in Model 1, and was further adjusted for maternal gestational diabetes, maternal asthma, maternal infection defined as not COVID-19, and maternal autoimmunity.

Table 3. Sex-stratified adjusted associations between neonatal cytokine/chemokine/growth factor concentrations and maternal COVID-19 infection status

		Male newborns (N= 268)			**Female newborns** (N= 277)		
Function	**Cytokine/ Chemokine/ growth factor**	**Estimate[a]**	**SE**	**P**	**Estimate[a]**	**SE**	**P**
Pro-inflammatory/ pro-inflammatory mediator	sCD40L	-0.16	0.17	0.34	0.07	0.09	0.47
	Eotaxin	0.03	0.07	0.68	0.04	0.06	0.48
	GROα	0.05	0.09	0.62	0.06	0.08	0.46
	IFNγ	0.13	0.14	0.38	0.14	0.16	0.37
	IL-1α	0.10	0.20	0.62	-0.06	0.21	0.79
	IL-1β	-0.13	0.27	0.63	0.09	0.27	0.75
	IL-2	0.29	0.35	0.41	0.69	0.37	0.06
	IL-3	0.09	0.14	0.51	0.15	0.13	0.26
	IL-5	-0.26	0.28	0.37	0.00	0.32	1.00
	IL-6	0.34	0.25	0.17	-0.19	0.28	0.50
	IL-7	-0.01	0.09	0.86	0.16	0.08	**0.04**
	IL-8	0.11	0.12	0.37	0.00	0.11	0.98
	IL-12p70	-0.13	0.14	0.38	0.09	0.15	0.56
	IL-15	-0.04	0.08	0.65	0.10	0.10	0.30
	IL-17E/25	-0.01	0.10	0.92	0.12	0.12	0.29
	IL-18	0.05	0.12	0.65	0.00	0.09	0.97
	IP-10	0.01	0.08	0.91	0.04	0.08	0.64
	MIP-1b	-0.03	0.09	0.75	0.02	0.08	0.78
	MCP-1	0.07	0.12	0.55	0.00	0.11	0.98
	MCP-3	0.03	0.06	0.64	0.03	0.06	0.58
	MDC	-0.06	0.10	0.54	0.02	0.08	0.80
	MIG	0.02	0.08	0.81	0.10	0.09	0.23
	RANTES	0.00	0.12	0.98	0.07	0.06	0.23
	TNFα	0.00	0.08	0.98	-0.01	0.09	0.94
Growth/proliferation factor	EGF	-0.20	0.29	0.50	0.12	0.27	0.65
	FGF-2	-0.13	0.14	0.37	-0.02	0.15	0.87
	FLT-3L	0.02	0.08	0.81	0.09	0.08	0.25
	G-CSF	0.10	0.31	0.75	-0.20	0.32	0.53
	GM-CSF	0.29	0.14	**0.04**	0.31	0.13	**0.01**

	M-CSF	0.11	0.11	0.34	0.01	0.10	0.96
	PDGF AB/BB	-0.03	0.11	0.80	0.04	0.08	0.64
	TGFα	-0.02	0.16	0.90	0.05	0.15	0.72
	VEGF	0.12	0.09	0.18	0.05	0.10	0.62
Anti-inflammatory	IL-1RA	0.05	0.11	0.68	-0.04	0.11	0.69
Repair	IL-22	0.19	0.10	0.06	0.15	0.11	0.21
	PDGF AA	-0.01	0.09	0.94	0.06	0.07	0.38
Pro- and anti-inflammatory	IL-9	0.05	0.08	0.51	0.09	0.08	0.25
	IL-27	0.03	0.48	0.94	0.46	0.48	0.34
	IFNa2	-0.05	0.09	0.58	0.02	0.08	0.79
Regulatory	IL-4	0.11	0.07	0.14	0.07	0.07	0.30
	IL-12p40	-0.03	0.22	0.88	0.71	0.26	**0.006**
	IL-13	-0.04	0.24	0.88	0.18	0.23	0.43

[a] Estimates represent adjusted differences between newborns born to COVID+ mothers and same-sex newborns born to COVID- mothers from multiple linear regression models fitted to natural log-transformed neonatal cytokine/chemokine/growth factor concentration, fitted separately to females and males. Among 268 male newborns, 223 were born to COVID+ mothers and 45 were born to COVID- mothers. Among female newborns, 237 were born to COVID+ mothers and 40 were born to COVID- mothers. The models included a term for maternal COVID-19 infection status during pregnancy and was adjusted for child birth season and year, age at bloodspot collection, maternal race, prepregnancy obesity status, length of child insurance, and Luminex plate number.

Table 4. Demographic and clinical characteristics of newborns and their mothers who had COVID-19 infection during pregnancy, stratified by child neurodevelopmental disorder status, IMPaCT COVID-19 study (N= 460)

	Child diagnosis		**P-value[a]**
	ND N=61	No-ND N=399	
Follow-up in months[b], mean (SD)	28.7 (3.9)	26.9 (3.8)	**0.0004**
Follow-up until first diagnosis/end of study in months[c], mean (SD)	19.1 (6.0)	26.9 (3.8)	**<.0001**
COVID-19 infection trimester, n (%)			0.25
Trimester 1	9 (15)	94 (24)	
Trimester 2	16 (26)	107 (27)	
Trimester 3	36 (59)	198 (49)	
Child sex, n (%)			**0.004**
Female	21 (34)	216 (54)	
Male	40 (66)	183 (46)	
Birth season and year[d], n (%)			0.06
Winter 2020	6 (10)	27 (7)	
Spring 2020	1 (2)	3 (1)	
Summer 2020	8 (13)	22 (5)	
Fall 2020	8 (13)	46 (11)	
Winter 2021	15 (25)	68 (17)	
Spring 2021	14 (23)	105 (26)	
Summer 2021	6 (13)	104 (26)	
Fall 2021	1 (2)	24 (6)	
Birth weight (g), mean (SD)	3308.0 (595.9)	3400.5 (485.8)	0.13
Age (hrs) at bloodspot collection, mean (SD)	24.7 (7.4)	24.4 (7.6)	0.36
Child insurance type, n (%)			0.66
Commercial	54 (89)	345 (87)	
Government	7 (11)	49 (12)	
Unknown	0 (0)	5 (1)	
Child insurance length (mos), mean (SD)	21.0 (4.2)	17.1 (7.0)	**<.0001**
Maternal race, n (%)			0.85
White	18 (30)	132 (33)	
Hispanic	28 (46)	176 (44)	
Other	15 (25)	91 (23)	
Maternal age (yrs), mean (SD)	32.1 (5.5)	31.4 (5.1)	0.28

Gestational age (wks), mean (SD)	38.7 (2.2)	39.3 (1.4)	**0.02**
Maternal prepregnancy asthma, n (%)	9 (15)	63 (16)	0.84
Maternal gestational diabetes, n (%)	8 (13)	40 (10)	0.46
Maternal prepregnancy obesity, n (%)	24 (39)	137 (34)	0.45
Maternal prepregnancy allergy, n (%)	14 (23)	71 (18)	0.33
Maternal autoimmunity, n (%)	7 (12)	34 (8)	0.34
Maternal infection defined as not COVID-19 during pregnancy, n (%)	45 (74)	256 (64)	0.14

[a] Group differences were assessed using Chi-square tests for categorical variables and Wilcoxon's rank-sum tests for continuous variables.

[b] Length of follow-up for all children is from birth to end of June 2023

[c] Length of follow-up for newborns later diagnosed with ND is from birth to the first ND diagnosis date. Length of follow-up for newborns with no-ND diagnosis is from birth to end of June 2023.

[d] Months were grouped by season as follows: Winter = December to February, Spring = March to May, Summer = June to August, Fall = September to November.

Table 5. Adjusted cytokine/chemokine concentrations in children with ND vs. those with no-ND, among COVID+ (N= 460)

Function	Cytokine/chemokine/ growth factor	Estimate	SE	P
Pro-inflammatory/pro-inflammatory mediator	sCD40L	-0.08	0.11	0.44
	Eotaxin	-0.07	0.05	0.14
	GROα	-0.08	0.07	0.21
	IFNγ	-0.19	0.11	0.08
	IL-1α	-0.02	0.15	0.90
	IL-1β	-0.30	0.20	0.14
	IL-2	-0.19	0.27	0.49
	IL-3	-0.02	0.10	0.87
	IL-5	0.09	0.22	0.68
	IL-6	-0.12	0.20	0.55
	IL-7	-0.07	0.06	0.25
	IL-8	-0.01	0.09	0.90
	IL-12p70	-0.15	0.11	0.18
	IL-15	-0.04	0.06	0.54
	IL-17E/25	-0.13	0.08	0.09
	IL-18	-0.05	0.08	0.51
	IP-10	-0.13	0.06	**0.03**
	MIP-1b	-0.05	0.06	0.40
	MCP-1	-0.03	0.09	0.77
	MCP-3	-0.07	0.05	0.14
	MDC	-0.07	0.07	0.35
	MIG	-0.11	0.06	0.08
	RANTES	-0.06	0.08	0.45
	TNFα	-0.09	0.07	0.18
Growth/proliferation factor	EGF	-0.36	0.21	0.09
	FGF-2	-0.11	0.11	0.30
	FLT-3L	-0.12	0.06	**0.04**
	G-CSF	-0.19	0.23	0.40
	GM-CSF	0.01	0.10	0.93
	M-CSF	-0.03	0.08	0.68
	PDGF AB/BB	-0.10	0.07	0.16
	TGFα	-0.05	0.11	0.63
	VEGF	-0.16	0.07	**0.02**
Anti-inflammatory	IL-1RA	-0.08	0.08	0.35

Repair	IL-22	-0.19	0.08	**0.02**
	PDGF AA	-0.08	0.06	0.21
Pro- and anti-inflammatory	IL-9	-0.04	0.06	0.53
	IL-27	0.07	0.35	0.85
	IFNa2	-0.06	0.06	0.36
Regulatory	IL-4	-0.08	0.05	0.14
	IL-12p40	0.04	0.18	0.81
	IL-13	0.00	0.17	0.99

Abbreviations: neurodevelopmental disorder (ND)

[a] Estimates represent adjusted differences between newborns with an ND diagnosis (N=61) and no-ND newborns (N=399) from multiple linear regression models fitted to natural log-transformed neonatal cytokine/chemokine/growth factor concentration. The model included a term for diagnosis (ND, no-ND) and was adjusted for child sex, birth season and year, follow-up length, age at bloodspot collection, gestational age, length of child insurance, maternal age and race, maternal infection defined as not COVID-19, and Luminex plate number.

Table 6. Sex-stratified adjusted associations between neonatal cytokine/chemokine/growth factor concentrations and child diagnosis, among COVID+ population (N= 460)

Function	Cytokine/ Chemokine/ growth factor	Male newborns (N= 233)			Female newborns (N= 237)		
		Estimate[a]	SE	P	Estimate[a]	SE	P
Pro-inflammatory/ pro-inflammatory mediator	sCD40L	-0.15	0.20	0.46	-0.13	0.11	0.24
	Eotaxin	-0.09	0.07	0.23	-0.13	0.07	0.07
	GROα	-0.10	0.10	0.30	-0.16	0.10	0.11
	IFNγ	-0.30	0.15	0.05	-0.18	0.19	0.32
	IL-1α	-0.26	0.22	0.24	0.22	0.25	0.39
	IL-1β	-0.61	0.29	**0.04**	-0.19	0.34	0.57
	IL-2	-0.41	0.37	0.27	-0.15	0.43	0.72
	IL-3	-0.11	0.15	0.47	0.09	0.16	0.60
	IL-5	-0.02	0.30	0.94	0.14	0.37	0.70
	IL-6	0.00	0.28	0.99	-0.62	0.32	0.05
	IL-7	-0.15	0.09	0.10	-0.05	0.09	0.61
	IL-8	0.02	0.14	0.87	-0.19	0.13	0.15
	IL-12p70	-0.31	0.16	0.05	-0.12	0.18	0.51
	IL-15	-0.06	0.09	0.47	0.02	0.11	0.87
	IL-17E/25	-0.15	0.10	0.14	-0.17	0.14	0.20
	IL-18	-0.14	0.13	0.27	-0.03	0.11	0.81
	IP-10	-0.16	0.08	0.06	-0.18	0.10	0.06
	MIP-1b	-0.06	0.10	0.56	-0.10	0.09	0.31
	MCP-1	-0.12	0.13	0.37	-0.05	0.13	0.70
	MCP-3	-0.09	0.07	0.17	-0.10	0.08	0.17
	MDC	-0.13	0.11	0.24	-0.05	0.10	0.61
	MIG	-0.15	0.09	0.12	-0.12	0.10	0.25
	RANTES	-0.09	0.14	0.52	-0.13	0.07	0.08
	TNFα	-0.06	0.09	0.52	-0.20	0.11	0.06
Growth/proliferation factor	EGF	-0.23	0.33	0.47	-0.30	0.31	0.33
	FGF-2	-0.01	0.15	0.95	-0.19	0.18	0.30
	FLT-3L	-0.13	0.09	0.13	-0.12	0.09	0.19
	G-CSF	-0.11	0.34	0.76	-0.62	0.38	0.10
	GM-CSF	-0.14	0.15	0.35	0.15	0.14	0.28
	M-CSF	-0.02	0.12	0.88	-0.08	0.12	0.48

	PDGF AB/BB	-0.11	0.12	0.35	-0.14	0.09	0.12
	TGFα	-0.06	0.17	0.74	-0.14	0.17	0.39
	VEGF	-0.08	0.09	0.39	-0.31	0.12	**0.009**
Anti-inflammatory	IL-1RA	-0.08	0.13	0.53	-0.07	0.13	0.56
Repair	IL-22	-0.20	0.10	**0.049**	-0.24	0.13	0.07
	PDGF AA	-0.12	0.10	0.21	-0.05	0.09	0.59
Pro- and anti-inflammatory	IL-9	-0.12	0.08	0.15	-0.03	0.10	0.73
	IL-27	0.04	0.51	0.94	-0.50	0.55	0.36
	IFNa2	-0.09	0.09	0.32	-0.11	0.10	0.27
Regulatory	IL-4	-0.10	0.08	0.22	-0.12	0.09	0.17
	IL-12p40	-0.07	0.25	0.79	0.18	0.30	0.54
	IL-13	-0.03	0.26	0.91	-0.11	0.28	0.68

Abbreviations: neurodevelopmental disorder (ND)

[a] Estimates represent adjusted differences between newborns with an ND diagnosis and same-sex no-ND newborns from multiple linear regression models fitted to natural log-transformed neonatal cytokine/chemokine/growth factor concentration, fitted separately to females and males. Among 233 COVID+ male newborns, 40 were newborns with ND diagnosis and 183 were newborns with no-ND diagnosis. Among 237 COVID+ female newborns, 21 were newborns with ND diagnosis and 216 were newborns with no-ND diagnosis. The model included a term for diagnosis (ND, no-ND) and was adjusted for birth season and year, age at bloodspot collection, gestational age, length of child insurance, maternal age and race, maternal infection defined as not COVID-19, and Luminex plate number.

Chapter 5:

The IMPaCT study— Characterizing Longitudinal Patterns of Maternal Immune and Metabolic Profiles during Pregnancy and the Association between Neurodevelopmental Outcomes in the Child

Abstract

Numerous studies have demonstrated strong associations between maternal immune dysregulation and abnormal neurodevelopment in offspring. While much of the supporting data come from animal models and retrospective epidemiological studies, longitudinal prospective clinical studies on characterizing maternal factors associated with maternal gestational inflammation and their association to specific neurodevelopmental outcomes in the child are scarce. We utilized maternal data and serum samples taken during the first (N= 2565) and second trimesters (N= 2565) both of which are critical periods for in fetal neurodevelopment. Analytes associated with immune dysregulation and metabolic disorder were profiled via 48-plex immune and 10-plex metabolic panel Multiplex assays. Maternal immune and metabolic conditions were also examined including allergy, asthma, infection, autoimmune diseases, preeclampsia/eclampsia, hypertension, diabetes, gestational diabetes, and pre-pregnancy obesity. The association between specific patterns of maternal conditions and child neurodevelopmental outcome of autism spectrum disorder (ASD) and delayed development (DD) were examined. Preliminary analyses of the immune panel showed that regardless of child diagnoses, first trimester of pregnancy levels differed compared to second trimester levels in a significant number of maternal immune markers. Throughout pregnancy, maternal immune marker patterns of mothers who went on to have children with ASD were significantly different from mothers who bore children with DD. The growth factors/innate immune responses (e.g., G-CSF, M-CSF, RANTES) were associated with a higher odds of an ASD diagnosis whereas, a DD diagnosis was associated with various types of immune markers related to pro-inflammatory (e.g., sCD40L, INFγ, IL-6, IL-13, IL-17E/IL-25, MIG, MIP-1α), anti-inflammatory (e.g., IL-10), and growth/proliferation (EGF, FGF-2, FLT-3L, VEGF-A). To our knowledge, this is the largest prospective and longitudinal study that includes extensive information on maternal medical history, demographics, gestational immune and metabolic profiles, that examines the association of these factors with a range of neurodevelopmental outcomes. This study will provide a comprehensive examination of maternal gestational health risk factors that are associated with an altered neurodevelopmental outcome in the offspring.

Introduction

Neurodevelopmental disorders (NDD) are a complex group of disabilities that can result through changes in the central nervous system (CNS) leading to behavioral changes and are thought to originate during the developmental period [1, 2]. Impairments in language and speech, learning, motor skills, behavior, and memory are characteristics of children who are diagnosed with NDD, and includes disorders such as autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), intellectual disability (ID), communication disorders, neurodevelopmental motor disorders, and specific learning disorders [1, 2]. Currently, as of 2021, 8.56% of the children in the United States are diagnosed with NDD [3] and the prevalence of NDD is likely to be increased even more. Understanding the dynamics of maternal-fetal interaction during gestation is critical to the assessment of potential contributing factors associated with NDD. Accumulating evidence suggests maternal immune dysregulation (MID) as one of the converging factors that can alter fetal brain development and increase risk for NDD during pregnancy [4-8]. Age, stress, obesity, asthma, gestational diabetes, and autoimmunity are associated with MID and cumulative exposure to these conditions can lead to epigenetic alterations that in the developing fetus [9-14]. These conditions can perturb homeostasis of both the number and type of immune cells in the maternal-fetal compartments, as well as the mediators these cells release such as cytokines and chemokines, all of which can lead to abnormal fetal neurodevelopment. Particularly, cytokines/chemokines are constitutively expressed throughout the fetal brain even in the absence of infection and they play an important role in directing and mediating growth, proliferation, and survival in neuronal, non-neuronal, and immune cells. Thus, it is of importance to characterize the types of MID and its magnitude that can lead to abnormal fetal development and NDD outcome in offspring [4-7, 15].

In this study, we utilized over 5,000 maternal samples taken during the first and second trimesters of pregnancy that included various demographic characteristics and health conditions, to determine the relationship between maternal health conditions and neurodevelopmental outcome of the offspring. We further assessed whether there are specific patterns of maternal health conditions associated with severity

or types of neurodevelopmental outcome in the offspring. To our knowledge, this study is the largest prospective longitudinal study that aimed to validate and characterize the association between maternal health conditions and MID in over 2,500 mothers.

Material and Methods

Participants

The study population (Immune and Metabolic Markers during Pregnancy and Child Development study; IMPaCT) included children born at Kaiser Permanente Northern California (KPNC) from January 2011 to January 2016 who survived to age of two, and their corresponding mothers who received healthcare during the 2 years prior to delivery. KPNC has one of the largest integrated healthcare systems with over 4.5 million members that have sociodemographic profiles representative of the local and state-wide California population.

All mothers consented to participate in the RPGEH pregnancy cohort, to donate their blood samples during the first and second trimesters of pregnancy, and to give permission to access their and their child's KPNC electronic health records (EHR). The current study utilized data on demographic and clinical characteristics of mothers and their children that were retrieved from medical records using the KPNC ENR in December 2019. Descriptive characteristics of the study sample are in Table 1. All study procedures were approved by the KPNC Institutional Review Board.

Child neurodevelopmental outcomes

The study population included children from three neurodevelopmental outcome groups of autism spectrum disorder (ASD, N=354), developmental delay (DD) including intellectual disability, learning disability, cerebral palsy (CP) language delay, and developmental delay (N=1263), and general population (GP) controls without ASD or DD diagnoses (GP, N=948). Depending on the time of diagnosis, Diagnostic and Statistical Manual (DSM) criteria -IV or -V and Autism Diagnostic Observation Schedule (ADOS) were used to diagnose ASD.

Children in the DD group had to have at least one of the diagnoses including intellectual disability, language delay, global delay, learning disorder, motor disorder, or CP, but no diagnosis of ASD recorded in their KPNC EHR. Among the children in the study birth cohort, those who had no diagnoses of ASD or DD recorded in their KPNC EHR were defined as GP control children and were randomly sampled.

Maternal health conditions

Diagnoses of maternal immune-mediated conditions during pregnancy (defined as the time between last menstrual period and date of delivery) were identified from the maternal inpatient and outpatient EHR. These included prenatal infections, asthma, allergy, autoimmune disease, gestational diabetes, preeclampsia, gestational hypertension, pre-existing chronic hypertension, diabetes, and obesity. Pre-pregnancy body mass index (BMI) that was recorded closest to the start of pregnancy was used to determine obesity. Obesity class I was defined as BMI= 30.0-34.9 kg/m^2, class II as BMI= 35.0-39/9 kg/m^2, and class III as BMI≥ 40 kg/m^2. BMI was imputed for mothers with missing BMI (8.4%) using fully conditional specification method.

Immune and metabolic profiling analyses

48 immune markers (e.g., cytokines and chemokines) and 13 metabolic markers were analyzed in serum samples (1st and 2nd trimesters) from mothers using Multiplex detection assays. The 48-plex immune assay included analytes of soluble CD40 ligand (sCD40L), epidermal growth factor (EGF), Eotaxin, fibroblast growth factor 2 (FGF-2), Fms-related tyrosine kinase 3 ligand (FLT-3L), fractalkine, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), growth-related alpha protein (GROα), interferon (IFN) α2, IFNγ, interleukin (IL) 1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IL-27, interferon gamma-induced protein 10 (IP-10), monocyte

chemoattractant protein (MCP) -1, MCP-3, macrophage colony-stimulating factor (M-CSF), macrophage-derived chemokine (MDC), monokine induced by gamma interferon (MIG/CXCL9), macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-AA, PDGF-AB/BB, regulated upon activation, normal T cell expressed and secreted (RANTES), transforming growth factor alpha (TGFα), tumor necrosis factor (TNF) α, TNFβ, and vascular endothelial growth factor (VEGF)-A. 13-plex metabolic panel consisted of C-peptide, active ghrelin, gastric inhibitory polypeptide (GIP), active glucagon-like peptide 1 (GLP-1), glucagon, insulin, leptin, pancreatic polypeptide (PP), gut hormone peptide YY (PYY), and amylin total. Three markers (IL-6, MCP-1, and TNFα) in the 13-plex metabolic panel were redundant from the 48-plex immune panel, and thus were removed in further analyses.

Statistical analysis

Analytes that fell below minimum level of detection (MLD) or had more than 40% of missing values were imputed with MLD/2. Data were natural log transformed prior to statistical analyses. Analyses are still on-going and includes Kmeans data clustering.

Results

Participant demographics

Maternal age ($p= 0.0093$), parity ($p= 0.0408$), plurality ($p< 0.0001$), season of first ($p< 0.0001$) and second ($p< 0.0001$) maternal blood draw, asthma ($p= 0.0031$), GDM ($p= 0.0302$), gestational age at delivery ($p< 0.0001$), and child sex ($p< 0.0001$) were significantly different between child diagnosis of ASD, DD, and GP (Table 1). Other demographic and clinical characteristics had no significant differences between diagnoses (Table 1).

Neurodevelopmental odds associated with maternal concentration of immune markers measured in first and second trimester

We first examined the unadjusted odds associated with a one-unit increase in the natural log-transformed concentrations of cytokines/chemokines for the first and second trimesters. We noted that that regardless of child neurodevelopmental outcome, more of the analytes associated with increased odds of child's neurodevelopmental diagnoses (both ASD and DD) were related to the first trimester rather than the second trimester (Table 2).

ASD vs. GP

In a comparison between mothers who had children with ASD compared to those with GP, we noted that G-CSF, M-CSF, and RANTES were the only growth factors and chemokine that differentiated between the two groups during the first trimester of pregnancy (Table 2). Higher levels of G-CSF (OR= 1.31, 95% Cl 1.07, 1.61) and M-CSF (OR= 1.24, 95% Cl 1.05, 1.46) were each associated with an approximately 1.31- and 1.24-fold increased odds of having an ASD child vs. GP child, respectively (Table 2). RANTES (OR= 2.14, 95% Cl 1.15, 3.98) was associated with 2.14-fold increased likelihood of ASD compared to TD (Table 2). None of these analytes emerged as significant in the second trimester of pregnancy. The chemokine IL-8 was the only analyte that differed between ASD vs. GP during the second trimester of pregnancy where higher levels of IL-8 were associated with a 1.23-fold increased odds of ASD compared to GP (OR= 1.23, 95% Cl 1.02, 1.49) (Table 2).

DD vs. GP

The cytokines/chemokines that showed persistent differences between DD vs. GP diagnoses throughout pregnancy were IL-6, MIG/CXCL9, and VEGF. The ones that differed between DD vs. GP during the first trimester of pregnancy were sCD40L, EGF, FGF, FLT-3L, IFNγ, IL-13, and IL-17E/IL-25 (Table 2). However, after preliminary statistical analyses, we demonstrated that higher levels of the cytokines/chemokines sCD40L (OR= 1.14, 95% Cl 1.03, 1.26), EGF (OR= 1.13, 95% Cl 1.04, 1.23), FGF

(OR= 1.09, 95% Cl 1.00, 1.19), FLT-3L (OR= 1.22, 95% Cl 1.01, 1.47), IFNγ (OR= 1.05, 95% Cl 1.00, 1.11), IL-13 (OR= 1.07, 95% Cl 1.01, 1.14), and IL-17E/IL-25 (OR= 1.16, 95% Cl 1.02, 1.33) were associated with increased likelihood of having a DD child compared to GP child during the first trimester of pregnancy.

During the second trimester, comparably a smaller number of cytokines/chemokines were associated with DD diagnosis compared to GP diagnosis. Higher levels of IL-1α (OR= 1.04, 95% Cl 1.00, 1.09), IL-8 (OR= 1.13, 95% Cl 1.00, 1.28), and IL-27 (OR= 1.21, 95% Cl 1.03, 1.42) were associated with increased odds of having a child with DD compared to GP (Table 2), whereas, lower level of IL-17A (OR= 0.76, 95% Cl 0.60, 0.98) was associated with increased odds of child with DD vs. GP (Table 2).

Discussion

While more descriptive statistical analyses are on-going, our preliminary unadjusted findings suggest that mothers whose child was later diagnosed with either ASD or DD have a distinct maternal immune profile compared to those whose child was in the GP group. Particularly, elevated concentrations of growth and proliferation factors were associated with mothers with children were subsequently diagnosed with ASD, whereas a larger variety of cytokines and chemokines were associated with mothers of children later diagnosed with DD. Although our preliminary findings did not account for maternal health conditions, our preliminary crude analysis implies that differences in mid-gestational immune profile of mothers with ASD or DD children might differentiate between the two neurodevelopmental disorders, suggesting differences in the underlying biology of a child. This presumption may be supported by Goines et al., where they found that increased mid-gestational serum levels of IFNγ, IL-4, and IL-5 were associated with 50% increased risk of ASD, whereas an alternative profile of increased mid-gestational IL-2, IL-4, and IL-6 were associated with increased risk of DD without autism [16]. Further, a study by Jones et al. also observed immunological distinction between mothers of children with ASD with intellectual

disability compared to those of children with DD [17]. Finally, Kim et al. showed that newborns later diagnosed with ASD and DD have dissimilar neonatal immune profiles compared to those that are typically developing [18]. These results imply that there is an etiologically distinct pathology with respect to immune dysregulation between the ASD and DD populations.

Our results indicate that it is primarily during the first trimester that much of the differences in maternal cytokines/chemokines levels seem to be occurring for mothers bearing either an ASD or DD child compared to those bearing typically developing children. In the context of the developing central nervous system (CNS), neurodevelopment is extremely complex during the first trimester. For example, neurulation, neurogenesis, microglial entry, and neuronal migration occur during the first trimester (0-13 gestational weeks) and continue to second trimester (14-27 gestational weeks) [19]. These then set the foundation for further development of the CNS including synaptogenesis, gliogenesis, and myelination [19]. Maternal IL-6, MIP-1α, and VEGF-A may be good examples of presetting fetal development favorable for child's DD diagnosis as our results show persistent association between these maternal cytokines/chemokines with higher odds of DD diagnosis throughout pregnancy. Not much is known about maternal MIP-1α and VEGF-A during pregnancy and its impact in offspring neurodevelopmental outcome. However, our result for IL-6 is in line with other studies where maternal IL-6 during pregnancy is associated with developmental delay-related neuroanatomical aspects of the brain (e.g., brain morphology, functional connectivity, structural connectivity) [20, 21]. To note, our results are unadjusted for covariates pertinent to changes in maternal cytokine/chemokine; thus, some of the association patterns with higher odds of ASD/DD diagnosis might be present and show persistent pattern throughout pregnancy after adjusting for covariates. With our study design, it remains unclear that the abnormal levels of maternally derived cytokines/chemokines can directly transfer to the fetal-placental environment. However, given our findings of abnormal levels of various cytokines/chemokines in mothers bearing ASD/DD child during the early stages of gestation compared to mothers bearing a

typically developing child, it is clear that aberrant levels and profiles of maternal cytokines/chemokines produced likely play a role in the early dynamics of aforementioned processes in brain development.

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Table 1. Characteristics of the IMPACT case-control study sample with maternal cytokine/chemokine measurements during pregnancy, Kaiser Permanente Northern California

Characteristic	All (N=2565) n (%)	ASD (N=354) n (%)	DD (N=1263) n (%)	GP (N=948) n (%)	p
Maternal Age					
Age at birth, mean (SD)	31.31 (5.20)	31.78 (5.41)	31.46 (5.27)	30.92 (5.00)	**0.0093**
Maternal Race					
Asian	534 (20.82)	76 (21.47)	255 (20.19)	203 (21.41)	0.7453
Black	148 (5.77)	26 (7.34)	76 (6.02)	46 (4.85)	
Hispanic	617 (24.05)	79 (22.32)	318 (25.18)	220 (23.21)	
Other	107 (4.17)	14 (3.95)	50 (3.96)	43 (4.54)	
Unknown	16 (0.62)	3 (0.85)	9 (0.71)	4 (0.42)	
White	1143 (44.56)	156 (44.07)	555 (43.94)	432 (45.57)	
Maternal Education					
Less High School	66 (2.57)	5 (1.41)	38 (3.01)	23 (2.43)	0.7013
High School	307 (11.97)	42 (11.86)	159 (12.59)	106 (11.18)	
College	1526 (59.49)	219 (61.86)	741 (58.67)	566 (59.70)	
Post-Graduate	422 (16.45)	52 (14.69)	210 (16.63)	160 (16.88)	
Unknown	244 (9.51)	36 (10.17)	115 (9.11)	93 (9.81)	
Parity					
0	1167 (45.50)	194 (54.80)	554 (43.86)	419 (44.20)	**0.0408**
1	863 (33.65)	97 (27.40)	428 (33.89)	338 (35.65)	
2	366 (14.27)	41 (11.58)	188 (14.89)	137 (14.45)	
3	106 (4.13)	16 (4.52)	57 (4.51)	33 (3.48)	
4+	34 (1.33)	4 (1.13)	19 (1.50)	11 (1.16)	
Unknown	29 (1.13)	2 (0.56)	17 (1.35)	10 (1.05)	
Plurality					**<0.0001**
Singleton	2465 (96.10)	336 (94.92)	1196 (94.70)	933 (98.42)	
Multiple[a]	100 (3.90)	18 (5.08)	67 (5.30)	15 (1.58)	
Gestational age at first maternal blood draw, mean (SD)	8.95 (2.10)	9.17 (1.99)	8.92 (2.13)	8.92 (2.09)	0.1408
Gestational age at second maternal blood draw, mean (SD)	17.44 (2.63)	17.46 (2.68)	17.45 (2.69)	17.43 (2.55)	0.9799
Season of first maternal blood draw					**<0.0001**
Jan-Mar	635 (24.76)	84 (23.73)	299 (23.67)	252 (26.58)	
April-June	613 (23.90)	100 (28.25)	259 (20.51)	254 (26.79)	
July-Sept	561 (21.87)	65 (18.36)	283 (22.41)	213 (22.47)	
Oct-Dec	565 (22.03)	66 (18.64)	270 (21.38)	229 (24.16)	
Missing	191 (7.45)	39 (11.02)	152 (12.03)	0 (0.00)	
Season of second maternal blood draw	552 (21.52)	68 (19.21)	247 (19.56)	237 (25.00)	**<0.0001**

Jan-Mar					
April-June	619 (24.13)	77 (21.75)	276 (21.85)	266 (28.06)	
July-Sept	549 (21.40)	73 (20.62)	233 (18.45)	243 (25.63)	
Oct-Dec	495 (19.30)	57 (16.10)	236 (18.69)	202 (21.31)	
Missing	350 (13.65)	79 (22.32)	271 (21.46)	0 (0.00)	
Maternal Immune-mediated conditions during pregnancy					
Allergy	393 (15.32)	62 (17.51)	201 (15.91)	130 (13.71)	0.1700
Asthma	422 (16.45)	76 (21.47)	215 (17.02)	131 (13.82)	**0.0031**
Autoimmune	262 (10.21)	32 (9.04)	136 (10.77)	94 (9.92)	0.5924
Infection	1261 (49.16)	185 (52.26)	623 (49.33)	453 (47.78)	0.3513
Maternal Cardio-metabolic conditions during pregnancy					
GDM	349 (13.61)	51 (14.41)	191 (15.12)	107 (11.29)	0.0302
Diabetes	36 (1.40)	5 (1.41)	18 (1.43)	13 (1.37)	0.9942
Obesity	685 (26.71)	109 (30.79)	350 (27.71)	226 (23.84)	0.0218
Obesity Class I	372 (14.50)	54 (15.25)	186 (14.73)	132 (13.92)	
Obesity Class II	186 (7.25)	30 (8.47)	99 (7.84)	57 (6.01)	0.0833
Obesity Class III	127 (4.95)	25 (7.06)	65 (5.15)	37 (3.90)	
Preeclampsia	194 (7.56)	33 (9.32)	99 (7.84)	62 (6.54)	0.2099
Hypertension	273 (10.64)	43 (12.15)	137 (10.85)	93 (9.81)	0.4519
Chronic	82 (3.20)	13 (3.67)	42 (3.33)	27 (2.85)	0.7052
Gestational	215 (8.38)	31 (8.76)	109 (8.63)	75 (7.91)	0.8027
Gestational Age at Delivery					
					<0.0001
<35 weeks (very preterm)	61 (2.38)	9 (2.54)	45 (3.56)	7 (0.74)	
35-37 weeks (preterm)	214 (8.34)	39 (11.02)	127 (10.06)	48 (5.06)	
>=38 weeks (term)	2290 (89.28)	306 (86.44)	1091 (86.38)	893 (94.20)	
Child Sex					
					<0.0001
Female	1030 (40.16)	77 (21.75)	469 (37.13)	484 (51.05)	
Male	1535 (59.84)	277 (78.25)	794 (62.87)	464 (48.95)	
Child Year of Birth					
2011	327 (12.75)	33 (9.32)	177 (14.01)	117 (12.34)	0.1212
2012	396 (15.44)	55 (15.54)	206 (16.31)	135 (14.24)	
2013	663 (25.85)	86 (24.29)	325 (25.73)	252 (26.58)	
2014	688 (26.82)	104 (29.38)	319 (25.26)	265 (27.95)	
2015	483 (18.83)	74 (20.90)	230 (18.21)	179 (18.88)	
2016	8 (0.31)	2 (0.56)	6 (0.48)	0 (0.00)	

ASD=Autism Spectrum Disorder; DD=Neurodevelopmental Disorders; GP=General Population controls.
[a] Only one child per multiple pregnancy was included in the analytic dataset.

Table 2. Odds associated with a one-unit increase in the natural log-transformed concentration of cytokines and chemokines measured in first and second trimester maternal serum, restricted to participants with both 1st and 2nd trimester measurements in the IMPACT Study

	ASD vs GP First Trimester	ASD vs GP Second Trimester	DD vs GP First Trimester	DD vs GP Second Trimester
Analyte	**OR_{adj} (95% CI)**	**OR_{adj} (95% CI)**	**OR_{adj} (95% CI)**	**OR_{adj} (95% CI)**
sCD40L	1.03 (0.88-1.20)	1.09 (0.93-1.28)	**1.14 (1.03-1.26)**	1.05 (0.95-1.17)
EGF	1.05 (0.93-1.19)	1.04 (0.94-1.16)	**1.13 (1.04-1.23)**	1.07 (0.99-1.15)
Eotaxin	0.80 (0.54-1.18)	0.66 (0.43-1.02)	0.99 (0.77-1.26)	0.96 (0.73-1.25)
FGF	0.96 (0.84-1.08)	0.89 (0.79-1.02)	**1.09 (1.00-1.19)**	1.03 (0.94-1.12)
FLT-3L	0.95 (0.71-1.27)	0.86 (0.65-1.14)	**1.22 (1.01-1.47)**	1.16 (0.97-1.39)
Fractalkine	0.98 (0.85-1.15)	0.99 (0.86-1.15)	1.07 (0.97-1.19)	1.02 (0.93-1.13)
G-CSF	**1.31 (1.07-1.61)**	1.20 (0.94-1.52)	1.08 (0.97-1.20)	1.12 (0.98-1.28)
GM-CSF	0.99 (0.92-1.06)	0.99 (0.92-1.07)	1.03 (0.99 (1.09)	1.04 (0.99-1.10)
GROa	1.09 (0.88-1.34)	1.10 (0.88-1.38)	1.09 (0.98-1.22)	0.99 (0.89-1.10)
IFNa2	1.02 (0.94-1.10)	1.03 (0.94-1.12)	1.02 (0.97-1.08)	0.99 (0.94-1.04)
IFNg	0.95 (0.88-1.02)	0.95 (0.87-1.04)	**1.05 (1.00-1.11)**	1.03 (0.98-1.09)
IL-1a	1.02 (0.95-1.09)	1.01 (0.94-1.08)	1.02 (0.97-1.06)	**1.04 (1.00-1.09)**
IL-1b	1.03 (0.93-1.14)	1.00 (0.87-1.15)	1.05 (0.98-1.12)	1.08 (0.98-1.19)
IL-1RA	1.02 (0.80-1.30)	0.94 (0.73-1.21)	1.08 (0.93-1.25)	1.03 (0.88-1.21)
IL-4	1.08 (0.93-1.25)	1.06 (0.91-1.23)	1.08 (0.99-1.17)	1.03 (0.95-1.12)
IL-5	1.08 (0.93-1.26)	1.07 (0.91-1.25)	1.06 (0.97-1.17)	1.01 (0.92-1.12)
IL-6	1.06 (0.96-1.18)	0.99 (0.89-1.09)	**1.09 (1.02-1.16)**	**1.07 (1.00-1.15)**
IL-7	0.99 (0.83-1.18)	0.96 (0.80-1.15)	1.02 (0.91-1.14)	1.05 (0.93-1.18)
IL-8	1.10 (0.91-1.33)	**1.23 (1.02-1.49)**	1.06 (0.93-1.19)	**1.13 (1.00-1.28)**
IL-9	0.99 (0.92-1.07)	1.02 (0.94-1.10)	1.03 (0.98-1.08)	1.00 (0.96-1.05)
IL-10	1.00 (0.95-1.05)	0.99 (0.94-1.05)	1.02 (0.99-1.06)	1.01 (0.98-1.04)
IL-12p40	0.96 (0.82-1.13)	0.93 (0.80-1.08)	1.09 (0.97-1.22)	1.02 (0.92-1.13)
IL-12p70	0.97 (0.92-1.02)	0.98 (0.93-1.03)	1.01 (0.98-1.05)	1.01 (0.98-1.04)
IL-13	1.02 (0.94-1.11)	1.02 (0.93-1.11)	**1.07 (1.01-1.14)**	1.03 (0.97-1.09)
IL-15	1.02 (0.89-1.18)	1.02 (0.87-1.19)	1.02 (0.94-1.12)	1.03 (0.93-1.14)
IL-17E/IL-25	1.15 (0.94-1.41)	1.03 (0.85-1.24)	**1.16 (1.02-1.33)**	1.12 (0.98-1.29)
IL-17F	1.03 (0.96-1.11)	1.00 (0.93-1.07)	1.02 (0.98-1.07)	1.03 (0.98-1.08)
IL-18	0.99 (0.84-1.18)	0.97 (0.81-1.15)	0.96 (0.86-1.07)	0.98 (0.87-1.10)
IL-27	1.16 (0.91-1.49)	1.14 (0.90-1.44)	1.13 (0.96-1.33)	**1.21 (1.03-1.42)**
IP-10	1.00 (0.75-1.33)	0.99 (0.74-1.32)	1.14 (0.94-1.38)	1.16 (0.96-1.42)

MCP-1	0.94 (0.63-1.40)	0.95 (0.63-1.41)	1.27 (0.98-1.64)	1.26 (0.97-1.64)
MCP-3	0.97 (0.76-1.24)	0.92 (0.72-1.18)	1.12 (0.96-1.32)	1.08 (0.92-1.27)
M-CSF	**1.24 (1.05-1.46)**	1.22 (0.99-1.49)	1.05 (0.96-1.15)	1.07 (0.95-1.21)
MDC	1.44 (0.85-2.45)	1.40 (0.82-2.38)	1.26 (0.91-1.74)	1.18 (0.85-1.64)
MIG/CXCL9	0.91 (0.70-1.19)	0.96 (0.74-1.25)	1.15 (0.97-1.35)	1.12 (0.95-1.32)
MIP-1	1.00 (0.90-1.11)	1.01 (0.91-1.14)	**1.07 (1.00-1.14)**	**1.07 (1.00-1.15)**
MIP-1b	0.93 (0.64-1.35)	0.96 (0.67-1.39)	1.09 (0.86-1.39)	1.07 (0.84-1.36)
PDGF-AA	0.95 (0.64-1.40)	0.92 (0.61-1.40)	1.22 (0.93-1.59)	1.14 (0.87-1.50)
PDGF-AB/BB	1.20 (0.65-2.23)	1.07 (0.59-1.94)	0.96 (0.64-1.43)	1.02 (0.69-1.51)
RANTES	**2.14 (1.15-3.98)**	1.63 (0.86-3.08)	1.09 (0.82-1.45)	1.06 (0.76-1.48)
TGFa	1.03 (0.85-1.23)	0.91 (0.74-1.11)	1.09 (0.97-1.23)	1.07 (0.94-1.22)
TNFa	0.92 (0.75-1.13)	0.97 (0.78-1.20)	1.06 (0.94-1.21)	1.02 (0.89-1.17)
TNFb	0.97 (0.93-1.02)	0.98 (0.93-1.04)	1.00 (0.97-1.04)	0.98 (0.95-1.02)
VEGF-A	1.05 (0.86-1.29)	1.14 (0.88-1.48)	**1.23 (1.09-1.40)**	**1.27 (1.07-1.52)**
For Missing more than 40% - Detected vs not detected				
IL-17A	0.73 (0.50-1.08)	0.71 (0.48-1.04)	0.90 (0.71-1.15)	**0.76 (0.60-0.98)**
IL-2	0.76 (0.54-1.05)	0.80 (0.58-1.11)	1.03 (0.84-1.27)	1.01 (0.82-1.24)
IL-22	1.14 (0.82-1.58)	1.09 (0.78-1.50)	1.03 (0.84-1.27)	1.04 (0.85-1.28)
IL-3	1.09 (0.49-2.40)	1.52 (0.69-3.38)	0.87 (0.49-1.54)	1.11 (0.62-1.98)

Logistic regression models were adjusted for gestational age at time of blood collection, maternal pre-pregnancy BMI, maternal age, maternal race/ethnicity, season of blood collection, and plate number.