Title
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Permalink
https://escholarship.org/uc/item/1ch6278c

Journal
Molecular psychiatry, 22(2)

ISSN
1476-5578

Authors
Jones, KL
Croen, LA
Yoshida, CK
et al.

Publication Date
2017-02-01

DOI
10.1038/mp.2016.77

Peer reviewed
Autism with Intellectual Disability is Associated with Increased Levels of Maternal Cytokines and Chemokines During Gestation

Karen L. Jones1,2, Lisa A. Croen3, Cathleen K. Yoshida3, Luke Heuer1,2, Robin Hansen2,4, Ousseny Zerbo3, Gerald N. DeLorenze3, Martin Kharrazi5, Robert Yolken6, Paul Ashwood2,7, and Judy Van de Water1,2
1Department of Internal Medicine, Division of Rheumatology, Allergy, and Clinical Immunology, University of California, Davis, California, USA
2MIND Institute, University of California, Davis, California, USA
3Division of Research, Kaiser Permanente Northern California, Oakland, California, USA
4Department of Pediatrics, University of California, Davis, California, USA
5Environmental Health Investigations Branch, California Department of Public Health, Richmond, California, USA
6Stanley Division of Developmental Neurovirology, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
7Department of Medical Microbiology and Immunology, University of California, Davis, California, USA

Abstract

Immune abnormalities have been described in some individuals with autism spectrum disorders (ASD) as well as their family members. However, few studies have directly investigated the role of prenatal cytokine and chemokine profiles on neurodevelopmental outcomes in humans. In the current study, we characterized mid-gestational serum profiles of 22 cytokines and chemokines in mothers of children with ASD (N=415), developmental delay without ASD (DD) (N=188), and general population (GP) controls (N=428) using a bead-based multiplex technology. The ASD group was further divided into those with intellectual disabilities (DQ<70) (ASD+ID, N=184) and those without (DQ≥70) (ASD-noID, N=201). Levels of cytokines and chemokines were compared between groups using multivariate logistic regression analyses, adjusting for maternal age, ethnicity, birth country, and weight, as well as infant gender, birth year, and birth month. Mothers of children with ASD+ID had significantly elevated mid-gestational levels of numerous cytokines and chemokines, such as GM-CSF, IFN-γ, IL-1α, and IL-6, compared to mothers of children with...
either ASD-noID, those with DD, or GP controls. Conversely, mothers of children with either ASD-noID or with DD had significantly lower levels of the chemokines IL-8 and MCP-1 compared to mothers of GP controls. This observed immunologic distinction between mothers of children with ASD+ID from mothers of children with ASD-noID or DD suggests that the intellectual disability (ID) associated with ASD might be etiologically distinct from DD without ASD. These findings contribute to the ongoing efforts toward identification of early biological markers specific to sub-phenotypes of ASD.

Introduction

Autism spectrum disorders (ASD) are a set of neurodevelopmental disorders classified by core impairments in socio-communicative behaviors that are accompanied by repetitive and stereotyped behaviors\(^1\). Currently, it is estimated that ASD affects 1 in 68 children in the United States, with the average age of diagnosis at approximately four years\(^2\). Despite increasing prevalence rates and awareness of the disorder, the etiology of ASD is unknown. While genetic factors are thought to play an important role in the etiology of ASD, recent evidence suggests that environmental influences, particularly during gestation or early postnatal periods, also contribute to the development of ASD\(^3\)–\(^5\).

One such environmental contributing factor for ASD is immune system dysregulation, which has been frequently described in individuals with ASD as well as their family members. For example, a family history of autoimmunity, particularly in mothers, has been significantly associated with an increased risk of ASD\(^6\)–\(^8\). Prenatal immune challenges, such as bacterial or viral infections, have also been identified as a potential risk factor of ASD\(^9\)–\(^11\). Immune associated findings reported in ASD include marked neuroinflammation in post-mortem brain tissues\(^12\)–\(^16\), immunoglobulin imbalances\(^17\)–\(^19\), increased numbers of monocytes\(^20\),\(^21\), altered cytokine and chemokine profiles\(^22\)–\(^25\), and autism-specific maternal antibodies reactive to fetal brain proteins\(^26\),\(^27\).

Under normal conditions, the maternal immune system is uniquely regulated during pregnancy to maintain a pathogen-free, yet non-inflammatory, environment for the developing fetus\(^28\),\(^29\). However, factors including cytokines, chemokines, and antibodies produced during gestation can have developmental consequences for the fetus. For example, some maternal cytokines may cross the placenta during gestation, as in the case of IL-6\(^30\)–\(^32\), or act on placental cells to stimulate the downstream production of immune mediators in the fetal compartment\(^33\). Maternal cytokines and chemokines influence several diverse aspects of typical neurodevelopment, including proliferation and differentiation of neural and glial cells\(^34\). However, fluctuations in the levels of these cytokines and chemokines either upwards or downwards is thought to alter the normal neurodevelopmental trajectory, possibly resulting in altered brain morphology and behavior in the offspring.

A limited number of studies have investigated the potential association between maternal mid-gestational cytokines and chemokines and ASD. We previously examined this relationship and found increased levels of IFN-\(\gamma\), IL-4, and IL-5 in 84 mothers of children with ASD relative to 159 mothers of general population (GP) control children\(^35\). In addition, we found increased mid-gestational levels of IL-2, IL-4, and IL-6 in 49 mothers of children...
with developmental delay (DD) compared to the mothers of GP controls. While these findings suggest that mid-gestational levels of certain cytokines may serve as early biomarkers for ASD and DD, the study had a limited sample size and requires replication in a larger sample. The objective of the current study was to further examine whether maternal mid-gestational cytokines and chemokines are associated with increased risk of ASD or other developmental delays in a significantly larger sample set utilizing a more sensitive and expansive immunoassay. Moreover, we aimed to determine whether different subtypes of ASD are associated with unique mid-gestational maternal cytokine and chemokine profiles.

Materials and Methods

Subjects
The study sample was obtained from the Early Markers for Autism (EMA) Study, a large population-based, nested case-control study designed to evaluate biomarkers for autism in mother-baby pairs using archived maternal mid-pregnancy and neonatal blood specimens. Women were eligible for inclusion in the EMA study if they delivered a live born infant from July 2000 to September 2003 in California and participated in the prenatal extended alpha-fetoprotein screening program (XAFP) in Orange, San Diego, or Imperial Counties. Three groups of children were identified: children with ASD, children with DD but not ASD, and GP controls. Children with ASD or DD were ascertained from regional centers (RC) operated by the California Department of Developmental Services (DDS), which coordinates services for persons with ASD and other developmental disabilities. After excluding all past or current DDS/RC clients, GP controls were randomly sampled from birth records linked to banked XAFP samples 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 Northern California.

Diagnostic Verification
Trained medical record abstractors reviewed and abstracted detailed diagnostic and clinical data from RC records for all children receiving services for ASD or DD according to a protocol initially developed by the Metropolitan Atlanta Developmental Disabilities Surveillance Program. An expert clinician, blinded to initial case status, reviewed abstracted data and determined ASD and DD status using Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria. Children with ASD were further categorized on the basis of cognitive status (presence or absence of intellectual disability (ID)) and onset type (early onset ASD, ASD with regression, or unknown). While comorbidity of ASD and ID are common, the presence of ID is not required for the diagnosis of ASD. Formerly diagnosed as mental retardation, ID is defined as “significant” delays in both cognitive and adaptive function. For diagnostic purposes and eligibility for many developmental and educational intervention services, “significant” is defined as two or more standard deviations from the mean on standardized tests of cognition and development (score <70). Thus, determination of cognitive status was based on composite scores on standardized cognitive and functional tests in the RC records (with ID: developmental/ cognitive and adaptive composite score <70; without ID: all scores ≥70 or some scores <70.
and others ≥70; unknown: no standardized scores in chart). ASD onset type was determined by parental report or clinical observations, as recorded in RC records, and categorized as early onset, regressive, or unknown. “Early onset ASD” was defined by no statement of loss of social and/or language skills, or early and sustained delays or plateauing of skills without actual loss. “ASD with regression” was defined as clear loss of previously acquired language and/or social skills. All children with DD had composite scores less than 70, as DD was defined as ID without ASD or Trisomy 21. The final analytic sample consisted of 415 children with ASD, 188 children with DD, and 428 GP controls (Table 1), representing the largest population-based sample size examining mid-gestational maternal cytokine and chemokines to date.

Specimen Collection

Maternal mid-pregnancy specimens were retrieved from the California Department of Public Health’s Project Baby’s Breath prenatal screening specimen archives. The archive includes maternal serum and blood cell pellet specimens collected for routine prenatal XAFP screening during 15–19 weeks gestation. Maternal specimens were collected in serum separator tubes by obstetrical care service providers or laboratories and underwent XAFP testing within 7 days of collection at a central laboratory (median time = 3 days). After 1–2 days of refrigeration, leftover specimens were stored at −20°C. Consent forms for the XAFP Screening program were obtained at the time of the test requisition and privacy notifications which stipulated that specimens and data from prenatal testing could be used for legitimate research purposes given appropriate IRB approval. Maternal specimens were packed on dry ice and shipped directly to our laboratory using overnight delivery, where they were stored at −80°C until their use in cytokine and chemokine measurements described below.

Cytokine and Chemokine Measurement

Maternal mid-gestational serum concentrations of 22 cytokines and chemokines were determined using a commercially available multiplex bead-based kit (Milliplex MAP Human Cytokine/Chemokine Kit; Millipore, Billerica, MA, USA) in accordance with the kit-specific protocols provided by Millipore. The following cytokines and chemokines were measured: granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)γ, interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, IFNγ-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, tumor necrosis factor (TNF)-α, Eotaxin, soluble IL-2 receptor alpha (sIL-2Ra), and IL-1 receptor antagonist (IL-1Ra). Briefly, 25 μL of serum was incubated with fluorescently-labeled capture antibody-coated beads in a 96-well filter bottomed plate on a plate shaker overnight at 4°C. After incubation, the sample-bead mix was removed and the plate washed twice using a vacuum manifold. Biotinylated detection antibodies were then added and incubated for 1 hour at room temperature with shaking. The reaction mixture was detected by the addition of streptavidin-phycoerythrin and incubated on a plate shaker at room temperature for 30 minutes. Following a repeat of the washing step, beads were re-suspended in sheath fluid for 5 minutes on the plate shaker. 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) with a five-parameter model used to calculate final concentrations and values.
(expressed in pg/mL). Reference samples were run on each plate to determine assay consistency, and all samples were run blinded to child developmental outcome.

**Statistical Methods**

Demographic differences between the ASD, DD, and GP groups were tabulated and t-tests (for means) as well as Chi squared tests (for frequencies) were calculated. To examine the association of maternal cytokine and chemokine levels during gestation with child developmental outcome logistic regression models were fit. As the distribution of the cytokine and chemokine concentration values were skewed, natural log transformations were used to reduce variance and outlier influence. For all values that were below the limit of detection (LOD), we assigned a value of LOD/2 prior to log transformation. Case vs. control status was regressed on natural log-transformed cytokine and chemokine levels with adjustment for several covariates related to the maternal blood draw (maternal weight and gestational age at time of draw) or associated with ASD in previous epidemiologic studies (maternal age, race, ethnicity and country of origin)\(^{35}\). Separate models were run for each cytokine and chemokine; no adjustments for multiple comparisons were made. \(P\) values of <0.05 for two-tailed tests were considered statistically significant.

Mid-gestational maternal cytokine and chemokine levels were additionally analyzed using principal component analysis (PCA) in order to better identify correlated maternal cytokine and chemokine levels and relate them to child developmental outcome. As our aim was to examine whether the groupings of cytokines and chemokines within principal components varied across child developmental outcome, we ran separate PCAs for each of the following groups: ASD+ID, ASD-noID, DD controls, and GP controls. Principal components with eigenvalues >1 were included in each model, and eigenvectors with a threshold of ±0.3 were included within each component.

**Results**

Descriptive characteristics of the study population are shown in Table 2. Compared to mothers of DD or GP controls, mothers of ASD cases were more likely to be older, non-Hispanic, and born in the United States. Further, ASD cases were more likely to be first born (primiparous) than DD or GP children, as well as have older fathers. In addition, compared to children with DD, children with ASD were more likely to be male, born in the spring or summer months, and less likely to be born as pre-term. Additionally, mothers of ASD children were more likely to weigh less at the time of XAFP blood draw than mothers of DD children. Compared to the GP control group, DD children were more likely to be female, born pre-term, born during winter months, and have younger parents. Mothers of DD children were also more likely to be Hispanic, born in Mexico, and weigh more at the time of XAFP blood draw than mothers of GP controls.

In the logistic regression model adjusted for covariates, no significant associations between maternal cytokine and chemokine levels were observed when comparing all children with ASD to children with DD or GP controls (Supplementary Table 1). Similarly, no significant differences relative to GP controls were observed regardless of whether the child had the regressive form or the early-onset form of the disorder (Supplementary Table 1). However,
in comparison to GP controls, higher mid-gestational maternal serum levels of GM-CSF, IL-1α, IL-6, and IFN-γ were significantly associated with an increased risk of ASD with ID (ASD+ID) (Figure 1). Trends for an increased risk of ASD+ID with higher levels of TNF-α and MIP-1α relative to GP controls were also observed, although these risk estimates did not reach statistical significance (OR_{TNF-α} = 1.16, 95% CI [1.00 – 1.36]; OR_{MIP-1α} = 1.08, 95% CI [1.00 – 1.16]). In contrast, elevated levels of IFN-γ, IL-8, and MCP-1 were associated with a decrease in risk of ASD without ID (ASD-noID) relative to GP controls (Figure 1).

Interestingly, the only significant associations in the DD group relative to GP controls was a decrease in risk with elevated levels of IL-8 and MCP-1 (Figure 1). While risk estimates did not reach statistical significance, a trend for a decreased risk of DD with higher levels of mid-gestational IL-1β relative to GP controls was also observed (OR_{IL-1β} = 0.91, 95% CI [0.83– 1.00]). There were no significant differences in levels of the remaining cytokines and chemokines of GP controls compared to ASD+ID, ASD-noID, or DD samples (Supplementary Table 2).

Higher levels of mid-gestational GM-CSF, TNF-α, IL-1α, IL-1β, IL-6, IFN-γ, IL-10, IL-1Ra, and MCP-1 were associated with an increased risk of ASD+ID compared to DD controls (Figure 1). Conversely, no significant differences for any cytokine or chemokine levels were observed for ASD-noID relative to DD controls (Supplementary Table 2).

Many differences in maternal cytokine and chemokine levels were observed between ASD+ID and ASD-noID cases. An increased risk of ASD+ID was associated with higher levels of the innate inflammatory cytokines TNF-α, IL-1α, IL-1β, and IL-6, and the chemokines IL-8, MCP-1, and MIP-1α. Higher levels of both the Th1 inflammatory cytokine IFN-γ and the Th2 cytokine IL-4 were also associated with an increased risk of ASD+ID relative to ASD-noID. Further, an increased risk of ASD+ID was significantly associated with higher levels of the growth factor cytokine GM-CSF, regulatory cytokine IL-10, and Th17 cytokine IL-17 compared to ASD-noID (Figure 1). Adjusted odds ratio and 95% confidence interval values for all ASD+ID, ASD-noID, and DD logistic regression analyses can be found in Supplementary Table 2.

Finally, separate PCAs for each study group were run in order to determine whether the groupings of cytokines and chemokines within each PC varied across child developmental outcome. Our results suggest that there is no obvious distinct cytokine or chemokine profile by child developmental outcome. Instead, clusters of maternal mid-gestational cytokines and chemokines remained consistent across all study groups. Descriptions of PCs can be found in Supplementary Table 3.

**Discussion**

The aim of this study was to characterize mid-gestational maternal serum profiles of cytokines and chemokines in mothers of children with ASD (with and without ID), of children with DD without ASD, and of children considered as GP controls. Our results suggest that significantly higher maternal levels of pro-inflammatory cytokines and chemokines during gestation are distinctly associated with an increased risk of having a child with ASD+ID. Further, this immunologic distinction between mothers of children with
ASD+ID and those with ASD-noID or DD suggests that the intellectual disability associated with ASD might be etiologically distinct from DD without ASD.

A limited number of studies have investigated the relationship between ASD and mid-gestational cytokines and chemokines, which can be measured using either maternal serum or amniotic fluid samples. In the only other study utilizing mid-gestational maternal serum samples, we previously found that higher levels of IFN-γ, IL-4, and IL-5 were significantly associated with increased risk of ASD, regardless of ID status, relative to GP controls\(^ {35}\). Our present study contrasts with this finding, as increases in risk of ASD were only seen when taking ID status into account. The discrepancies in our findings are most likely due to the previous study’s smaller sample size as well as the increased sensitivity of the cytokine and chemokine detection assay used in the current study. In assessing amniotic fluid samples, Abdallah and colleagues found increased levels of the chemokine MCP-1\(^ {38}\) and the cytokines IL-4, IL-10, TNF-α, and TNF-β in ASD cases relative to controls\(^ {39}\). While these studies utilized the large Danish Birth Cohort study population, they did not take ID status into account in their statistical analyses. Further, while amniotic fluid samples are reflective of a mid-gestational environment, they are considered to be more representative of the fetal rather than maternal immune profile\(^ {40}\). Despite these differences, together these studies suggest that higher levels of mid-gestational cytokines and chemokines, whether maternal or fetal in origin, are associated with an increased risk of ASD relative to controls.

While the exact mechanism(s) by which circulating maternal cytokines and chemokines affect fetal neurodevelopment remain to be elucidated, numerous studies have shown that they play critical roles in the normal development of the central nervous system. Chemokines have been shown to regulate the migration, proliferation, and differentiation of neuronal cells, and are involved in the communication between neurons and microglia\(^ {41}\). For example, studies have suggested that the chemokines IL-8 and MCP-1 may direct the migration and differentiation of neural stem/progenitor cells in early neurodevelopment\(^ {42}\). This is of particular interest to the current study, as elevated mid-gestational maternal levels of IL-8 and MCP-1 were significantly associated with a decrease in risk of having a child with either DD or ASD-noID relative to GP controls. However, due to the high degree of interaction between several chemokines and their receptors, it has been challenging for researchers to determine the exact roles of specific chemokines during neurodevelopment. Conversely, numerous studies have been able to determine the roles of specific cytokines during neurodevelopment, such as influencing neurogenesis, neuronal and glial cell migration, proliferation, differentiation, and synaptic maturation and pruning\(^ {41,43}\). For example, IFN-γ has been shown to have a major regulatory effect on neural precursor activity in both the developing and adult brain\(^ {44}\). The role of cytokines and chemokines during development are highly variable, and depend on the timing, duration, region, and intensity of exposure. In addition to their roles in neurodevelopment, cytokines and chemokines have been shown to play important roles in establishing and maintaining pregnancy. Over the course of gestation, there is a gradual shift from the inflammatory cell-mediated Th1 response toward the humoral-mediated Th2 response\(^ {28,45,46}\). Decreased response in the pleiotropic pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 have also been observed in the progression of pregnancy\(^ {36}\). Thus, our finding that mothers of children with ASD+ID have higher levels of mid-gestational cytokines and chemokines suggests a
shift in balance in the pattern usually observed in pregnancy and may be indicative of an immune activation. This alteration in gestational immune environment may then lead to alterations in the neurodevelopmental trajectory of the developing fetus, which may subsequently result in to the altered behavioral phenotype characteristic of children with ASD+ID.

While our findings may be indicative of an immune activation that affects developmental programming, maternal immune activation represents only one of several pathways that can result in differences in maternal cytokines observed in the present study. For example, several environmental toxicants such as heavy metals, pesticides, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) can cause both neural and immune dysfunction and have been associated with increased risk of ASD\(^47\). The potential source(s) of increased mid-gestational maternal cytokine and chemokine levels may further interact with other potential risk factors of ASD, such as parental genetic contribution. For instance, some genes that have been associated with ASD may cause inappropriate immune responses\(^47, 48\). Furthermore, the observed correlation between maternal cytokine and chemokines with risk of ASD+ID may be coincidental in nature. Thus, readers should be careful to not infer causation through our observed correlation between mid-gestational maternal cytokine and chemokine levels and increased risk of ASD+ID. The exact mechanism(s) responsible for the observed findings remain to be elucidated.

The results of this study should be considered in light of the following limitations. First, maternal samples were available from only one time point during pregnancy, limiting our ability to examine other critical windows of neurodevelopment. Future longitudinal studies that assess the maternal immune profile throughout pregnancy and fetal neurodevelopment will help to better understand this potential relationship. Second, maternal peripheral blood samples are not necessarily representative of the immune profile at the maternal-fetal interface. This interface is best represented through the use of placental or amniotic samples, which were not available in this study. However, our study provides insight on the global maternal immune profile during a developmentally relevant time period using the largest sample size to date. Third, long term storage of samples is known to result in the degradation of some cytokines and chemokines\(^49\). While our study utilizes samples that have been stored for an extended period of time before their use, all samples were collected and stored for a similar length of time prior to their analysis. Thus while the cytokine and chemokines levels observed in the present study will have degraded and decreased compared to levels at initial collection, the degree of degradation should be anticipated to be comparable across all sample types and therefore all child developmental outcome groups. Furthermore, the unnecessary degradation of samples was prevented by avoiding multiple freeze thaws and storing samples long term in an \(-80^\circ\text{C}\) freezer until assayed. Fourth, we did not have access to any maternal clinical information such as presence of allergy, asthma, or infection in the present study. We therefore were unable to investigate the contributing role these factors may have had on the maternal immune profile and subsequent association with increased risk of ASD+ID. Finally, the diagnostic assessment of cases and controls was not directly confirmed clinically using standardized measures, but instead was made on the basis of medical record abstraction reviewed by expert clinicians.
Despite these limitations, our study was strengthened by the use of a highly sensitive immunoassay in the detection of cytokines and chemokines. Further, our multivariate analyses were adjusted for several covariates, thus reducing the likelihood of confounding factors influencing the specificity of our findings. Finally, the inclusion of the DD group further supports the specificity of our findings.

In conclusion, we found that elevations in maternal cytokine and chemokine levels during mid-gestation were associated with an increased risk of ASD+ID. This maternal immune profile was associated with increased risk of ASD+ID not only in comparison to GP controls, but also to ASD-noID as well as DD controls. As the majority of these cytokines are typically downregulated during mid-gestation, our results suggest that a shift in the immune balance during pregnancy may lead to alterations in the neurodevelopment trajectory of the developing child, ultimately resulting in the development of ASD+ID. This unique mid-gestational immune profile suggests a possible etiological distinction from ASD without ID as well as ID without ASD. We also found elevated mid-gestational levels of IL-8 and MCP-1 were significantly associated with decreased risk of both DD and ASD-noID relative to GP controls. Continued research efforts will aim to establish the longitudinal relationship of the maternal immune profile with subsequent clinically validated behavioral outcomes in the child. Further, ongoing studies are investigating the potential relationships between maternal mid-gestational immune profiles and additional risk factors of ASD, both genetic and environmental. Finally, future research is required to understand the potential mechanism(s) of action by which the maternal mid-gestational immune profile may act in order to alter the trajectory of neurodevelopment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by grants 3R01ES016669 from National Institute of Environmental Health Sciences; 5R01MH072565 from the National Institute of Mental Health, and the NICHD funded IDDRC 054 (U54HD079125). We thank Yanjun Cui and Lori Haapanen for their data collection and management efforts. Banked specimens were provided by Project Baby’s Breath (M Kharrazi and G DeLorenze, Co-Principal Investigators) under the direction of the California Genetic Disease Screening Program. The views expressed are those of the authors and do not necessarily represent those of the California Department of Public Health.

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### Figure 1. Mothers of children with ASD and intellectual disability (ASD+ID) had significantly elevated inflammatory T cell and innate immune cell cytokines and chemokines

The alterations of these particular cytokines and chemokines, which are normally downregulated during mid-gestation, suggest a lack of immune regulation that is typically associated with pregnancy. Cell values represent *p*-values obtained during adjusted logistic regression analyses. Logistic regression models were adjusted for gestational age at time of draw, maternal weight, age, race, ethnicity and country of origin. Highlighted cells represent significant logistic regression findings. Red highlighting denotes an increased risk relative to the comparison group was significantly associated with elevated mid-gestational levels of

| Cytokine               | ASD+ID vs. GP | ASD-noID vs. GP | DD vs. GP | ASD+ID vs. DD | ASD-noID vs. DD | ASD+ID vs. ASD-noID |
|------------------------|---------------|-----------------|-----------|---------------|-----------------|---------------------|
| GM-CSF                 | 0.042         | 0.074           | 0.565     | 0.041         | 0.266           | 0.004               |
| TNF-α                  | 0.055         | 0.385           | 0.375     | 0.014         | 0.423           | 0.022               |
| IL-1α                  | 0.042         | 0.277           | 0.749     | 0.006         | 0.589           | 0.011               |
| IL-1β                  | 0.093         | 0.251           | 0.055     | 0.009         | 0.919           | 0.015               |
| IL-6                   | 0.008         | 0.468           | 0.213     | 0.012         | 0.675           | 0.003               |
| IFN-γ                  | 0.044         | 0.045           | 0.870     | 0.020         | 0.427           | 0.003               |
| IL-2                   | 0.358         | 0.380           | 0.440     | 0.427         | 0.425           | 0.102               |
| IL-12p40               | 0.838         | 0.768           | 0.993     | 0.843         | 0.824           | 0.998               |
| IL-12p70               | 0.822         | 0.382           | 0.148     | 0.606         | 0.230           | 0.930               |
| IL-4                   | 0.214         | 0.613           | 0.956     | 0.652         | 0.083           | 0.044               |
| IL-13                  | 0.584         | 0.426           | 0.986     | 0.528         | 0.634           | 0.401               |
| IL-10                  | 0.154         | 0.129           | 0.226     | 0.046         | 0.988           | 0.022               |
| IL-17                  | 0.253         | 0.172           | 0.492     | 0.386         | 0.476           | 0.035               |
| IL-1Ra                 | 0.263         | 0.384           | 0.177     | 0.014         | 0.614           | 0.077               |
| IL-8                   | 0.432         | 0.014           | 0.003     | 0.073         | 0.688           | 0.002               |
| IP-10                  | 0.886         | 0.747           | 0.894     | 0.872         | 0.870           | 0.503               |
| MCP-1                  | 0.625         | 0.011           | 0.032     | 0.015         | 0.967           | 0.001               |
| MIP-1α                 | 0.051         | 0.107           | 0.588     | 0.086         | 0.224           | 0.003               |
| MIP-1β                 | 0.142         | 0.660           | 0.539     | 0.129         | 0.939           | 0.139               |
| Eotaxin                | 0.395         | 0.349           | 0.599     | 0.194         | 0.887           | 0.144               |
| IL-7                   | 0.340         | 0.823           | 0.335     | 0.223         | 0.527           | 0.222               |
| sIL-2Ra                | 0.609         | 0.343           | 0.659     | 0.920         | 0.537           | 0.486               |
the indicated cytokines and chemokines. Blue highlighting indicates a reduced risk was significantly associated with elevated mid-gestational levels relative to the comparison group.
Table 1
Classification of autism cases in the Early Markers for Autism study

| Autism spectrum disorder subgroups | Number of subjects |
|-----------------------------------|--------------------|
| Total                             | 415                |
| Onset type                        |                    |
| Early onset                       | 299                |
| Regressive                        | 102                |
| Unknown                           | 14                 |
| Intellectual disability           |                    |
| Yes                               | 184                |
| No                                | 201                |
| Unknown                           | 30                 |
## Table 2

Characteristics of the Early Markers for Autism study population

| Characteristics            | ASD (N = 415) | DD (N = 188) | GP (N = 428) | ASD vs. GP | ASD vs. DD | DD vs. GP |
|----------------------------|---------------|--------------|--------------|------------|------------|-----------|
|                            | n             | %            | n            | %          | p value    | p value    | p value    |
| Child’s sex²               |               |              |              |            |            |           |            |
| Male                       | 343           | 82.7%        | 106          | 56.4%      | 0.9102     | < 0.0001   | < 0.0001   |
| Female                     | 72            | 17.3%        | 82           | 43.6%      | 0.5873     | 0.5800     | 0.3105     |
| Parity                     |               |              |              |            |            |           |            |
| Singleton                  | 405           | 97.6%        | 182          | 96.8%      | 0.5873     | 0.5800     | 0.3105     |
| Multiple                   | 10            | 2.4%         | 6            | 3.2%       | 0.0109     | 0.0025     | 0.2863     |
| Maternal age (Years)       |               |              |              |            |            |           |            |
| <20                        | 14            | 3.4%         | 26           | 13.8%      | 0.0432     | < 0.0001   | 0.0003     |
| 20–24                      | 58            | 14.0%        | 43           | 22.9%      | 0.0432     | < 0.0001   | 0.0003     |
| 25–29                      | 109           | 26.3%        | 52           | 27.7%      | 0.0066     | < 0.0001   | 0.0022     |
| Mean maternal age, years (+/− SD) | 30.01 (5.67) | 27.18 (6.25) | 28.70 (5.40) |            |            |           |            |
| Maternal Age (Years)       |               |              |              |            |            |           |            |
| <20                        | 14            | 3.4%         | 26           | 13.8%      | 0.0432     | < 0.0001   | 0.0003     |
| 20–24                      | 58            | 14.0%        | 43           | 22.9%      | 0.0432     | < 0.0001   | 0.0003     |
| 25–29                      | 109           | 26.3%        | 52           | 27.7%      | 0.0066     | < 0.0001   | 0.0022     |
| 30–34                      | 154           | 37.1%        | 44           | 23.4%      | 0.0432     | < 0.0001   | 0.0003     |
### Table: Characteristics

| Characteristics                        | ASD (N = 415) | DD\(^1\) (N = 188) | GP (N = 428) | ASD vs. GP | ASD vs. DD | DD vs. GP |
|---------------------------------------|---------------|---------------------|--------------|------------|------------|------------|
|                                       | n             | %                   | n            | %          | p value    | p value    | p value    |
| ≥35                                   | 80            | 19.3%               | 23           | 12.2%      | 0.0026     | 0.0001     | 0.0843     |
| Mean paternal age, years (+/- SD)     | 32.76 (6.11)  | [N = 398]           | 30.43 (7.55) | [N = 171]  | 31.47 (6.12) | [N = 412]  | 0.0026     | 0.0001     | 0.0843     |
| Paternal Age (Years)                  |               |                     |              |            |            |            |            |
| <20                                   | 7             | 1.8%                | 8            | 4.7%       | 8          | 1.9%       | 0.0892     | < 0.0001   | 0.0316     |
| 20–24                                 | 29            | 7.3%                | 32           | 18.7%      | 50         | 12.1%      | 0.0892     | < 0.0001   | 0.0316     |
| 25–29                                 | 87            | 21.9%               | 44           | 25.7%      | 96         | 23.3%      | 0.0892     | < 0.0001   | 0.0316     |
| 30–34                                 | 120           | 30.2%               | 39           | 22.8%      | 127        | 30.8%      | 0.0892     | < 0.0001   | 0.0316     |
| ≥35                                   | 155           | 38.9%               | 48           | 28.1%      | 131        | 31.8%      | 0.0892     | < 0.0001   | 0.0316     |
| Maternal Weight at XAFP Blood draw (lbs) | 151.91 (36.04) | [N = 402]       | 158.98 (37.12) | [N = 179] | 150.70 (34.02) | [N = 416] | 0.6149     | 0.0275     | 0.0070     |
| Gestational Age at XAFP Blood draw (days) | 119.20 (8.84) | [N = 402]       | 119.99 (9.23) | [N = 179] | 118.68 (8.40) | [N = 416] | 0.3811     | 0.3182     | 0.0849     |
| Gestational Age at Birth (weeks)      | 39.29 (0.21)  | [N = 402]           | 38.36 (0.32) | [N = 179]  | 39.85 (0.25) | [N = 416]  | 0.0869     | 0.0128     | 0.0007     |
| <33 (weeks)                           | 9             | 2.2%                | 15           | 8.4%       | 7          | 1.7%       | 0.8336     | 0.0011     | 0.0001     |
| 33–<37                                | 39            | 9.7%                | 23           | 12.8%      | 39         | 9.4%       | 0.8336     | 0.0011     | 0.0001     |
| ≥37                                   | 354           | 88.1%               | 141          | 78.8%      | 370        | 88.9%      | 0.8336     | 0.0011     | 0.0001     |
| Birth Season\(^2\)                   |               |                     |              |            |            |            |            |
| Spring (March – May)                  | 129           | 31.08%              | 51           | 27.13%     | 132        | 30.84%     | 0.9688     | 0.0479     | 0.0482     |
| Summer (June – August)                | 116           | 27.95%              | 41           | 21.81%     | 116        | 27.10%     | 0.9688     | 0.0479     | 0.0482     |
| Autumn (September – November)        | 94            | 22.65%              | 44           | 23.40%     | 103        | 24.07%     | 0.9688     | 0.0479     | 0.0482     |
| Winter (December – February)         | 76            | 18.31%              | 52           | 27.66%     | 77         | 17.99%     | 0.9688     | 0.0479     | 0.0482     |

\(^1\) Trisomy 21 cases were excluded from the DD group.

\(^2\) GP controls were frequency matched to ASD cases on child’s sex, birth month and birth year.

Bolding indicates p-values <0.05.

Abbreviations: ASD: autism spectrum disorder; DD: developmental delay; GP: general population.