# Neonatal Cytokine Profiles Associated With Autism Spectrum Disorder

## *Supplemental Information*

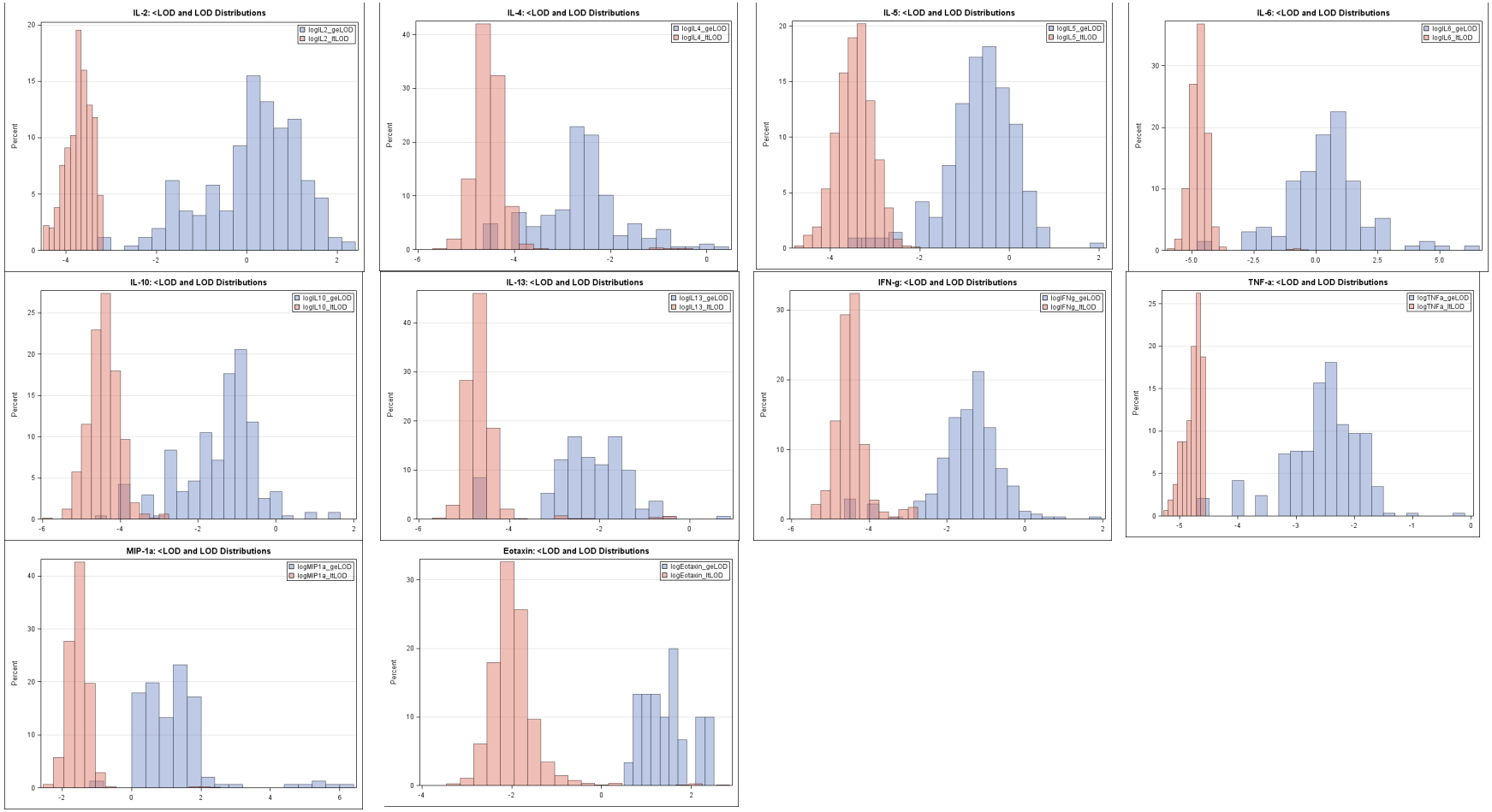


**Figure S1. Distributions of cytokine and chemokine values below and above levels of detection (LOD).** Values <LOD were imputed by a multiple imputation procedure (left distribution, in red). Values in the detectable range are shown in blue. The y-axis represents the percent of values with a given value (denoted on x-axis) in the distribution of imputed <LOD values and separately in the distribution of LOD values. The upper bound limits were restricted to LOD value for IL-2 and TNF-α only. Both distributions have a normal shape and there is little to no overlap. The top row shows the following cytokines (left to right): IL-2, IL-4, IL-5, and IL-6; the next row shows IL-10, IL-13, IFN-γ, and TNF-α; and the bottom row shows MIP-1α and Eotaxin.

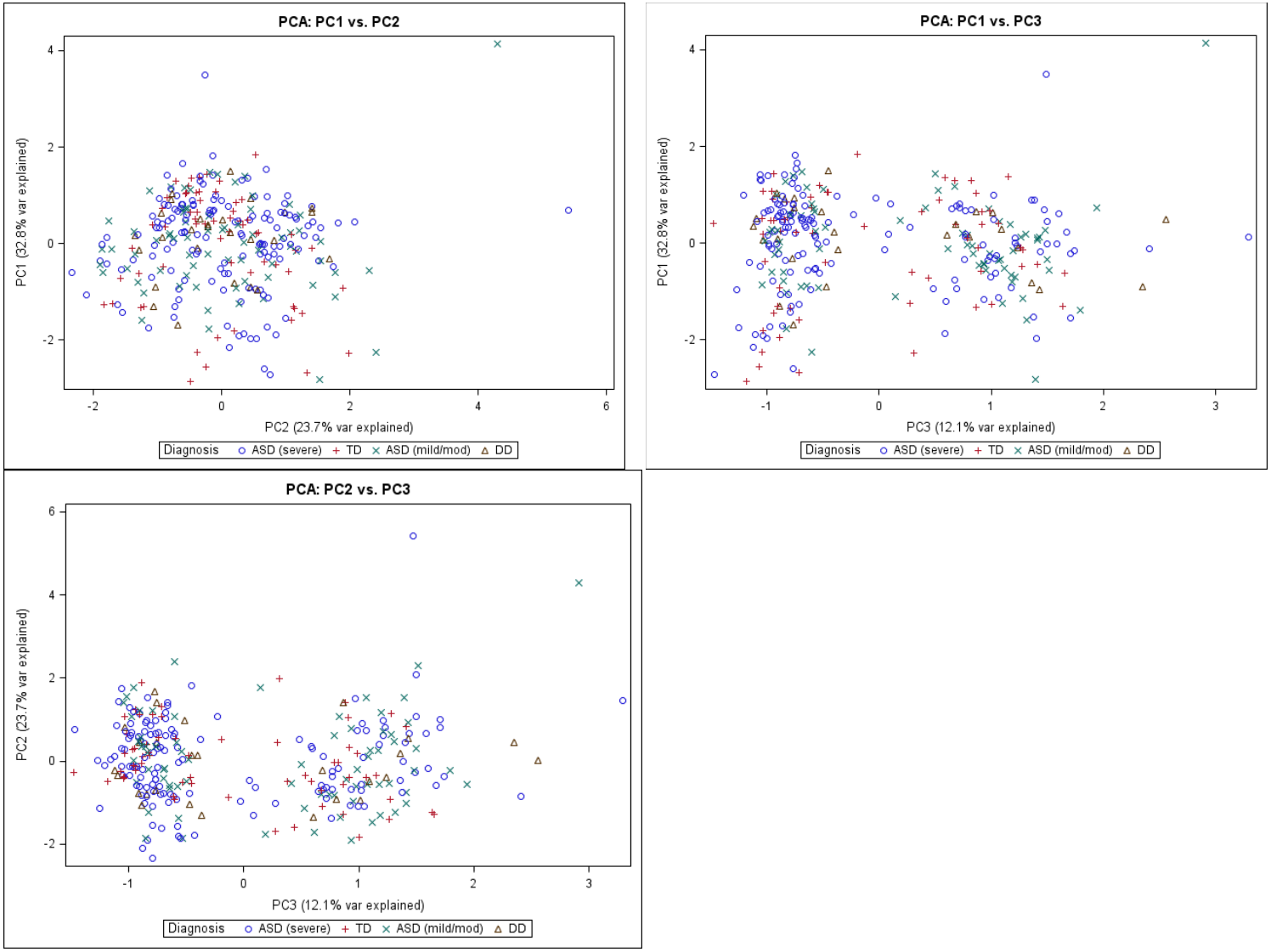


**Figure S2. Plots of principal component scores.** PC1 is comprised of cytokines involved in adaptive immune responses (e.g., IL-2, IL-4, IL-12); PC2 includes chemokines; and PC3 consists of cytokines involved in innate immune responses (IL-1β, IL-6, IL-10). ASD severity is defined using ADOS severity scores, where ≥7 indicates severe and <7 indicates mild to moderate symptoms.

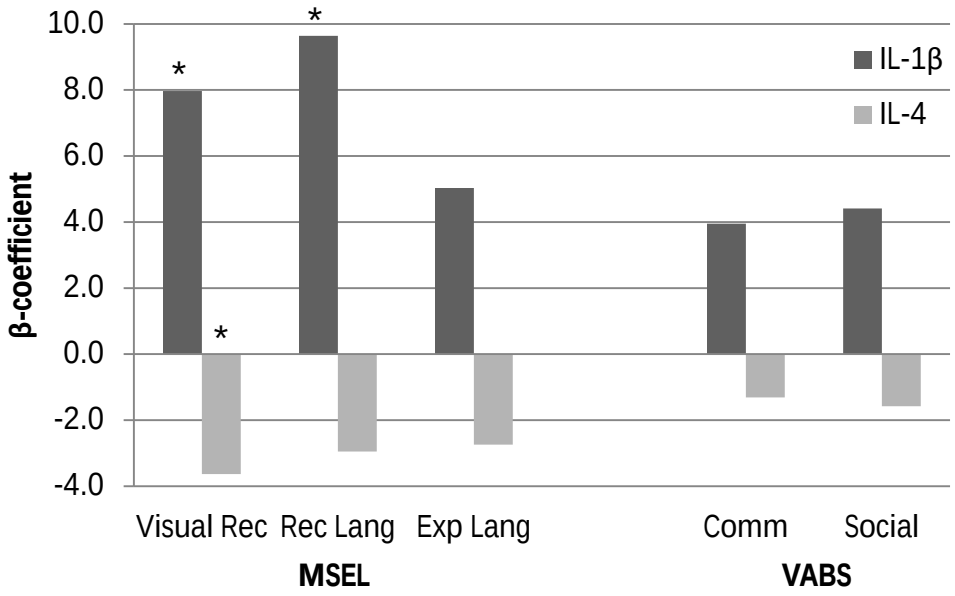


**Figure S3. Cognitive and adaptive scores of 2-5 year old males with ASD in relation to their neonatal IL-1β and IL-4 concentrations**. This figure illustrates the mean change in cognitive and adaptive scores for a 1-unit increase in ln-transformed cytokine (pg/mg total protein). Bars with an asterisk (*) denote statistically significant associations ($P < 0.05$); *P*-values were adjusted for multiple comparisons. Mullen Scales of Early Learning (MSEL) measures cognitive development and subscales include Visual Reception (non-verbal cognitive ability), Receptive Language (language comprehension), and Expressive Language (language production); Vineland Adaptive Behavior Scales (VABS) subscales include Communication and Socialization. The scores have been converted to developmental quotients, and higher scores represent better performance.

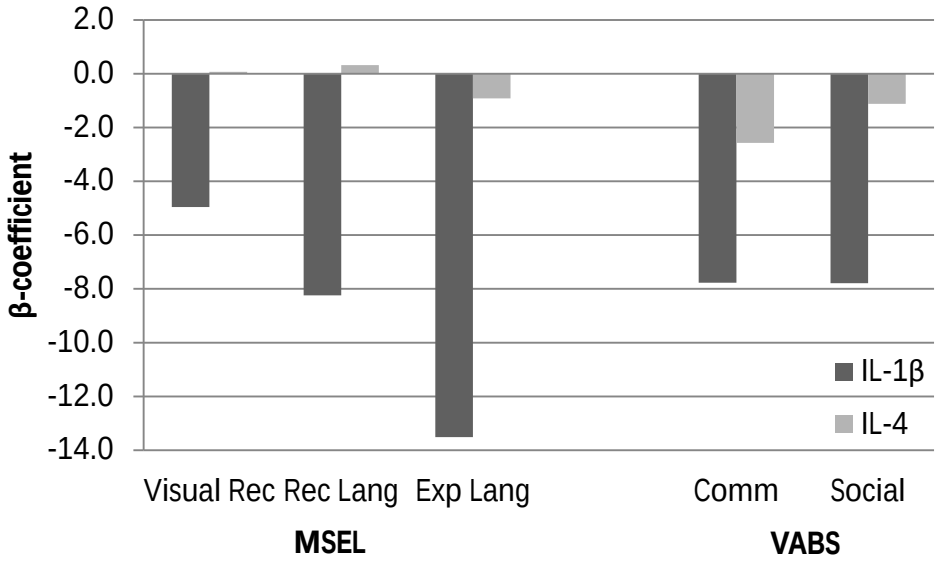


**Figure S4. Cognitive and adaptive scores of 2-5 year old children without ASD (DD and TD controls) in relation to their neonatal IL-1β and IL-4 concentrations**. This figure illustrates the mean change in cognitive and adaptive scores for a 1-unit increase in ln-transformed cytokine (pg/mg total protein). Bars with an asterisk (*) denote statistically significant associations (*P* <0.05); *P*-values were adjusted for multiple comparisons. Mullen Scales of Early Learning (MSEL) measures cognitive development and subscales include Visual Reception (non-verbal cognitive ability), Receptive Language (language comprehension), and Expressive Language (language production); Vineland Adaptive Behavior Scales (VABS) subscales include Communication and Socialization. The scores have been converted to developmental quotients, and higher scores represent better performance.

**Table S1: Distribution of cases and controls across plate numbers, *N* = 303**

| | ASD (*n* = 214) | | DD (*n* = 27) | | TD (*n* = 62) | | |
|---|---|---|---|---|---|---|---|
| | ***n*** | **%** | ***n*** | **%** | ***n*** | **%** | ***P*-value[a]** |
| Cytokine plate number | | | | | | | 0.88 |
| 1 | 37 | 17 | 6 | 22 | 9 | 14 | |
| 2 | 37 | 17 | 6 | 22 | 9 | 14 | |
| 3 | 10 | 5 | 0 | 0 | 6 | 10 | |
| 4 | 28 | 13 | 3 | 11 | 6 | 10 | |
| 5 | 33 | 15 | 5 | 19 | 11 | 18 | |
| 6 | 38 | 18 | 3 | 11 | 12 | 20 | |
| 7 | 31 | 15 | 4 | 15 | 9 | 14 | |
| | | | | | | | |
| Chemokine plate number | | | | | | | 0.92 |
| 1 | 37 | 17 | 6 | 22 | 9 | 15 | |
| 2 | 38 | 18 | 4 | 15 | 13 | 21 | |
| 3 | 38 | 18 | 6 | 22 | 10 | 16 | |
| 4 | 32 | 15 | 3 | 11 | 7 | 11 | |
| 5 | 36 | 17 | 2 | 8 | 12 | 19 | |
| 6 | 33 | 15 | 6 | 22 | 11 | 18 | |

[a]*P*-values calculated with Chi-square test.

**Table S2. Principal components analysis loadings, *N* = 303**

| Cytokine | PC 1 | PC 2 | PC 3 |
|---|---|---|---|
| IL-2 | **0.66** | 0.52 | . |
| IL-4 | **0.61** | . | . |
| IL-5 | **0.61** | . | . |
| IL-12 | **0.65** | . | . |
| IL-13 | **0.72** | . | . |
| IFN-γ | **0.65** | . | . |
| TNF-α | **0.65** | . | . |
| IL-8 | . | **0.51** | . |
| MIP-1α | . | **0.86** | . |
| MIP-1β | . | **0.56** | . |
| RANTES | . | **0.46** | . |
| IL-1β | . | . | **0.54** |
| IL-6 | . | . | **0.99** |
| IL-10 | 0.48 | . | **0.54** |
| Variance explained | 32.8% | 23.7% | 12.1% |

PC, principal component.

[a]Cytokines/chemokines measured in 303 newborns were ln-transformed and normalized for total protein (pg/mg total protein); values below levels of detection were imputed (single imputation); components that explained ≥10% of total variance were retained; loadings <|0.40| are not shown (MCP-1, IP-10, and Eotaxin had loadings <0.40 and were excluded).

**Table S3: Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD, DD, and TD, *N* = 303[a]**

| | ASD vs DD | | | ASD vs TD | | | DD vs TD | | |
|---|---|---|---|---|---|---|---|---|---|
| **Cytokine** | **OR** | **95% CI** | ***P*-value[b]** | **OR** | **95% CI** | ***P*-value[b]** | **OR** | **95% CI** | ***P*-value[b]** |
| IL-1β | 0.84 | (0.46, 1.52) | 0.9200 | 1.74 | (0.99, 3.07) | 0.3296 | 2.07 | (0.95, 4.52) | 0.8067 |
| IL-2 | 1.12 | (0.86, 1.45) | 0.9200 | 1.17 | (0.97, 1.41) | 0.3296 | 1.05 | (0.78, 1.40) | 0.8067 |
| IL-4 | 1.07 | (0.75, 1.52) | 0.9457 | **1.33** | **(1.01, 1.75)** | 0.3296 | 1.24 | (0.82, 1.89) | 0.8067 |
| IL-5 | 1.23 | (0.91, 1.65) | 0.9200 | 1.06 | (0.87, 1.29) | 0.7437 | 0.86 | (0.62, 1.19) | 0.8067 |
| IL-6 | 0.96 | (0.82, 1.12) | 0.9200 | 1.00 | (0.89, 1.12) | 0.9933 | 1.05 | (0.87, 1.25) | 0.8067 |
| IL-10 | 0.90 | (0.67, 1.21) | 0.9200 | 1.02 | (0.83, 1.24) | 0.9286 | 1.13 | (0.81, 1.58) | 0.8067 |
| IL-12 | 1.16 | (0.71, 1.89) | 0.9200 | 1.32 | (0.96, 1.83) | 0.3296 | 1.14 | (0.67, 1.96) | 0.8067 |
| IL-13 | 0.83 | (0.62, 1.13) | 0.9200 | 1.06 | (0.85, 1.31) | 0.7437 | 1.27 | (0.90, 1.79) | 0.8067 |
| IFN-γ | 1.04 | (0.73, 1.50) | 0.9457 | 1.20 | (0.96, 1.50) | 0.3296 | 1.15 | (0.77, 1.71) | 0.8067 |
| TNF-α | 1.02 | (0.60, 1.71) | 0.9457 | 1.13 | (0.78, 1.62) | 0.7437 | 1.11 | (0.61, 2.00) | 0.8067 |
| IL-8 | 0.81 | (0.51, 1.31) | 0.9200 | 1.20 | (0.77, 1.88) | 0.7437 | 1.48 | (0.81, 2.71) | 0.8067 |
| MCP-1 | 1.47 | (0.71, 3.05) | 0.9200 | 0.88 | (0.51, 1.52) | 0.7437 | 0.60 | (0.26, 1.40) | 0.8067 |
| MIP-1α | 0.99 | (0.76, 1.28) | 0.9457 | 1.04 | (0.87, 1.25) | 0.7437 | 1.31 | (0.52, 3.29) | 0.8067 |
| MIP-1β | 0.95 | (0.43, 2.10) | 0.9457 | 1.24 | (0.68, 2.26) | 0.7437 | 1.18 | (0.37, 3.42) | 0.8067 |
| IP-10 | 1.08 | (0.59, 1.98) | 0.9457 | 1.21 | (0.79, 1.84) | 0.7437 | 1.11 | (0.56, 2.20) | 0.8067 |
| RANTES | 1.66 | (0.68, 4.08) | 0.9200 | 1.47 | (0.81, 2.67) | 0.5304 | 0.89 | (0.32, 2.42) | 0.8125 |

CI, confidence interval; OR, odds ratio.

[a] Logistic regression models were adjusted for season of birth, gestational age, years from blood spot collection to elution, and child's sex; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); values below levels of detection were imputed by multiple imputation.

[b] *P*-values were corrected for multiple comparisons (16 biomarkers)

**Table S4: Adjusted odds ratios comparing neonatal cytokine and chemokine concentrations in ASD with severe symptoms, ASD with mild to moderately severe symptoms, and DD, *N* = 241[a]**

| | $ASD_{sev}$ vs. DD | | | $ASD_{mild}$ vs. DD | | |
|---|---|---|---|---|---|---|
| **Cytokine or Chemokine** | **OR** | **95% CI** | ***P*-value[b]** | **OR** | **95% CI** | ***P*-value[b]** |
| IL-1β | 0.57 | (0.27, 1.21) | 0.6988 | 1.25 | (0.62, 2.55) | 0.9683 |
| IL-2 | 1.15 | (0.88, 1.51) | 0.6988 | 1.05 | (0.79, 1.40) | 0.9683 |
| IL-4 | 1.12 | (0.78, 1.61) | 0.8654 | 0.98 | (0.67, 1.45) | 0.9687 |
| IL-5 | 1.23 | (0.91, 1.67) | 0.6988 | 1.21 | (0.88, 1.68) | 0.8677 |
| IL-6 | 0.90 | (0.76, 1.06) | 0.6988 | 1.05 | (0.88, 1.24) | 0.9683 |
| IL-10 | 0.87 | (0.64, 1.18) | 0.6988 | 0.96 | (0.70, 1.33) | 0.9687 |
| IL-12 | 1.13 | (0.68, 1.87) | 0.8654 | 1.21 | (0.71, 2.06) | 0.9683 |
| IL-13 | 0.83 | (0.61, 1.13) | 0.6988 | 0.85 | (0.61, 1.18) | 0.8677 |
| IFN-γ | 0.96 | (0.66, 1.38) | 0.8654 | 1.29 | (0.85, 1.94) | 0.8677 |
| TNF-α | 1.01 | (0.59, 1.73) | 0.9600 | 1.01 | (0.57, 1.79) | 0.9687 |
| IL-8 | 0.78 | (0.48, 1.27) | 0.6988 | 0.89 | (0.52, 1.53) | 0.9683 |
| MCP-1 | 1.45 | (0.69, 3.07) | 0.6988 | 1.51 | (0.67, 3.40) | 0.8677 |
| MIP-1α | 0.97 | (0.74, 1.26) | 0.8654 | 1.02 | (0.77, 1.36) | 0.9687 |
| MIP-1β | 0.83 | (0.36, 1.90) | 0.8654 | 1.23 | (0.52, 2.93) | 0.9683 |
| IP-10 | 0.90 | (0.47, 1.70) | 0.8654 | 1.58 | (0.80, 3.12) | 0.8677 |
| RANTES | 1.49 | (0.60, 3.71) | 0.6988 | 2.04 | (0.79, 5.26) | 0.8677 |

CI, confidence interval; OR, odds ratio.

[a] Logistic regression models were adjusted for season of birth, gestational age, years from blood spot collection to elution, and child's sex; cytokines/chemokines were ln-transformed and normalized for total protein (pg/mg total protein); values below levels of detection were imputed by multiple imputation; 241 participants comprised the following groups: 141 ASD (severe), 73 ASD (mild), and 27 DD; ASD severity was defined using ADOS severity scores, where ≥7 indicated severe and <7 indicated mild to moderate symptoms.

[b] *P*-values were corrected for multiple comparisons (16 biomarkers).

**Table S5: Behavioral and developmental characteristics of 2-5 year old children with ASD in relation to their neonatal IL-1β and IL-4 concentrations, *N* = 214**

| | | IL-1β | | | IL-4 | | |
|---|---|---|---|---|---|---|---|
| | **Int** | **β** | **SE** | ***P*-value[c]** | **β** | **SE** | ***P*-value[c]** |
| *Aberrant Behavior Checklist*[a] | | | | | | | |
| Irritability | 2.756 | 0.054 | 0.078 | 0.8889 | -0.001 | 0.036 | 0.9877 |
| Lethargy/Social Withdrawal | 2.318 | -0.035 | 0.085 | 0.8889 | -0.050 | 0.040 | 0.9124 |
| Stereotypy | 1.874 | 0.000 | 0.113 | 0.9990 | 0.022 | 0.052 | 0.9877 |
| Hyperactivity | 3.012 | 0.038 | 0.065 | 0.8889 | -0.010 | 0.031 | 0.9877 |
| | | | | | | | |
| *Mullen Scales of Early Learning*[b] | | | | | | | |
| Visual Reception | 63.58 | **7.13** | **2.54** | **0.0138** | **-3.05** | **1.27** | 0.0855 |
| Receptive Language | 53.50 | **8.32** | **2.90** | **0.0138** | -1.84 | 1.45 | 0.3431 |
| Expressive Language | 46.76 | 4.42 | 2.56 | 0.1065 | -2.24 | 1.28 | 0.3431 |
| | | | | | | | |
| *Vineland Adaptive Behavior Scales*[b] | | | | | | | |
| Communication | 48.69 | 3.60 | 2.49 | 0.15 | -0.36 | 1.24 | 0.7754 |
| Socialization | 46.25 | 4.08 | 2.33 | 0.08 | -1.28 | 1.17 | 0.3431 |

β, β-coefficient (estimate); Int, intercept; SE, standard error.

[a]Negative binomial regression models were adjusted for maternal education (≤High school, Some college [referent], ≥Bachelor degree) and child's age at enrollment (centered) with predictors ln-transformed IL-1β and IL-4 (pg/mg total protein); values below levels of detection were imputed by multiple imputation; 191 participants completed an Aberrant Behavior Checklist (ABC); the β-coefficient represents the change in the (natural) log of ABC raw score count for a 1-unit increase in a ln-transformed cytokine (pg/mg total protein), with a positive β indicating greater behavior problems.

[b]Linear regression models were adjusted for maternal education (≤High school, Some college [referent], ≥Bachelor degree) with predictors ln-transformed IL-1β and IL-4 (pg/mg total protein); values below levels of detection were imputed by multiple imputation; 211 participants had Mullen and Vineland assessments; the β-coefficient represents the change in behavioral/developmental developmental quotient (DQ) for a 1-unit increase in a ln-transformed cytokine (pg/mg total protein), with a positive β indicating better performance in a given behavioral/developmental domain.

[c]*P*-values were corrected for multiple comparisons (4 behavior scores (ABC); 5 developmental scores (MSEL,VABS)).

**Table S6: Behavioral and developmental characteristics of 2-5 year old males with ASD in relation to their neonatal IL-1β and IL-4 concentrations, *N* = 189**

| | | IL-1β | | | IL-4 | | |
|---|---|---|---|---|---|---|---|
| | **Int** | **β** | **SE** | ***P*-value[b]** | **β** | **SE** | ***P*-value[b]** |
| *Mullen Scales of Early Learning*[a] | | | | | | | |
| Visual Reception | 63.39 | **7.97** | **2.66** | **0.0078** | **-3.63** | **1.33** | **0.0360** |
| Receptive Language | 52.44 | **9.64** | **3.03** | **0.0078** | -2.95 | 1.52 | 0.0915 |
| Expressive Language | 46.44 | 5.03 | 2.69 | 0.0905 | **-2.74** | **1.36** | 0.0915 |
| | | | | | | | |
| *Vineland Adaptive Behavior Scales*[a] | | | | | | | |
| Communication | 45.53 | 3.95 | 2.56 | 0.1249 | -1.31 | 1.29 | 0.3083 |
| Socialization | 45.43 | 4.41 | 2.44 | 0.0905 | -1.58 | 1.23 | 0.2491 |

β, β-coefficient (estimate); Int, intercept; SE, standard error.

[a]Linear regression models were adjusted for maternal education (≤High school, Some college [referent], ≥Bachelor degree) with predictors ln-transformed IL-1β and IL-4 (pg/mg total protein); values below levels of detection were imputed by multiple imputation; 186 male participants had Mullen and 187 had Vineland assessments; the β-coefficient represents the change in behavioral/developmental developmental quotient (DQ) for a 1-unit increase in a ln-transformed cytokine (pg/mg total protein), with a positive β indicating better performance in a given behavioral/developmental domain.

[b]*P*-values were corrected for multiple comparisons (5 developmental scores).

**Table S7: Behavioral and developmental characteristics of 2-5 year old children without ASD (DD and TD controls) in relation to their neonatal IL-1β and IL-4 concentrations, *N* = 89**

| | | IL-1β | | | IL-4 | | |
|---|---|---|---|---|---|---|---|
| | **Int** | **β** | **SE** | ***P*-value[b]** | **β** | **SE** | ***P*-value[b]** |
| *Mullen Scales of Early Learning*[a] | | | | | | | |
| Visual Reception | 72.13 | -4.96 | 6.38 | 0.4390 | 0.07 | 3.01 | 0.9809 |
| Receptive Language | 57.41 | -8.24 | 6.61 | 0.4390 | 0.32 | 3.11 | 0.9809 |
| Expressive Language | 42.18 | -13.51 | 6.84 | 0.2580 | -0.92 | 3.23 | 0.9809 |
| | | | | | | | |
| *Vineland Adaptive Behavior Scales*[a] | | | | | | | |
| Communication | 51.70 | -7.77 | 7.60 | 0.4390 | -2.57 | 3.62 | 0.9809 |
| Socialization | 59.57 | -7.79 | 8.38 | 0.4390 | -1.12 | 3.95 | 0.9809 |

β, β-coefficient (estimate); Int, intercept; SE, standard error.

[a]Linear regression models were adjusted for maternal education (≤High school, Some college [referent], ≥Bachelor degree) with predictors ln-transformed IL-1β and IL-4 (pg/mg total protein); values below levels of detection were imputed by multiple imputation; 89 participants had Mullen and Vineland assessments; the β-coefficient represents the change in behavioral/developmental developmental quotient (DQ) for a 1-unit increase in a ln-transformed cytokine (pg/mg total protein), with a positive β indicating better performance in a given behavioral/developmental domain.

[b]*P*-values were corrected for multiple comparisons (5 developmental scores).