Title

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Neonatal immune signatures differ by sex regardless of neurodevelopmental disorder status: macrophage migration inhibitory factor (MIF) alone reveals a sex by diagnosis interaction effect

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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

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Declaration of interest

The authors have no conflicts of interest to declare.

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.

Keywords

newborn; immune; cytokine; chemokine; MIF; sex; sex by diagnosis; neurodevelopment; neurodevelopmental disorder

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 −20°C 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 −80°C 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 4°C 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 −80°C.

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 nomeclature 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 C6KINE 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 differs 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 C6KINE, 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., C6KINE, 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, particularly in male children, and lower levels of MIF in male 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 sigantures 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 diagnoses 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- At birth, female and male newborns are born with significantly different immune profiles (e.g., cytokines, chemokines) regardless of diagnosis including autism spectrum disorder, developmental delay, and typical development.
- In this study, macrophage migration inhibitory factor (MIF) was the only chemokine that displays sex by diagnosis interaction effect in which its neonatal concentration differences between female and males depend on diagnosis, with males displaying higher levels than females in the control group only.

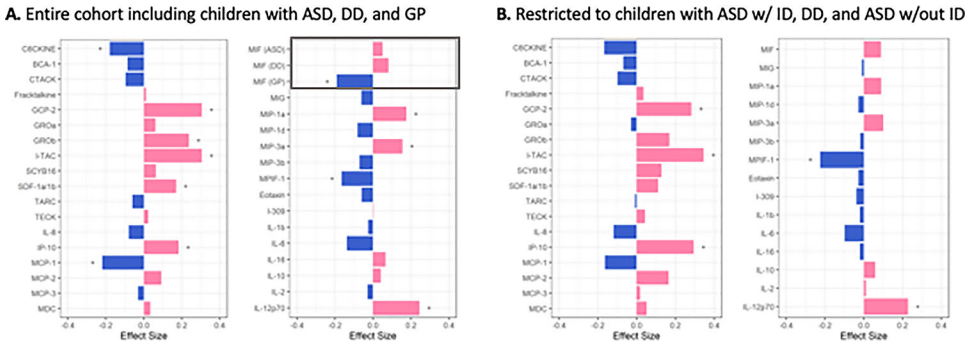

Figure. 1. Sex differences in cytokine/chemokine levels at birth.

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). (A) MIF was the only chemokine that showeddemonstrated a sex by diagnosis interaction effect (gray box outline); separate bars are used to represent sex differences in each group (ASD, DD, and GP). Only in the GP control group male children had a higher concentration of this chemokine than female children. Cytokines/chemokines with significant sex difference ($p < 0.05$) are marked with asterisks(*).

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						
C6CKINE	−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

	Model 1[a]			Model 2[b]		
	Estimate	***SE***	***p*-value**	**Estimate**	***SE***	***p*-value**
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.