Archival Report

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An Exploratory Examination of Neonatal Cytokines and Chemokines as Predictors of Autism Risk: The Early Markers for Autism Study

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ABSTRACT

BACKGROUND: The identification of an early biomarker for autism spectrum disorder (ASD) would improve the determination of risk, leading to earlier diagnosis and, potentially, earlier intervention and improved outcomes.

METHODS: Data were generated from the Early Markers for Autism study, a population-based case-control study of prenatal and neonatal biomarkers of ASD. Newborn bloodspots of children with ASD (n = 370), children with developmental delay (n = 140), and general population (GP) controls (n = 378) were analyzed for 42 different immune markers using a Luminex multiplex platform. Comparisons of immune marker concentrations between groups were examined using logistic regression and partial least squares discriminant analysis.

RESULTS: Children with ASD had significantly increased neonatal levels of interleukin-6 (IL-6) and IL-8 compared with GP controls. An increase in IL-8 was especially significant in the ASD group with early onset compared with the GP group, with an adjusted odds ratio of 1.97 (95% confidence interval, 1.39–2.83; p = .00014). In addition, children with ASD had significantly elevated levels of eotaxin-1, interferon-γ, and IL-12p70 relative to children with developmental delay. We observed no significant differences in levels of immune markers between the developmental delay and GP groups.

CONCLUSIONS: Elevated levels of some inflammatory markers in newborn bloodspots indicated a higher degree of immune activation at birth in children who were subsequently diagnosed with ASD. The data from this exploratory study suggest that with further expansion, the development of neonatal bloodspot testing for cytokine/chemokine levels might lead to the identification of biomarkers that provide an accurate assessment of ASD risk at birth.

Keywords: Autism, Bloodspot, Chemokine, Cytokine, Developmental delay, Neonatal

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder defined by social and communication deficits and repetitive behaviors. ASD is currently estimated to occur in 1% to 2% of children in the United States (1–4). While a number of genetic risk factors have been identified for ASD, recent evidence has shown that a little more than half of the risk for developing ASD can be attributed to genetic mutations and over 40% of the risk is likely due to unknown environmental factors (5,6). There is evidence that a subset of children with ASD display alterations in immune function including differences in immune cell numbers/function, immunoglobulin levels, and cytokine/chemokine levels relative to typically developing control subjects (7). While activation of the immune system is classically associated with defense against invading pathogens, there is now evidence that the immune system plays a significant role in neurodevelopment (8) and in the regulation of neural plasticity throughout life (9,10).

Cytokines and chemokines are cell-signaling molecules used by the immune system to orchestrate the appropriate response to physiological challenges. Most are highly pleiotropic, serving as immune mediators, growth factors, and chemotactic signals for cellular migration during development (7,11,12). Differences in circulating cytokine/chemokine levels are among the most commonly reported immune abnormalities in individuals already diagnosed with ASD (13,14). The numerous studies on immune dysregulation in ASD suggest that cytokine/chemokine profiles at birth may be useful biomarkers for predicting risk of ASD (7,15).

A reliable ASD diagnosis is typically not given until at least 2 years of age (16–18). The most effective treatment currently available is behavioral intervention, and its success depends on initiating treatment as early as possible (19). Neonatal bloodspots are potentially useful for biomarker discovery. Several large statewide and national programs conducting universal newborn screening for genetic and metabolic disorders collect and store newborn bloodspots. These archives provide researchers the ability to retrospectively analyze newborn samples for children subsequently diagnosed with

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various developmental outcomes. Several studies, including from our own group, have examined newborn bloodspots for cytokine/chemokine differences as potential biomarkers for ASD (20,21). Inconsistent results between these studies can likely be attributed to small study sample sizes, differences in outcome definition and covariate inclusion, and assay sensitivity. In our previous study (20), a significant number of samples had signal levels that fell below the threshold of detection, limiting the interpretability of the results. However, a high-sensitivity assay has since been developed with an expanded set of cytokines and chemokines for analysis.

The current study was designed to examine whether cytokine/chemokine levels in newborn screening bloodspots could provide early markers for ASD risk in a larger cohort of individuals than previously analyzed, utilizing a recently expanded high-sensitivity immunoassay. Moreover, we aimed to examine whether different behavioral subsets within the ASD group are associated with unique cytokine/chemokine profiles in newborn bloodspots.

METHODS AND MATERIALS

Study Population

Previous publications have described the study population in detail (20,22). In brief, our samples were obtained from a large population-based, nested case-control study, the Early Markers for Autism (EMA) study, which was designed to investigate archived biological samples for markers of exposure and susceptibility to ASD. All study subjects were born between March 2000 and July 2003 in the same 3 counties in Southern California to women who participated in California's prenatal screening program and for whom both a prenatal maternal blood sample and a newborn bloodspot were available for analysis. The sociodemographic characteristics of the screened women, which represented 70% of all pregnant women, were similar to the characteristics of all women in study counties and birth years. Two behavioral groups—ASD and developmental delay without ASD (DD) were initially identified from the California Department of Development Services (DDS) system of 21 regional centers (RCs), which coordinates services for persons with ASD and other developmental disabilities. A general population (GP) control group was randomly sampled from the birth certificate files after excluding all past or current DDS/RC clients and frequency matched to ASD cases by sex, birth month, and birth year. This study was approved by the institutional review boards of the California Health and Human Services Agency and Kaiser Permanente of Northern California.

Diagnostic Validation

For children identified from the DDS/RCs as having ASD or DD, medical record abstractors compiled detailed diagnostic and clinical data from the RC records according to a protocol developed by the Metropolitan Atlanta Developmental Disabilities Surveillance Program (23). All children were between 4.5 and 9 years of age at time of record review and abstraction. A developmental pediatrician subsequently performed an expert clinical review of abstracted data to confirm the initial DDS/RC diagnosis. A final study classification of ASD was given if DSM-IV criteria were met. Final classification of DD was based on standardized cognitive and adaptive test scores found in RC records, with composite scores of <70 categorized as DD, while all scores ≥ 70 or some scores <70 and others ≥ 70 was categorized no DD. Additionally, those classified as ASD were further categorized as early onset (EO) (no statement of loss of social and/or language skills) or regressive (Reg) (clear loss of previously acquired language and/or social skills) and also classified according to presence or absence of intellectual disability (YesID and NoID, respectively). Intellectual disability was defined as developmental/cognitive score and adaptive composite score <70, as done for DD. The final study population consisted of 378 GP children, 140 children with DD, and 370 children with ASD that were further broken down into EO/Reg and YesID/NoID subgroups as reported in Table 1.

Table 1. Classification of Study Subjects

Behavioral Classification	Subjects, *n*
General Population	378
Developmental Delay	140
ASD	370
ASD Subgroup Classifications	
Onset type	
Early onset	269
Regressive	92
Unknown	9
Intellectual disability	
Yes	163
No	180
Unknown	27

ASD, autism spectrum disorder.

Specimen Collection

Capillary blood was collected at birth by heel stick method and spotted onto standardized filter paper for routine newborn screening of various endocrine, metabolic, and genetic disorders. After collection, specimens were transported without temperature control by courier to a regional screening laboratory for testing. Any bloodspots remaining were then catalogued and stored at -20°C by the California Department of Public Health. All bloodspots included in this study were collected within 72 hours of birth.

Blood Spot Elution

Dried bloodspot samples were received as three 3-mm punches per subject in a single well of 96-well plates and stored at -80°C until elution. For elution, each sample received 200 μL of elution buffer (0.5% bovine serum albumin in 50-mL phosphate-buffered saline with 1 tablet of Roche Complete Protease Inhibitor Cocktail [Roche Diagnostics Operations, Indianapolis, IN]) and was placed on a plate shaker overnight at 4°C. The eluates were then isolated from the filter paper spots, and a small 4-μL aliquot was used for bicinchoninic acid assay (Thermo Scientific, Rockford, IL) determination of total protein to normalize cytokine/chemokine levels against blood sample quantity variation.

Table 2. Characteristics of the Early Markers for Autism Study Population

				p Value		
	GP Group (*n* = 378)	DD Group (*n* = 140)	ASD Group (*n* = 370)	ASD vs. GP	ASD vs. DD	DD vs. GP
Birth Type						
C-section	104 (27.5)	40 (28.6)	104 (28.1)	.856	.280	.150
Vaginal	274 (72.5)	100 (71.4)	266 (71.9)			
Sex						
Male	311 (82.3)	79 (56.4)	303 (81.9)	.891	.000[a]	.000[a]
Female	67 (17.7)	61 (43.6)	67 (18.1)			
Maternal Education						
Less HS graduate	93 (24.6)	57 (40.7)	55 (14.9)	.000[a]	.000[a]	.000[a]
HS graduate	102 (27.0)	34 (24.3)	75 (20.3)			
College	128 (33.9)	40 (28.6)	168 (45.4)			
Postgraduate	55 (14.6)	9 (6.4)	72 (19.5)			
Paternal Education						
Less HS graduate	82 (21.7)	52 (37.1)	54 (14.6)	.002[a]	.000[a]	.000[a]
HS graduate	98 (25.9)	42 (30.0)	71 (19.2)			
College	136 (36.0)	39 (27.9)	166 (44.9)			
Postgraduate	62 (16.4)	7 (5.0)	79 (21.4)			
Birth Season						
Winter	70 (18.5)	36 (25.7)	69 (18.6)	.993	.151	.036[a]
Spring	119 (31.5)	43 (30.7)	115 (31.1)			
Summer	105 (27.8)	32 (22.9)	106 (28.6)			
Fall	84 (22.2)	29 (20.7)	80 (21.6)			
Maternal Birth Place						
United States	186 (49.2)	64 (45.7)	183 (49.5)	.11	.000[a]	.000[a]
Mexico	108 (28.6)	59 (42.1)	85 (23.0)			
Other	84 (22.2)	17 (12.1)	102 (27.6)			
Birth Year						
2000	73 (19.3)	33 (23.6)	65 (17.6)	.883	.235	.154
2001	98 (25.9)	37 (26.4)	92 (24.9)			
2002	154 (40.7)	53 (37.9)	158 (42.7)			
2003	53 (14.0)	17 (12.1)	55 (14.9)			
Maternal Ethnicity						
Hispanic	171 (45.2)	96 (68.6)	140 (37.8)	.04[a]	.000[a]	.000[a]
Non-Hispanic	207 (54.8)	44 (31.4)	230 (62.2)			
Paternal Ethnicity						
Hispanic	163 (43.1)	96 (68.6)	141 (38.1)	.163	.000[a]	.000[a]
Non-Hispanic	215 (56.9)	44 (31.4)	229 (61.9)			
Gestational Age, Days	277.484 ± 12.881	275.786 ± 14.944	275.535 ± 13.681	.508	1.000	.921
Age at Blood Spot Collection, Hours	31.947 ± 11.775	32.957 ± 13.979	31.624 ± 11.885	1.000	.952	.990
Birth Weight, g	3455.352 ± 480.406	3283.157 ± 543.831	3491.03 ± 499.439	.979	.001[a]	.013[a]
Maternal Age, Years	28.75 ± 5.44	26.87 ± 6.32	29.91 ± 5.63	.093	.000[a]	.017[a]
Paternal Age, Years	31.61 ± 6.01	30.36 ± 7.25	32.76 ± 6.02	.172	.002[a]	.456
Maternal Weight, lbs	149.89 ± 33.83	156.94 ± 36.96	151.51 ± 34.45	.12	.053	.0093[a]

Values are *n* (%) or mean ± SD. All *p* values were determined by χ^2 test and analysis of variance.
ASD, autism spectrum disorder; DD, developmental delay; GP, general population; HS, high school.
[a]$p < .05$.

Immune Marker Measurement

Immediately following overnight elution, neonatal levels of peripheral blood immune markers were determined using commercially available Luminex multiplex magnetic bead assays (Bio-Rad Laboratories, Hercules, CA). We combined a Bio-Plex Pro Human Chemokine kit (Bio-Rad Laboratories) containing a mix of 40 different immune markers (Supplemental Table S1) with 2 individual single-plex beads, interleukin (IL)-12p70 and IL-13 (Bio-Rad Laboratories). The assay was run according to the manufacturer's directions.

Table 3. Significant Adjusted Odds Ratios for Immune Markers in the Early Markers for Autism Study

Immune Markers	ASD vs. GP	ASD-NoID vs. GP	ASD-YesID vs. GP	ASD-EO vs. GP	ASD-Reg vs. GP	ASD vs. DD	ASD-NoID vs. DD	ASD-YesID vs. DD	ASD-EO vs. DD	ASD-Reg vs. DD	ASD-YesID vs. ASD-NoID
IL-8	1.7 (1.2–2.4)[b]	1.6 (1.1–2.4)[a]	1.8 (1.2–2.8)[b]	1.9 (1.3–2.8)[c]							
IL-6	1.4 (1.0–2.1)[a]										
IL-12p70		2.2 (1.1–4.7)[a]				2.9 (1.4–6.1)[b]	4.0 (1.6–9.5)[b]	2.4 (1.0–5.7)[a]	3.2 (1.4–7.0)[b]		
IFN-γ Q2						2.3 (1.2–4.3)[b]		2.6 (1.3–5.2)[b]	2.2 (1.1–4.2)[a]	2.7 (1.2–6.3)[a]	
Eotaxin-1 Q4		1.8 (1.1–3.1)[a]				2.2 (1.2–4.1)[b]	2.9 (1.4–5.8)[b]		2.0 (1.0–3.8)[a]	3.0 (1.3–6.8)[b]	
Eotaxin-1 Q3						2.1 (1.1–3.9)[a]	2.1 (1.0–4.3)[a]	2.1 (1.1–4.1)[a]	2.1 (1.1–3.9)[a]		
Eotaxin-1 Q2		1.8 (1.0–3.1)[a]					2.3 (1.1–4.5)[a]				0.5 (0.2–0.8)[a]
6CKINE							2.1 (1.0–4.1)[a]		1.9 (1.0–3.8)[a]		
IL-13 Q4							2.1 (1.1–4.1)[a]				
TNF-α >10%						0.4 (0.1–0.9)[a]					
IL-4 ≥90%			2.2 (1.2–4.2)[a]								
GCP-2		2.2 (1.0–4.6)[a]					3.1 (1.2–8.0)[a]				
CTACK											0.5 (0.2–0.9)[a]
BCA-1					2.8 (1.1–7.5)[a]						
Eotaxin-3					2.4 (1.1–5.6)[a]						
IL-10					2.7 (1.1–7.0)[a]						
MIP-1a					2.3 (1.0–5.4)						
MCP-4 >10%										0.3 (0.1–0.8)[a]	

Significant adjusted odds ratios representing 1-SD change and 95% confidence intervals from Supplemental Table S2. For quartile analyses, Q1 was used as the reference for Q2, Q3, and Q4. Odds ratios ≥90% use <90% as the reference. Odds ratios >10% use ≤10% as the reference.

6CKINE, chemokine (C-C motif) ligand 21 (CCL21); ASD, autism spectrum disorder; BCA-1, B cell-attracting chemokine 1; CTACK, cutaneous T cell–attracting chemokine; DD, developmental disorder; EO, early onset; GCP-2, granulocyte chemotactic protein 2; GP, general population; IFN, interferon; IL, interleukin; MCP-4, monocyte chemotactic protein; MIP-1a, macrophage inflammatory protein 1 alpha; NoID, absence of intellectual disability; Q, quartile; Reg, regressive; TNF, tumor necrosis factor; YesID, presence of intellectual disability.

[a].05 > $p \geq$ .01.

[b].01 > $p \geq$ .001.

[c]$p <$ .001.

Briefly, 50 μL of bloodspot eluate was incubated with fluorescently labeled capture antibody-coated beads in a 96-well plate on a plate shaker for 1 hour at room temperature. After incubation, the sample-bead mix was removed and washed, and biotinylated detection antibodies were added 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 resuspended in sheath fluid for 5 minutes on the plate shaker. Plates were read on a Bio-Plex 200 system (Bio-Rad Laboratories) and analyzed using Bio-Plex Manager software (Bio-Rad Laboratories) with a 5-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 Analysis

Sociodemographic factors were compared across groups using chi-square tests for categorical variables and analysis of variance for continuous variables. All immune markers were normalized for sampling variation in blood collection by dividing total protein content in the eluate as determined by bicinchoninic acid assay. Those that fell below the minimum level of detection were assigned a value of minimum level of detection/2. Data were then natural log transformed to reduce variance and outlier influence. The range for each analyte and median for each behavioral group, as well as the percentage of samples that fell below the minimum level of detection, are reported in Supplemental Table S1. Immune markers that were highly skewed or had more than 40% nondetects were divided into quartiles (Supplemental Table S2) and dichotomized as follows: <10% versus 11% to 100%; >90% versus 0% to 89%. Our previous study on neonatal blood spots indicated that the most prominent risk effects were found in these strata (20). A single cytokine (granulocyte macrophage colony-stimulating factor) was detected in <50% of samples and was therefore divided into detect versus nondetect groups for analysis. The majority of analytes were not categorized into quartiles or dichotomized and were analyzed continuously. Odds ratio represents a 1-SD change from the comparison group.

We performed unadjusted and adjusted logistic regression analyses with the child's diagnostic group as the outcome of interest and each immune marker as the predictor. We made comparisons among the 3 main behavioral classifications, ASD cases versus GP controls, ASD cases versus DD cases, and DD cases versus GP controls. In addition, the ASD group was further broken down into those children with and without intellectual disability (ASD-YesID and ASD-NoID) and those with EO ASD (ASD-EO) or Reg ASD (ASD-Reg). Comparisons were then made between each of these subgroups versus GP controls and versus DD cases.

We ran a separate model for each immune marker and included covariates of interest in the adjusted model that were chosen a priori, including birth type (C-section vs. vaginal), gender, gestational age at birth, birth weight, postnatal age at bloodspot collection, maternal and paternal age, maternal and paternal education, birth season, maternal birth place, child's birth year, maternal and paternal race, and Bio-Plex plate number. All analyses were run using SPSS version 22 (IBM Corp., Armonk, NY).

Partial least squares discriminant analysis (PLS-DA) was performed to examine whether different combinations of multiple cytokines could be used to differentiate between child developmental outcomes. Initially, linear regression analysis was performed on each transformed immune marker individually using the covariates stated above to generate residuals for use in the PLS-DA. Eotaxin-2, epithelial neutrophil-activating protein 78, granulocyte macrophage colony-stimulating factor, eotaxin-1, interferon-γ (IFN-γ), IL-4, monocyte chemoattractant protein 4 (MCP-4), and IL-13 all violated assumptions of linearity in the linear regression model and were therefore excluded from the PLS-DA. The PLS-DA was computed using the web-based MetaboAnalyst software in accordance with the protocol by Xia and Wishart (24). Analysis was performed using leave-one-out cross-validation and prediction accuracy performance measure for determining the number of latent variables. The permutation statistic was performed using prediction accuracy during training with 2000 permutations.

RESULTS

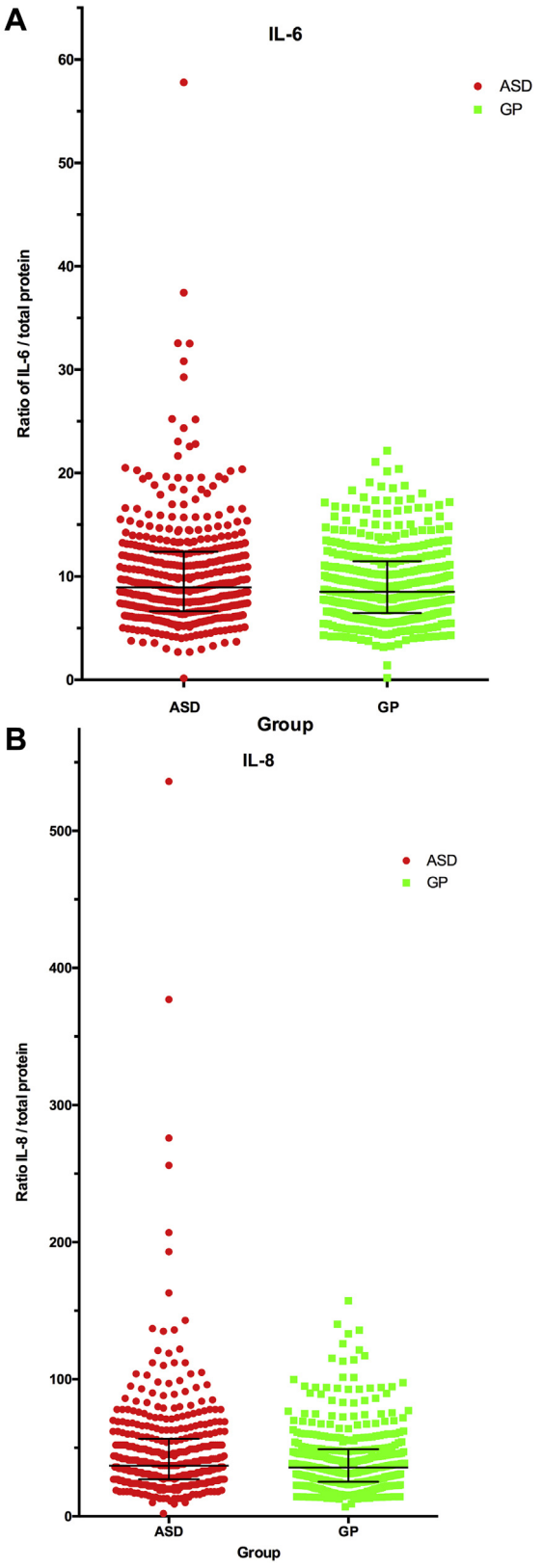
Population Characteristics

There were no differences among the GP, DD, and ASD groups with respect to maternal weight, method of delivery (C-section vs. vaginal), gestational age, age at blood collection, or year of birth (Table 2). The ASD and GP groups were sex-matched by design to reflect the male bias in ASD, but the DD group was not, resulting in a skew toward female children in that group. Birth weight was significantly lower in the DD group than in the ASD and GP groups. With respect to parental demographics, children in the ASD group was more likely to have parents who were more highly educated and non-Hispanic when compared with children in the GP group. In contrast, children in the DD group were more likely to be born in winter; have parents who were younger, less educated, and of Hispanic heritage; and have mothers who were heavier and born in Mexico when compared with children in the GP group.

Immune Markers and Diagnosis

Only 3 analytes including epithelial neutrophil-activating protein 78, eotaxin-2, and granulocyte macrophage colony-stimulating factor had >10% of samples with undetectable levels (Supplemental Table S1) and were thus analyzed as dichotomous variables. Results of all logistic regression analyses for all study group comparisons are reported in Supplemental Table S2. Table 3 displays analytes with statistically significant findings for any study group comparisons.

ASD Cases Versus GP Controls. Compared with GP controls, there was increased risk of ASD associated with higher levels of the inflammatory chemokine IL-8 and cytokine IL-6 (Table 3; Figure 1).



ASD-YesID and ASD-NoID Cases Versus GP Controls. We further divided the ASD group into subgroups based on intellectual disability to determine if cytokines contributed differently to risk of phenotypically distinct populations within the spectrum. The inflammatory chemokine IL-8 was the only chemokine for which higher levels were associated with significantly increased odds of both ASD-NoID and ASD-YesID compared with GP controls. Higher levels of inflammatory cytokine IL-12p70, and chemokines eotaxin-1 (quartile 2 [Q2] and Q4 vs. Q1), and granulocyte chemotactic protein 2 were associated with increased odds for the ASD-NoID group only, and the highest levels of IL-4 (≥90% vs. <90%) were uniquely associated with ASD-YesID.

ASD-Reg or ASD-EO Cases Versus GP Controls. We further examined if there were individual cytokines that contributed to risk of developing either EO or Reg ASD when compared with GP controls. We found an increased risk associated with elevated IL-8 in the ASD-EO group relative to GP controls (Table 3). The ASD-Reg group was unique as a subphenotype within ASD, with an increase in the chemokines B cell-attracting chemokine 1 and eotaxin-3, and the regulatory cytokine IL-10, in association with elevated risk relative to the GP controls.

ASD Cases Versus DD Controls. When compared with DD controls, increased odds of developing ASD were associated with significantly higher levels of the inflammatory cytokines IL-12p70 and IFN-γ (Q2 vs. Q1), and the chemokine eotaxin-1 (Q3 and Q4 vs. Q1), but lower levels of tumor necrosis factor alpha (≤10% vs. >10%) (Table 3).

ASD-YesID and ASD-NoID Cases Versus DD Controls. Both the ASD-NoID and ASD-YesID groups demonstrated increased odds corresponding to higher levels of IL-12p70 and eotaxin-1 (Q3 vs. Q1) relative to DD controls (Table 3). The odds of ASD-NoID were elevated with higher levels of eotaxin-1 (Q4 and Q2 vs. Q1), 6CKINE (chemokine [C-C motif] ligand 21 [CCL21]) (T cell chemoattractant with angiogenic properties), IL-13 (Q4 vs. Q1), and granulocyte chemotactic protein 2 when compared with DD controls. The odds of ASD-YesID were elevated with higher levels of IFN-γ (Q2 vs. Q1) and eotaxin-1 (≥90% vs. <90%) when compared with DD controls (Table 3).

ASD-Reg or ASD-EO Cases Versus DD Controls. Similar to the ASD group as a whole, we found increased odds of ASD-EO associated with higher levels of IL-12p70, IFN-γ (Q2 vs. Q1), eotaxin-1 (Q4 and Q3 vs. Q1), and 6CKINE relative to the DD group (Table 3). The risk of developing ASD-Reg relative to DD is also increased with higher levels of IFN-γ and eotaxin-1 (Q4 vs. Q1), but unlike ASD-EO, decreased risk with higher levels of

Figure 1. Representative scatterplot of data from autism spectrum disorder (ASD) and general population (GP) subjects for **(A)** interleukin-6 (IL-6) and **(B)** IL-8. Data are presented as a ratio of the cytokine concentration/total protein concentration of the bloodspot eluate. This was done to normalize differences based on total protein content of the eluate.

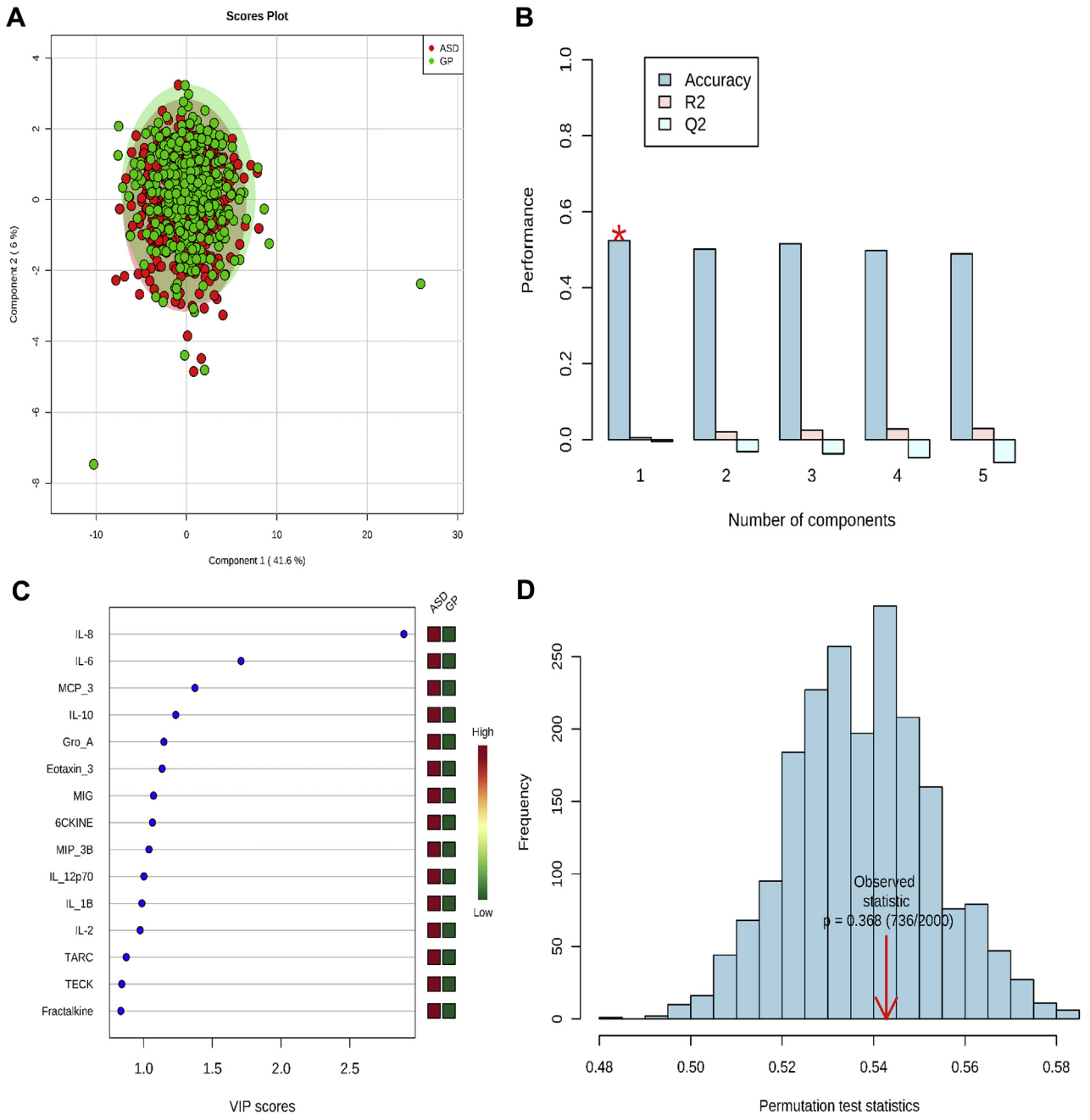


Figure 2. Representative multivariate analysis between autism spectrum disorder (ASD) and general population (GP) groups on linear regression residuals using partial least squares discriminant analysis to determine combined cytokine profiles. **(A)** Partial least squares discriminant analysis 2-dimensional score plot. **(B)** Bar plots showing the 3 performance measures using different numbers of components. The red asterisk indicates the measure used for this analysis. **(C)** The top 15 immune markers ranked by Variable Importance in Projection (VIP) score. **(D)** The results of permutation tests summarized in a histogram with the observed statistic highlighted by red arrow. 6CKINE, chemokine (C-C motif) ligand 21 (CCL21); Gro-α, growth-regulated oncogene alpha; IL, interleukin; MCP, monocyte chemotactic protein; MIG, monkine induced by gamma interferon; Q, quartile; R2, cross-validation value; TARC, thymus and activation regulated chemokine; TECK, thymus-expressed chemokine.

MCP-4 (chemoattractant for monocytes and T cells) (>10% vs. ≤10%) (Table 3).

DD Cases Versus GP Controls. We observed no significant differences in any of the immune marker levels measured for the DD group relative to the GP controls (Supplemental Table S2).

ASD-YesID Cases Versus ASD-NoID Cases. When considering the ASD-YesID phenotype relative to the

Table 4. Permutation Statistics for All Group Interactions by Partial Least Squares Discriminant Analysis

Group	*p* Value
ASD vs. GP	.380
DD vs. GP	.990
ASD-NoID vs. GP	.470
ASD-YesID vs. GP	.847
ASD-EO vs. GP	.710
ASD-Reg vs. GP	.950
ASD vs. DD	.410
ASD-NoID vs. DD	.470
ASD-YesID vs. DD	.847
ASD-EO vs. DD	.390
ASD-Reg vs. DD	.780
ASD-YesID vs. ASD-NoID	.183
ASD-Reg vs. ASD-EO	.423

ASD, autism spectrum disorder; DD, developmental disorder; EO, early onset; GP, general population; NoID, absence of intellectual disability; Reg, regressive; YesID, presence of intellectual disability.

ASD-NoID phenotype within the ASD group, we found that elevated levels of eotaxin-1 and cutaneous T cell-attracting chemokine were associated with lower odds of ASD-YesID compared with ASD-NoID (Table 3).

PLS-DA Multivariate Analysis

Individual immune marker data, independent of diagnosis, were subjected to a linear regression analysis with the same covariates as the logistic regression model above. Residuals generated through this analysis were used as adjusted data points for inclusion in the PLS-DA model. Figure 2 is a representative analysis comparing the GP and ASD groups, showing the individual subjects plotted in component space, the cross-validation method, permutation statistic, and the top 15 Variable Importance in Projection scores. The Variable Importance in Projection scores are a weighted sum of squares of the PLS loadings that takes into account the amount of Y-variance explained by each component. In Figure 2, when comparing ASD versus GP data, we find IL-8 and IL-6 as the most important variables, replicating our findings in the logistic regression analysis, with all other group comparisons summarized in Table 4. Using the available immune marker data, we were unable to differentiate between behavioral groups when plotted in component space. Likewise, a significant number of randomizations outperformed the predictive power of our behavioral group labels in the permutation test statistic. These results suggest that while there are individual cytokine and chemokine levels that are significantly different between ASD and GP controls, there were no obvious combinations of cytokine and/or chemokines that could differentiate across case status.

DISCUSSION

The goal of this study was to examine cytokine and chemokine levels in neonatal bloodspots as potential early markers for ASD risk. The high-sensitivity assays used herein proved to be an efficient detection method, as only 3 analytes remained at undetectable levels in >10% of the samples, delivering the most extensive analysis of newborn cytokines and chemokines to date. Most notably, we found an increased risk of ASD associated with select markers of immune activation when compared with GP and DD controls, but surprisingly no such associations for the DD group compared with GP controls.

In previous work using a less sensitive assay, Zerbo *et al.* (20) found increased levels of MCP-1 and decreased levels of RANTES in the newborn bloodspots of ASD cases compared with GP controls. In addition, they also found decreased neonatal levels of both macrophage inflammatory protein 1 alpha and RANTES in DD cases compared with GP controls. In the present study, MCP-1 levels were found to be comparable between all groups, whereas levels of macrophage inflammatory protein 1 alpha were slightly higher in the ASD-Reg group compared with the GP controls. Unfortunately, RANTES was unavailable for inclusion in the high-sensitivity kit used in the present study, so no direct comparisons between studies can be made for this chemokine.

In comparison with previous studies on neonatal bloodspots from non-EMA sample populations, this study finds an increased risk of ASD with intellectual disability associated with elevated levels of IL-4 compared with GP controls and IFN-γ, when compared with DD controls, which is in direct contradiction with the findings of Abdallah *et al.* (25), in which they noted decreased levels of IFN-γ, IL-4, and IL-10. However, these data are in agreement with a study by Krakowiak *et al.* (21), which demonstrated an increase in IL-4 in individuals with ASD associated with increased odds of what is referred to as severe ASD (Autism Diagnostic Observation Schedule comparison score $\geq$7). We find a similar tendency in this study, with an increase in IL-4 uniquely associated with greater odds of ASD-YesID, what some would consider a more impaired form of ASD. The inconsistencies in findings between this study and previous studies can likely be attributed to advancements in the Luminex methodology and reagents. In addition, the current study had significantly more samples per group compared with the 3 previous studies on neonatal blood spots, providing more statistical power to the analysis.

Our findings of increased risk of ASD associated with elevated neonatal levels of the inflammatory cytokines/chemokines IL-8 (ASD cases compared with GP controls), IL-12p70 and eotaxin-1 (ASD cases without intellectual disability compared with GP controls), and IFN-γ (ASD cases [total and YesID] relative to DD controls) are of particular interest. The chemokines IL-8 and eotaxin-1 are upregulated in response to local innate immune activation, thereby recruiting neutrophils and eosinophils that phagocytize debris and aid in tissue remodeling, respectively. The results for IL-8 suggest early differences in immune function between those children with EO ASD and those with the Reg form of the disorder. Increased IL-8 in neonatal serum is also associated with other neurological disorders including cerebral palsy (26,27) and seizures induced by hypoxic-ischemic encephalopathy (28). Likewise, children experiencing a traumatic brain injury have dramatically increased IL-8 in the cerebrospinal fluid and measurable levels in serum that correlate with unfavorable outcomes (29). Given these numerous associations between IL-8 and damage to the central nervous system, our data may implicate IL-8 as an indicator of early neuroimmune

dysfunction in children with ASD, especially those that experience an early onset of symptoms.

IL-12p70 is an innate immune cytokine that initiates inflammation and drives the development of IFN-γ-producing T cells, thereby perpetuating a proinflammatory environment. While very little is known about the exact mechanism by which cytokines may influence neurodevelopment and ASD-related behavioral outcomes, several other studies have found the presence of proinflammatory cytokines to be associated with ASD (7,12,30). Previously, circulating levels of IL-6, IL-8, IL-12, and eotaxin-1 were elevated in children with ASD postdiagnosis between 2 and 5 years of age (31,32). These related findings suggest that blood levels of these inflammatory cytokines could be a persistent phenotype of the ASD population. Studies are underway to explore this further.

We also performed a multivariate analysis on select immune markers to assess whether combinations of immune markers would be able to predict behavioral outcome. The PLS-DA method was unable to clearly distinguish between behavioral groups. Thus, our results suggest that there is no obvious neonatal cytokine or chemokine combined profile that predicts child developmental outcome.

It would be of further interest to explore the relationship between altered cytokine/chemokine levels in newborn blood spots and the maternal cytokine profile during gestation. A preliminary analysis using the maternal samples linked to our neonatal specimens was limited by the lack of measurement of the same analyte in both maternal and neonatal samples; the 15 cytokines/chemokines that did overlap had no correlation (33). While the initial exploratory analyses were not conclusive, we will continue to address the relationship between the maternal and neonatal cytokine profile in ongoing studies.

Limitations of this study, and the EMA study population in general, include the method by which behavioral groups were classified. Subjects from the EMA study did not undergo standardized clinical evaluation by the research team, but rather diagnosis was dependent on expert review of abstracted medical records as part of diagnostic eligibility for developmental services from regional centers. Because we did not have deep phenotypic data on the children in this study population, we examined associations by standard subgroups (onset type, intellectual disability status) and did report some differences across subgroup. However, a more refined examination of ASD phenotype in relation to neonatal biological markers is clearly important and could be addressed in other study populations with prospective data collection and longitudinal follow-up of children. Our analyses were limited by the information routinely collected on birth certificates, which did not include many potentially relevant environmental factors. However, there is no evidence that these factors are associated with neonatal cytokine/chemokine levels, and as such, they are unlikely to confound the associations we observed. While no consistent IQ test was performed on all children, intellectual disability status was determined in a consistent manner based on the cognitive assessment that was performed. In addition, the environmental conditions of bloodspot samples were not controlled during transportation, which might have led to degradation of sample integrity. However, this limitation applies equally to all bloodspots, so while actual levels measured in the samples may have been subjected to this degradation, the relative levels of cytokines and chemokines between individual samples should not be affected. Finally, as newborn bloodspots are taken at a single time point, we are limited in that these data reflect a snapshot of the infant-circulating cytokines and chemokines at birth. Future studies that evaluate the child's immune function over time would be of great interest.

Despite these limitations, our study was strengthened by the use of a highly sensitive assay for immune markers, which resulted in an increase in the number of cytokines and chemokines detected compared with previous studies. Further, our study utilized a significantly larger sample size than previous studies, allowing for the examination of different behavioral subgroups within the ASD population. The findings from this exploratory study require replication and extension in future studies to examine the involvement of immune molecules in the development of ASD in early life.

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